A STUDY OF THE HEPATIC GLUTATHIONE S-TRANSFERASES OF SALMONID FISH.

by

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DECLARATION OF ORIGINALITY

I declare that this Thesis represents my work and was composed by me.

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ABBREVIATIONS

APS	= Ammonium persulphate
Δ-5-A	= Δ-5-Androstene-3,17-dione
ANS	= 8-Anilino-1-naphthalenesulphonic acid
BSP	= Bromosulphophthalein
CDNB	= 1-Chloro-2,4-dinitrobenzene
DCNB	= 1,2-Dichloro-4-nitrobenzene
ENPP	= 1,2-Epoxy-3-(p-nitrophenoxy)propane
ETHA	= Ethacrynic acid
GSH	= Reduced glutathione
GSSG	= Oxidized glutathione
mBrB	= monobromobimane
NBC	= p-Nitrobenzyl chloride
NPEB	= p-Nitrophenethyl bromide
PMSF	= Phenylmethylsulphonyl fluoride
SDS-PAGE	= Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBO	= Trans-4-phenyl-3-buten-2-one
TEMED	= N,N,N`,N`-tetramethylethylene diamine

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ABSTRACT

A STUDY OF THE HEPATIC GLUTATHIONE S-TRANSFERASES OF SALMONID FISH

1. The hepatic glutathione S-transferases (GSTs) from the rainbow trout were purified using S-hexylGSH affinity chromatography and chromatofocusing into at least 5 cationic forms (eluted in the range pH 9-6) and 1 anionic form (eluted with salt). The anionic form was further separated into 3 components by hydroxyl-apatite chromatography. At least half of the anionic material did not bind to the S-hexylGSH affinity column. GSH affinity chromatography was used, but altered the pI's of the enzymes possibly because of mixed- disulphide formation. The substrate and ligand-binding specificities of the GSTs were tested using fluorimetric and spectrophotometric techniques. In these tests, the anionic form differed to the cationic forms. The enzymes did not obey Michaelis-Menten kinetics; empirical half-saturation concentrations for GSH and 1-chloro-2,4-dinitrobenzene (CDNB) were higher for the anionic form than for the cationic forms. The enzymes were further characterized on SDS-PAGE. Trout from two fish-farms had different relative proportions of the cationic enzymes. Antibodies raised to fractions containing the cationic forms cross-reacted with a number of non-GST proteins.

2. The hepatic GSTs from the Atlantic salmon were purified using affinity chromatography and chromatofocusing. At least 10 cationic and 1 anionic form were separated from fish from salt-water. In addition, a number of enzymes did not bind to the affinity columns used. The GSTs from fish from fresh-water differed to those from fish from salt-water in that they did not bind to a S-hexylGSH affinity column and bound only to a GSH affinity column. The GST's from fish from both waters had

identical electrophoretic mobilities and pI's. Limited proteolysis did not show any differences between them. The substrate specificities, empirical half-saturation concentrations for GSH and CDNB, and amino acid compositions were determined for the salt-water forms. N-terminal amino acid composition determinations were attempted for the salt-water forms. Antibodies raised to the cationic and anionic forms from salt-water fish reacted specifically with a number of proteins from salmon and trout.

3. Salmonid fish have several soluble hepatic GSTs with differing physical and enzymic properties.

SECTION 1

INTRODUCTION

SECTION 1: INTRODUCTION

1.01: PREAMBLE

The purpose of the present study was to investigate the structure and function of the glutathione S-transferases (EC 2.5.1.18) in salmonid fish and to find out what differences occur between these enzymes and those of other species.

The glutathione S-transferases are found in high concentrations in tissue such as the liver, comprising 10% and 3% respectively of the hepatic cytosolic protein in rats and humans (Jakoby & Keen, 1977) and are present in the same order of magnitude in the rainbow trout (Nimmo et al., 1979). The enzymes are believed to play an important role in the detoxication and removal of xenobiotics such as industrial effluents, fossil fuels, pesticides and the products of waste-water treatment, which have been shown to accumulate in the environment (Gibson, 1968). Fish, because of their ecological niche, can be subjected to intense xenobiotic loadings (Hawkes, 1980) and might therefore be expected to have evolved mechanisms for dealing with these. In addition, in Scotland the farming of salmonid fish is becoming increasingly important economically, in particular the Atlantic salmon (*Salmo salar*) and the rainbow trout (*Salmo gairdneri*). The fish are fed with pelleted food which may sometimes be contaminated with the food-spoilage mould *Aspergillus flavus* which produces aflatoxins. These are activated by microsomal hydroxylases to 2,3-diol epoxides (Coulombe et al., 1984) which are potent mutagens (Loveland et al., 1983). The correlation between the high incidence of hepatomas in hatchery-reared rainbow trout and the presence of aflatoxins in the feed was first demonstrated by Adamson (1967) and has since been the subject of much research.

It was thought important to determine whether or not salmonids have any effective means of detoxifying potentially hazardous xenobiotics. In addition, it is interesting from a comparative point of view to see how the glutathione S- transferases in salmonids compare with those of rats and humans, about which a great deal has been written, and to relate their properties to the physiology of the species concerned.

In mammals, the hepatic glutathione S-transferases have been said to have a second function, namely to aid the transport of relatively insoluble compounds such as bromosulphophthalein (BSP) and bilirubin across the hepatocyte (Wolkoff, 1980). *In vitro* this function is expressed as the ability of the glutathione S-transferases to bind reversibly and non-covalently to the compounds.

Levine et al. (1971) in a phylogenetic study of the capacity of vertebrates to remove the organic anion BSP from plasma to the liver, found that this function was either absent or minimal in elasmobranchs and teleosts. This function was associated with the presence of two cytoplasmic protein fractions Y and Z (Levi et al., 1969). Fraction Y was not seen in fish species but appeared in all the lung breathing vertebrates investigated. This fraction was subsequently shown to be associated with glutathione S- transferase activity (Litwack et al., 1971).

It appeared that fish had no Y fraction, yet were shown to possess substantial glutathione S-transferase activity (Grover & Sims, 1964; Bend & Fouts, 1973). Nimmo et al. (1979) demonstrated the presence of glutathione S-transferase activity in a wide range of tissues in *Salmo gairdneri* but found capacity to bind lithocholic acid associated only with the Z fraction (Nimmo et al., 1980). It is therefore of interest to see what properties the glutathione S-transferases of salmonids possess and how these differ from those of other species.

1.02: HISTORICAL INTRODUCTION

In 1879 Baumann & Preusse showed that when monohalogenobenzenes were administered to dogs they were recovered as unstable N-acetylated S-substituted cysteine derivatives in the urine. Such compounds became known as mercapturic acids and were isolated from the urine of various species. The involvement of the tripeptide glutathione in the formation of mercapturates was indicated by Barnes et al. (1959), who showed that hepatic glutathione was depleted by the administration of mercapturate precursors. Bray et al. (1959a) demonstrated the *in vitro* formation of S-(p-chlorobenzyl)glutathione from p-benzyl chloride by rat liver cytosol and its subsequent hydrolysis to the cysteine derivative. Later Bray et al. (1959b) demonstrated the *in vivo* N-acetylation of S-substituted cysteine derivatives.

Booth et al. (1960a) showed that the rate of reaction was increased by the addition of rat liver cytosol and that the enzyme(s) involved were specific for glutathione but not for the other substrate. This led to the partial purification (Booth et al., 1961) of the enzyme in rat liver cytosol responsible for the conjugation of glutathione (GSH) with 1,2-dichloro-4-nitrobenzene (DCNB), BSP and a number of other substrates (Combes & Stakelum, 1961; Grover & Sims, 1964).

The protein(s) became known as glutathiokinase (Al-Kassab et al., 1962) and were shown to replace the nitro-group of 4-nitropyridine-N-oxide with GSH (Al-Kassab et al., 1963). Johnson (1963) showed that an enzyme catalysing the conjugation of GSH with aliphatic halogen compounds could be separated from the glutathiokinase activity and it was suggested that the enzymes be called GSH S-aryltransferases.

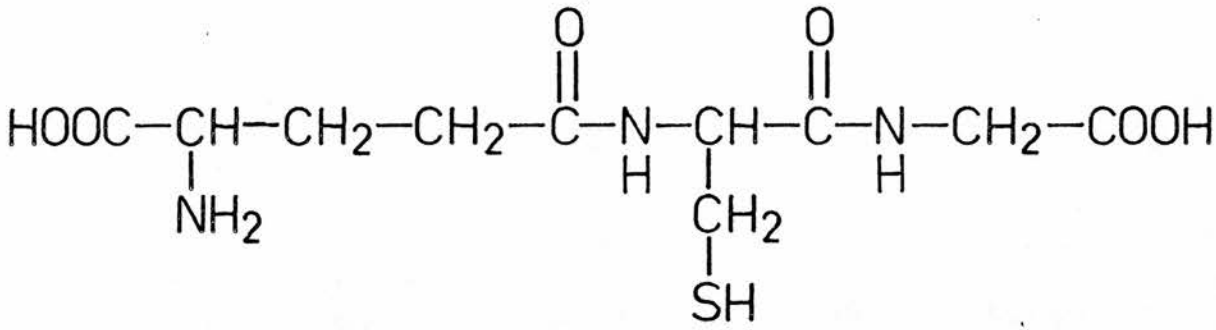
The discovery of separate proteins catalysing the conjugation of GSH with epoxides (Boyland & Williams, 1965), alkyl compounds such iodomethane (Johnson, 1966), benzyl chloride (Suga et al., 1967) and alpha-beta unsaturated carbonyl compounds (Boyland & Chasseaud, 1968) led to the introduction of the terms GSH S-epoxide, S-alkyl, S-arylalkyl and S-alkene transferases.

Subsequently Habig et al. (1974) showed that the different enzymes from rat liver had broad and overlapping substrate specificities and that the nomenclature was rather ambiguous; thereafter the proteins became known as the GSH S-transferases. The enzymes separated by Jakoby's group were assigned the names A, B, C, D and E based on their reverse order of elution from a carboxymethyl cellulose cation exchange column and were detected by their ability to conjugate DCNB with GSH.

Later Habig et al. (1976) using a more sensitive substrate called 1-chloro-2,4-dinitrobenzene (CDNB) found a transferase eluting from the cation-exchange column at an even higher salt concentration than GSH S-transferase A and named it GSH S-transferase AA. This compound has now become the standard substrate for the detection of GSH S-transferase activity. Since 1974 many new enzymes have been discovered, both in rats and in other species, new systems of purification and nomenclature have been introduced and detailed information is available on the structure and mode of action of the enzymes. This information will be presented in greater detail in later sections, but first a little must be said on the role of the tripeptide GSH as a co-substrate for the reaction, and on its relevant chemistry.

Fig. 1.03

The structure of glutathione



γ-Glutamylcysteinylglycine

1.03: THE CHEMISTRY OF GLUTATHIONE

Glutathione (γ-L-glutamyl-L-cysteinylglycine; Fig.1.03) is an abundant non-protein thiol found in a wide variety of species such as bacteria, yeast, potatoes (Schroeder & Woodward, 1939), insects (Lipke & Chalkley, 1962) and animals (Woodward, 1935). This component of most living cells was first isolated from yeast by Hopkins (1921) who thought it to be a dipeptide comprising glutamate and cysteine. Hopkins (1929) later found that it was in fact a tripeptide containing in addition a glycine residue. GSH is found at concentrations as high as 5-10mM in cells such as rat hepatocytes (Kosower & Kosower, 1978) and has a number of functions.

One of these is as an intracellular reductant. It achieves this by maintaining protein thiol groups required for catalysis and by producing the reducing power for many reactions within the cell, protecting the cell against peroxides and free radicals by the action of GSH peroxidase which catalyses the oxidation of GSH with peroxides to oxidized GSH (GSSG). GSH is also involved in transhydrogenation reactions and participates as a coenzyme in several reactions. Furthermore, GSH is known to form mixed disulphides with other thiols and protein thiols (Jocelyn, 1972), for example a protein thiol group reacting with GSH disulphide (GSSG) would form a mixed disulphide and release free GSH. This type of reaction can occur both enzymically and non-enzymically (Mannervik & Eriksson, 1973).

GSH has a net negative charge at physiological pH's due to an excess of of carboxyl groups over amino groups. Its unique properties are determined by the γ-glutamyl peptide bond between the N-terminal glutamate and the cysteine residue and by the reactive cysteinyl thiol group. The published pKa's for the various groups show variation (Jocelyn,

1972). The values for the cysteinyl thiol group are usually in the range 8.6 - 9.3 (Kaplowitz, 1980), for the glutamate amino group 9.1 - 9.5 (Boyland & Chasseaud, 1969; Kosower & Kosower, 1976) and for the glutamate and glycine carboxyl groups around 2.5 and 3.7 respectively.

GSH is hydrophilic and thus increases the solubility of hydrophobic moieties to which it may become conjugated. The γ-glutamyl peptide bond gives the tripeptide a degree of specificity because it cannot be cleaved by alpha-carboxypeptidases but can be by γ-glutamyl transpeptidase, an enzyme involved in the first stage of mercapturic acid formation which specifically cleaves the glutamate residue from the GSH-conjugate. The linkage also protects the cysteinyl sulphydryl group from rapid oxidation. In addition, GSH is involved in amino acid transport, transpeptidation, secretory processes and leukotriene interconversion (Meister, 1981; Hammarström, 1983).

The formation of GSH-conjugates and their subsequent degradation via the mercapturic acid pathway is dependent on the reactive cysteinyl thiol group. The high pKa of this group (about 9.3) means that at physiological pH's it is protonated and less likely to react covalently with potential substrates. The GSH S-transferases are believed to act by binding GSH and lowering the pKa of the thiol group enhancing its nucleophilicity (Keen et al., 1976; Jakoby, 1978). This facilitates attachment to the electrophilic centre of the second substrate which probably occupies a non-specific binding site in an adjacent hydrophobic region of the enzyme. The conjugation reaction and other functions of the GSH S-transferases are dealt with in the next section (1.04).

1.04: THE PHYSIOLOGICAL ROLES OF THE GLUTATHIONE S-TRANSFERASES

The GSH S-transferases play three roles in detoxication known collectively as the "Triple Threat" (Jakoby & Keen, 1977). The first involves the conjugation of GSH with electrophilic compounds and also includes a number of reactions where GSH participates but does not form a conjugate or is not consumed at all. In addition these enzymes bind non-covalently to hydrophobic compounds which are not substrates for the previous reaction, acting as transport and storage proteins until the compounds are removed and metabolized by other systems. A final role for the transferases is covalent binding to highly reactive electrophilic compounds in a so-called "suicide" reaction. This reaction is believed to take place to protect vital cellular constituents such as nucleic acids from damage by potential alkylating agents. These two binding functions may account for the high intracellular concentration of these enzymes in the body.

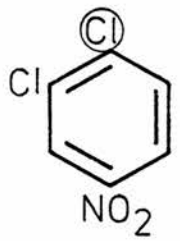
1.04.01: Catalytic activity of the glutathione S-transferases

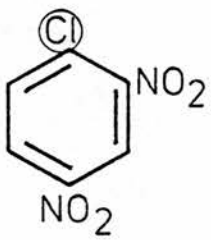
The conjugation of electrophilic substrates with GSH is the first step in mercapturic acid formation (Boyland & Chasseaud, 1969). The resultant GSH adduct is hydrolysed by γ-glutamyl transpeptidase (Booth et al., 1960b) with the removal of the N-terminal glutamic acid residue. Subsequent cleavage of the glycine residue by a dipeptidase and the addition of an acetyl group by an N-acetylase leads to the formation of mercapturic acids (N-acetylated cysteine derivatives) which are then excreted in the urine and faeces. GSH- conjugates formed in the liver are removed in the bile and either excreted directly or reabsorbed by the gut. They are then further metabolised and recycled. However, Booth et al. (1960a) demonstrated N-acetylation in

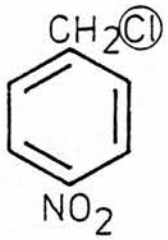
Fig. 1.04.01a

Substrates for glutathione S-transferases. These substrates undergo replacement reactions with GSH. Leaving groups are circled.

1-chloro-2,4-nitrobenzene

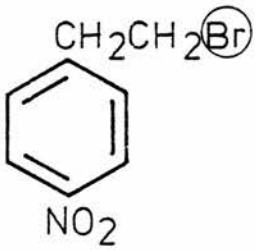


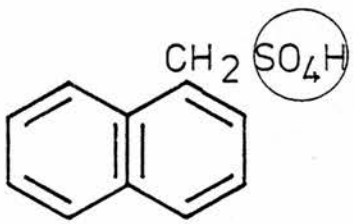




1,2-dichloro-4-nitrobenzene

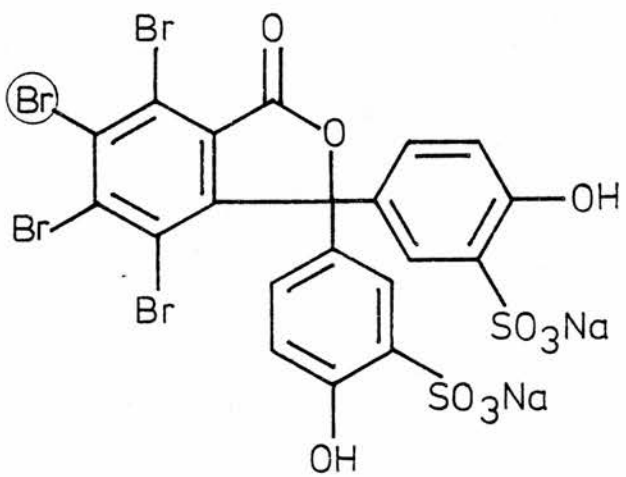
p-nitrobenzyl chloride





p-nitrophenethyl bromide

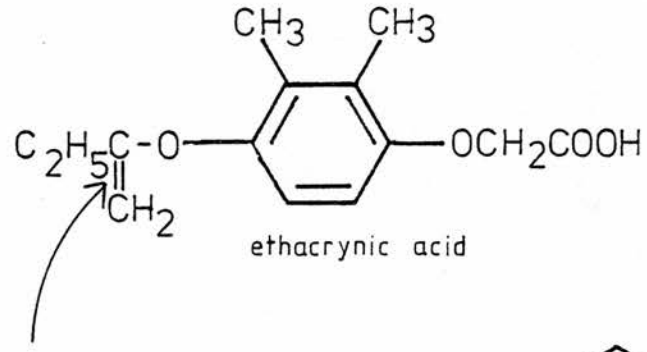
1-menaphthyl sulphate



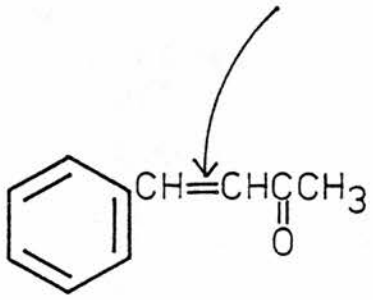
bromosulphophthalein

Fig.1.04.01b

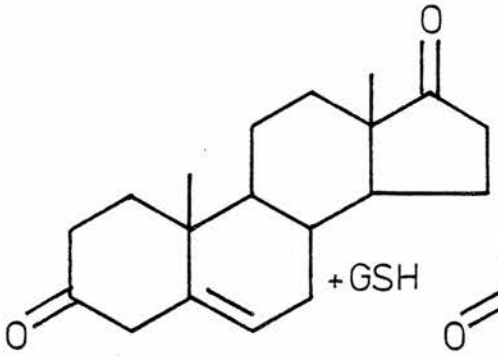
Two substrates which undergo an addition reaction with GSH across an α,β-unsaturated double bond (arrowed). In addition a classical nucleophilic reaction with p-nitrophenyl acetate and the Δ^5-ketosteroid isomerase reaction with Δ^5-androstene-3,17-dione are shown.



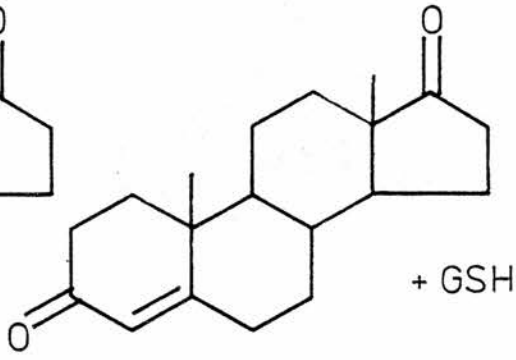
ethacrynic acid



trans-4-phenyl-3-buten-2-one



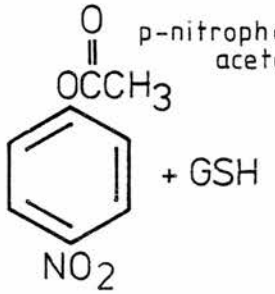
Δ^5-androstene-3,17-dione



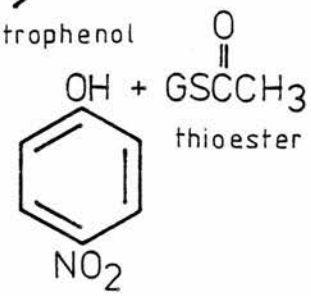
Δ^4-androstene-3-,17-dione

GSH S-transferase

p-nitrophenyl acetate



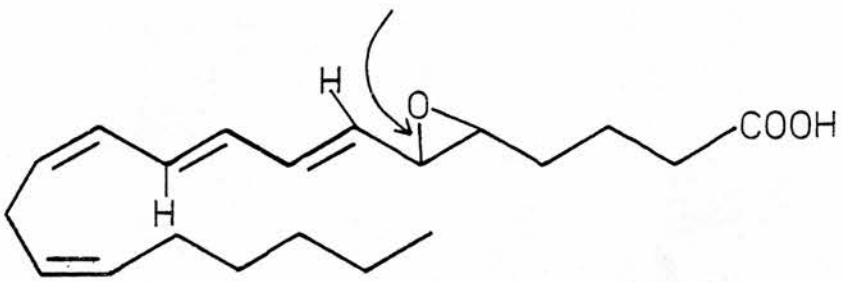
p-nitrophenol



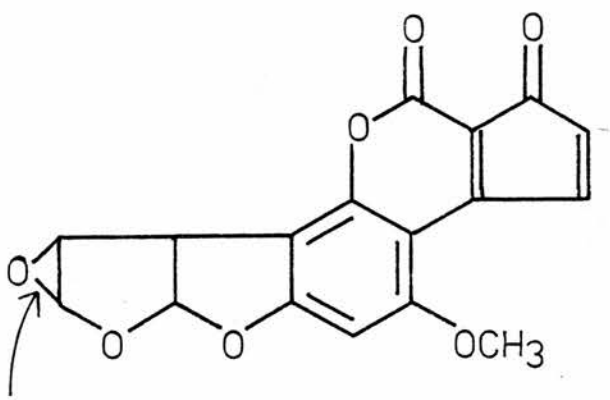
thioester

Fig. 1.04.01c

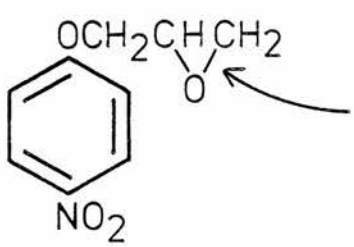
Substrates for glutathione S-transferases. The addition of GSH across epoxide groups is indicated with an arrow.



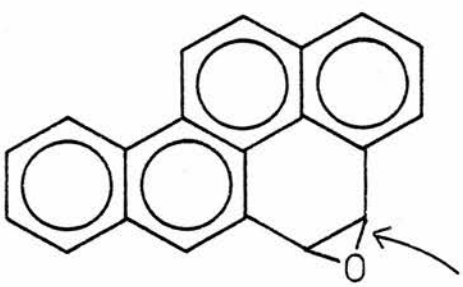
leukotriene A_4



aflatoxin B -2,3-oxide



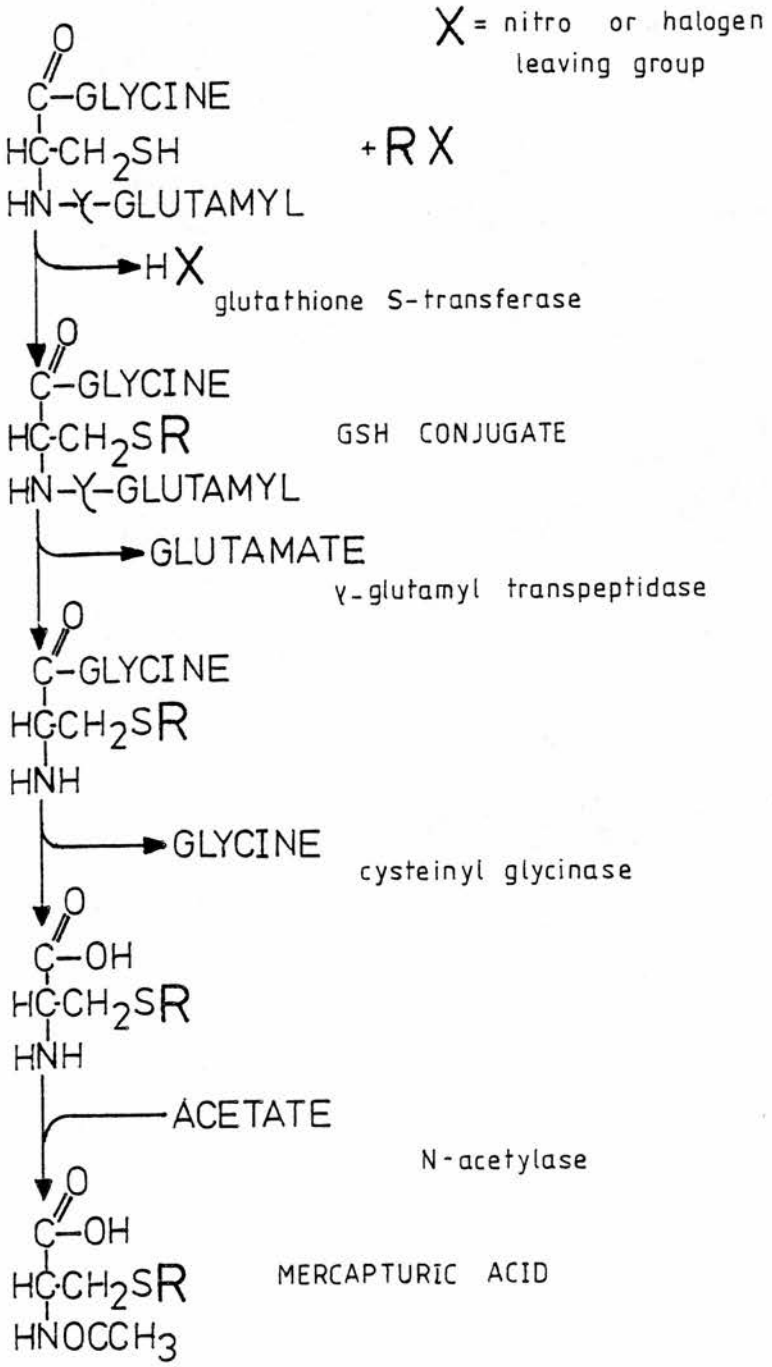
1,2-epoxy-3-(p-nitrophenoxy)propane



benzo(a)pyrene-4,5-oxide

Fig. 1.04.01d

Typical reaction sequence showing the formation of a glutathione conjugate and its further metabolism via the mercapturate pathway.



liver slices and Lay & Menn (1979) found mercapturic acids in fish bile, an indication that perhaps the whole reaction sequence can occur in the liver. Similarly Roubal et al. (1977) showed the presence of napthyl mercapturate in the liver of Coho salmon after intraperitoneal injection of napthalene. An alternative explanation is that mercapturates formed in the gut are reabsorbed and removed from the blood circulation by the liver.

Many electrophilic compounds have been used as substrates for the GSH transferases (Habig et al., 1974; Benson & Talalay, 1976; Keen & Jakoby, 1978), most of these being synthetic halogenated nitrobenzenes. Often the non-enzymic rate of reaction is quite rapid and difficult to distinguish from that catalysed by the transferases. Some substrates are naturally-occurring such as leukotriene A_4 which is converted to leukotrienes C_4 and D_4 by addition of GSH across an epoxide group (Hammarström, 1983). The leukotriene reactions have recently been investigated by Bach et al. (1984) who have shown that the enzyme responsible is a particulate GSH transferase which differs from the form isolated from rat liver by Morgenstern et al. (1979). A number of substrates and reactions are illustrated in Figs. 1.04.01a, b, c and d.

There appear to be several classes of reaction, the first being a substitution whereby labile halogen or nitro leaving groups of aromatic compounds are replaced by thioether linkage with GSH, examples being bromosulphophthalein, 1-chloro-2,4-dinitro- and 1,2-dichloro-4-nitrobenzenes. The reactivity of the electrophilic centre is governed by the electronic and steric effects of neighbouring groups. The second type of reaction involves the addition of GSH across an epoxide group such as that of 1,2-epoxy-3-(p-nitrophenoxy)propane or across an α-β unsaturated ketone site such as that of trans-4-phenyl-3-buten-2-one. A third reaction type is the reduction (Habig et al., 1975) of compounds such as nitroglycerine. In this

reaction nitrite and GSSG are the ultimate products following the reaction of the GSH thiolate ion with an electrophilic nitrogen to form a GSH-adduct which is attacked non- enzymically by another GSH molecule. Two related reactions are the thiolysis reaction with p-nitrophenyl acetate (Keen & Jakoby, 1978) to form p-nitrophenol and a thiol ester and the GSH-dependent ketosteroid isomerase reaction (Benson & Talalay, 1976) in which a Δ-5 ketone group is isomerized to a Δ-4 group without consumption of GSH.

Another catalytic function is the reduction of organic peroxides to hydroxides, in which GSH is oxidized to form GSSG (Lawrence & Burk, 1976; Prohaska & Ganther, 1977). This selenium-independent GSH peroxidase activity differs from that catalysed by the seleno-enzyme GSH peroxidase (Mills, 1957) in that it will only work with organic peroxides and lipid hydroperoxides (Burk et al., 1978).

A more diverse form of catalytic activity has been proposed for GSH transferases isolated from the sap of the rubber tree *Hevea brasiliensis* (Balabaskaran & Muniandy, 1984) which are believed to be identical to the GSH dependent rubber transferase enzymes (Archer & Cockbain, 1969) responsible for the incorporation of isopentenyl-pyrophosphate into the rubber polymer.

Because there are such a wide range of substrates for the transferases and because of the very high intracellular concentration of the enzymes themselves (in the millimolar range), they possess the potential to play a major role in detoxication reactions within the body. But under conditions of GSH depletion, the binding of GSH to the transferases (Sugiyama & Kaplowitz, 1984) may result in less free GSH being available for the catalytic function. Because of their size (M_r greater than 400) the GSH-adducts are preferentially excreted by the biliary system (Milburn, 1976) or further metabolized to mercapturates in the kidney prior

to urinary or faecal excretion. They are more soluble and therefore less likely to be reabsorbed on passage through the gut. This role is however, just one of three major functions in detoxication for this group of enzymes.

1.04.02: The role of the glutathione S-transferases in transport and storage

Interest in the binding capacity of these proteins began when two protein fractions designated Y and Z were discovered in rat liver (Levi et al., 1969; Reyes et al., 1971) which were able to bind bilirubin and BSP. The Y fraction was quantitatively the more important of the two (Kamisaka et al., 1975a) because it was present in higher concentrations. A third fraction X was also found but its binding properties were less specific and believed to be associated with lipoproteins. Meanwhile, Ketterer et al. (1966) and Litwack et al. (1971) were working on proteins binding to cortisol metabolites and azo-dye carcinogens in seemingly unconnected studies. However, once Y fraction had been purified to apparent homogeneity and antibodies raised to it (Fleischner et al., 1972), it became clear that all the groups were working with the same protein(s). Because of its binding capacity for a wide range of ligands such as steroids, thyroid hormones and polyaromatic hydrocarbons it was named ligandin. Ligandin itself was a basic protein of about 45000 daltons mass (Listowsky et al., 1976) comprising two subunits (Ya 22,000 and Yc 25,000, Bass et al., 1977). By this time the GSH transferases were already known to be dimeric proteins (Habig et al., 1974).

The binding of BSP to GSH was then observed to be an enzymic process (Kaplowitz et al., 1973). Assaying the fractions from gel-filtration of rat liver cytosol for ligandin activity and GSH transferase activity using DCNB as substrate showed that ligandin and the GSH S-transferase activity co-eluted, indicating identity.

Purified ligandin and glutathione S-transferase B were

seen to exhibit similar substrate specificities and binding capacities and to have identical amino acid compositions. However, Bass et al. (1977) suggested that the Ya and Yc subunits from ligandin were the subunits from two homodimeric proteins and Carne et al. (1979) fractionated ligandin into proteins comprising YaYa and YaYc subunits. This was in disagreement with Bhargava et al. (1978a) who indicated that ligandin was a heterodimer. Finally, Hayes et al. (1979) isolated two proteins from the Y fraction which bound lithocholate and had GSH transferase activity. Ligandin was shown to be the YaYa homodimer, the other protein was shown to be transferase B, a YaYc heterodimer. In later work (Hayes et al., 1981) the YaYc heterodimer was shown to form from a mixture of ligandin (YaYa) and transferase AA (YcYc) by hybridization *in vitro* in the presence of guanidine HCl.

The term ligandin has been used to encompass the whole family of GSH transferases (Maruyama et al., 1983) as the binding of non-substrate ligands is not restricted to ligandin alone. Binding to organic anions is a property of most but not all of the rat and human transferases (Kamisaka et al., 1975b; Wolkoff, 1980). In particular most attention has been focussed on bile salt binding (Tipping et al., 1976; Hayes et al., 1979, 1980; Vessey & Zakim, 1981). The binding of lithocholate to transferase B was shown by Hayes et al. (1981) to be a property of the Ya subunit and the binding of cholate to be associated with proteins having the Ya and Yc subunits (Hayes et al., 1980). The Ya subunit was shown to possess a high affinity site for lithocholic acid, whilst both Ya and Yc subunits had only a low affinity site for cholic acid. Later work however, has shown that Yb type subunits can bind cholic acid and that many different rat GSH S-transferases can bind bile-salts with differing affinities (Hayes, 1983).

The binding of non-substrate ligands to the transferases has often been studied on the basis of the inhibition of enzymic activity. Boyer et al. (1984) showed

that the extent of the inhibition observed was pH dependent. At lower pH's, inhibition of proteins with Ya and Yc subunits was increased, whilst the YcYc homodimer was not effectively inhibited at either high or low pH's, indicating a dual binding/catalytic activity. Changes in pH are believed to effect conformational changes on enzyme-inhibitor complexes (Vander Jagt et al., 1982), conformers at higher pH's retain catalytic activity whilst those at lower pH's lose activity. The physiological significance of this is that under cholestatic conditions, a drop in intracellular pH may allow cellular damage by electrophiles as GSH transferase activity may be significantly inhibited.

The high affinity shown by certain transferases for organic anions and their high concentration suggests a transport role in the liver, with a binding function similar to that shown by albumin (Wolkoff, 1980). It is believed that they influence hepatic uptake by binding to organic anions to prevent back-diffusion into the plasma. However, Gregus & Klassen (1982) found that biliary secretion of substrate ligands increased differentially over the secretion of non-substrate ligands after induction of ligandin by butylated hydroxyanisole. This suggests that biliary secretion is due more to the catalytic activity of ligandin than to the binding properties of this protein. Furthermore Boyer et al. (1983) demonstrated that GSH transferases did not influence the rate of release of non-polar compounds from membranes, so were unlikely to influence the influx of ligands across the hepatocyte.

The transferases may be involved in the enterohepatic circulation of bile-salts (Strange, 1981). They may prevent endogenous and exogenous anions from partitioning into membranes or sub-cellular organelles, particularly during cholestasis, but as yet no direct physiological significance has been found for bile-salt binding by these proteins. There are also some factors which may have been overlooked

by researchers in this field. It would be interesting to see how the binding properties of these enzymes differ when measured in concentrated solutions (such as those experienced intracellularly) and in dilute phosphate buffers (as used for assaying transferase activity). In addition, within the cell a great number of potential ligands may be present all competing for the available binding sites; as yet no data are available to show how *in vitro* binding differs from *in vivo* binding. As the GSH S-transferases are found in most tissues it is suprising that little attention has been paid to the different properties of enzymes from different tissues (Mannervik, 1984) with respect to bile-salt binding. Clearly organs such as the lung and spleen are not expected to be involved in the enterohepatic circulation and might predictably therefore have GSH transferase forms which do not bind bile-salts if this function is as important as the volume of research suggests. Finally little attention seems to have been paid to the fact that the rat does not have a gall bladder. This affects the flow of bile into the gut (regular rather than feeding-initiated) and therefore alters the enterohepatic circulation.

1.04.03: Covalent binding to highly reactive electrophiles

Highly reactive ligands such as the azo-dyes (Ketterer et al., 1967) often react directly with GSH transferases to form a stable covalent linkage. Many non-carcinogenic compounds are activated by microsomal enzyme systems (Heidelberger, 1975) to form carcinogenic epoxide derivatives. The most potent carcinogens are diol-epoxides (Sims & Grover, 1974) formed from polyaromatic hydrocarbons, which react covalently with nucleic acids to produce malignant transformations (Heidelberger, 1975). Aflatoxin B_1, a secondary metabolite from the food-spoilage mould *Aspergillus flavus* is one of the most potent carcinogens known. It is almost certainly activated to a 2,3-oxide form by microsomal enzymes. This ultimate carcinogen is so highly reactive (Swenson et al., 1975) that attempts to isolate or

synthesize it have proved unsuccessful. A synthetic model (aflatoxin B_1-2,3-dichloride) indicated that the alkylation of nucleic acids was the initial reaction in tumour induction. The sacrificial role that the transferases play may well be important in the prevention of chemical carcinogenesis.

Thus the role of the GSH transferases in detoxication is diverse. They appear to act as storage/transport proteins by binding non-covalently to a wide range of hydrophobic compounds. Compounds which are sufficiently electrophilic react with GSH (itself activated by these proteins) to form soluble GSH-conjugates in a reaction catalysed by the enzymes. The conjugates can then be removed from the body. Really reactive electrophiles react with GSH alone or covalently to the proteins themselves. Their wide tissue distribution and high cytosolic concentration are probably due to the diversity of reaction in which they partake. It has been suggested (Grover, 1982) that their original role was not the detoxication of xenobiotics, simply because of their wide species and tissue distribution. It remains to be seen if these original functions can be elucidated, the discovery of natural substrates may well lead to this. It is unfortunate that so much of the research carried out on these proteins has concentrated on *in vitro* rather than *in vivo* studies. The extent to which these proteins bind, are inhibited and catalyse reactions probably differs considerably in the cell from the observations recorded in spectrophotometer cuvettes. However, to obtain information on the kinetics and mode of action of enzymes, such methods must be used.

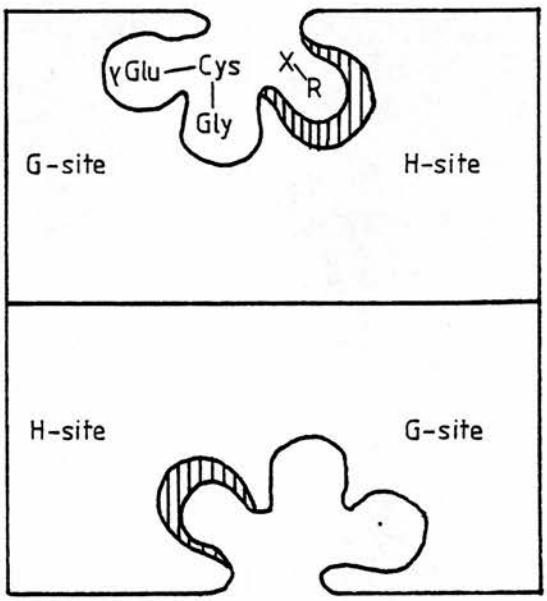
1.05: MECHANISM OF ACTION AND KINETICS

Pabst et al. (1974) described the mechanism by which GSH transferase A catalysed the conjugation of electrophilic compounds with GSH. Below a GSH concentration of 0.1mM, the electrophilic substrate bound first in a ping-pong mechanism. At higher concentrations an ordered Bi-Bi sequence of addition occurred (Cleland, 1963), in agreement with the mechanism proposed for transferase M by Gillham (1973). (An ordered sequence (Cleland, 1963) is a reaction where substrates add on in obligatory order and products leave in a likewise manner, whereas in a ping-pong mechanism one or more products are released before all the reactants have added to the enzyme so that the protein oscillates between two or more stable forms during the reaction.) However, Mannervik & Askelöf (1975) presented evidence that the mechanism for transferase A was not ping-pong and a random steady-state mechanism was suggested (Jakobson et al., 1977; 1979) with GSH binding first. The derivation of various rate equations supports this evidence (Mannervik, 1984). A similar mechanism was suggested for insect larval GSH transferases by Clark et al., (1984) demonstrating the generality of the sequential reaction scheme for the transferases.

Rat liver ligandin possesses a high and a low affinity binding site for bilirubin and Bhargava et al. (1978b) were able to show (using chemical modifications) that catalytic activity and the low affinity binding site were distinct from the high affinity binding site (Vander Jagt et al., 1982). At saturating concentrations of bilirubin, the ligand then competes for the lower affinity non-specific binding site. Binding at the high affinity site was associated with quenching of the intrinsic fluorescence of a tryptophan residue (Ketley et al., 1975). This has been confirmed by Pickett et al. (1984a) who found only one such residue in a

Fig. 1.05

A model for the GSH transferase active sites (Mannervik, 1984).



G-site = GSH binding site

H-site = hydrophobic binding site

sequenced Ya subunit, which was surrounded by hydrophobic amino acids.

Non-substrate ligands can compete with the electrophilic substrate for the low affinity binding site, and if they bind in the absence of GSH a conformer is formed which is catalytically inactive. However, the Yc subunit appears to have dual catalytic/binding activity as complexes that are formed between the enzyme, the non-substrate ligand and the substrate remain catalytically active (Boyer et al., 1984). The formation of inactive conformers with bile-salts appears to be a pH dependent effect as at lower pH's the degree of inhibition is increased, particularly for enzymes with Ya type subunits. It is postulated that this effect is due to a change in the ionization states of the bile-salts (Boyer et al., 1984), reflected in a change in the ratio of inactive:active conformers (Vander Jagt et al., 1982).

Little information is available on the active site of these enzymes. The GSH binding site is known to be highly specific (Pabst et al., 1974) and is located adjacent to the second substrate binding site. A model has been recently proposed for the active site of the GSH transferases (Mannervik, 1984; Fig 1.05) which is based on the fact that the two subunits of the enzyme appear to function independently from one another. This leads to the question of why the GSH S-transferases have two subunits ?; to which there may be several answers. These enzymes are present at millimolar concentrations within the cell, to carry out a function of some kind which requires a high enzyme concentration. By being dimeric proteins, the transferases can supply double the catalytic activity for a set number of protein molecules, a factor which may be important in helping the cell maintain its osmotic pressure. A second possibility concerns the binding function of the

transferases. The rate at which a substance diffuses in dilute solution is proportional to the square root of its relative molecular mass. It follows from this that a GSH transferase dimer with a M_r 47,000 which can bind two ligand molecules is a slightly more effective transporting agent than a monomer of M_r 25,000 which can only bind half as many ligand molecules. Of course, this assumes that diffusion in the cytosol follows the same law.

As more sequencing of the GSH S-transferases is completed and the shape and positions of binding sites become apparent, then the whole mode and mechanism of reactions for this group of enzymes will become clearer. Until then, the postulated mechanisms for catalytic and binding activity will remain unproven.

The above work all refers to the enzymes from rat liver. This is presumably because rodents have a very efficient detoxication system and are easily obtained.

1.06: GLUTATHIONE S-TRANSFERASES IN RATS

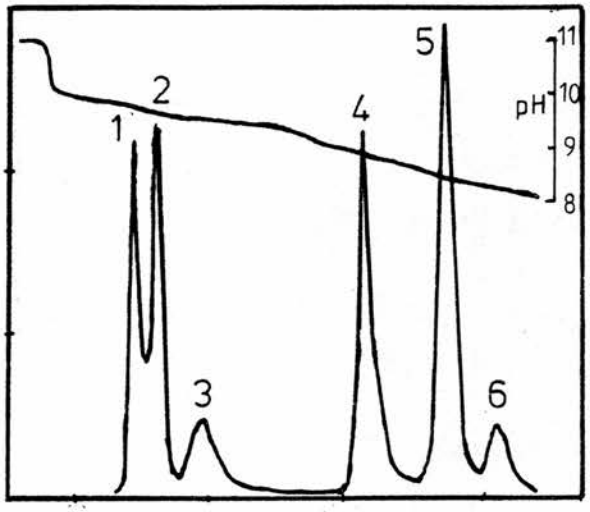
As well as the basic GSH transferases (E, D, C, B, A and AA) separated by Habig et al. (1974, 1976) which did not bind to DEAE cellulose at pH 7.2 (Scully & Mantle, 1980), a more acidic protein was discovered called transferase M (Gilham, 1973) because of its activity with 1-menaphthyl sulphate.

The transferases were shown to comprise two out of three subunits called Ya, Yb and Yc, with M_rs of 22,000, 23,500 and 25,000 respectively (Bass et al., 1977; Hayes et al., 1979). The binding protein ligandin was thought to have identity with GSH transferase B (Listowsky et al., 1976) and to comprise Ya and Yc subunits. Work by Bass et al. (1977) and Hayes et al. (1979) however, showed that ligandin contained only the Ya subunit, that transferase B was a YaYc heterodimer and that these subunits showed immunological similarities. It was suggested (Bass et al.,

Table 1.06

Chromatofocusing and nomenclature of rat liver glutathione S-transferases

(Mannervik and Jensson, 1982)



Peak	New Name	Old Name	Subunits
1	L_2	Ligandin	Ya Ya
2	BL	B	Ya Yc
3	B_2	AA	Yc Yc
4	A_2	A	Yb_1Yb_1
5	AC	C	Yb_1Yb_2
6	C	D*	Yb_2Yb_2

(* Hayes 1983)

1977; Hayes et al., 1979; Sculley & Mantle, 1980; Sculley & Mantle, 1981) that the multiple forms of subunit may be products of the proteolytic cleavage of the YcYc homodimer (transferase AA). This followed reports of interconversion of the proteins on storage (Listowsky et al., 1976; Daniel et al., 1977). However, this theory of post-translational modification was later abandoned following investigations using peptide mapping and immunotitration (Hayes & Clarkson, 1982) and cloning techniques (Pickett et al., 1982).

A new nomenclature system was introduced by Mannervik & Jensson (1982) after purification of the basic rat enzymes using S-hexylGSH affinity chromatography (Mannervik & Guthenberg, 1981) and chromatofocusing (Sluyterman & Elgersma, 1978; Sluyterman & Wijdenes, 1978). The combination of these techniques separated the basic enzymes from rat liver into at least six fractions (Jensson et al., 1982). The peaks were divided into two groups of three proteins, the first three to be eluted from the column comprising Ya and Yc subunits and reacting to antibodies raised against transferase B (YaYc). The second group which reacted with antibodies raised to transferase C (YbYb) contained two types of Yb subunit (Yb_1 and Yb_2). These could be distinguished on the basis of substrate specificities and differential inhibition by a number of inhibitors (Jensson et al., 1982; Yalcin et al., 1983). Transferase C (Yb_1Yb_2) was shown to hybridize *in vitro* from transferase D and transferase A (Yb_2Yb_2 and Yb_1Yb_1 respectively) by Hayes (1981) in the presence of guanidine HCl.

A summary of the new nomenclature and a comparison with the old nomenclature can be seen in Table 1.06.

In addition to the basic transferases mentioned above, a number of neutral ones have also been discovered. The neutral transferases from rat liver were separated from the basic forms on DEAE cellulose pH 7.6 (Hayes & Chalmers, 1983) and were subsequently purified to forms Q, R, S, T and

U. Transferase S contained a new subunit Yn, of slightly faster electrophoretic mobility than the Yb type subunits (M_r 23,500) of Q, R, T and U (transferases Q and D appear to be identical).

An additional GSH transferase is transferase X (Freidberg et al., 1983; Frey et al., 1983) which has a relatively high activity with DCNB and potentially mutagenic polyaromatic hydrocarbons. It reacts with antisera raised to transferases A and C and is claimed to be distinct from GSH transferase D because its pI (obtained using isoelectric focusing) differs from the pI obtained using chromatofocusing. This point is debatable because the pH at which a protein is eluted from a chromatofocusing column does not neccessarliy coincide with the protein's pI (Sluyterman & Elgersma, 1978). However the difference in substrate specificities between transferases X and D does indicate a distinction. As both proteins are Yb_2 homodimers, the total number of Yb type subunits remains a question. Recent work by Hayes (1984, personal communication) suggests as many as five Yb type subunits which further complicates the issue.

The confusion in the nomenclature becomes even more apparent when another transferase X is considered (Hales et al., 1978; Sculley & Mantle, 1981). This protein is a Ya homodimer which Hayes & Clarkson (1982) called transferase YaYa. However, Sheehan and Mantle (1984) have isolated two kinetically distinct forms of YaYa protein which suggests the presence of more than one type of Ya subunit and agrees with cloning data where sequence homology for cloned Ya subunits is extensive but incomplete. In addition, sequencing of the Ya subunit (Pickett et al., 1984b) has shown that the M_r of the Ya subunit is really 25,547 rather than 22,000. The M_r's of the Yb type (23,500) and Yc type (25,000) subunits have also recently been estimated at approximately 26,500 and 28,000 respectively using sequencing data (Mannervik, 1984). This means that the

whole series of M_r's obtained from polyacrylamide gel electrophoresis will have to be re-evaluated. Because of the confusion over nomenclature, it was suggested at a recent meeting of the Biochemical Society (Dundee, 1983) that the GSH S- transferases should be identified by the name of the organism from which they were isolated, and that each subunit type be assigned a number, leading to the identification of each enzyme by a species name and two numbers. Whether or not this nomenclature is accepted remains to be seen.

As far as the tissue distribution of the rat GSH transferases is concerned, these enzymes have been isolated and purified in many tissues (Hales et al., 1979; Sculley & Mantle, 1981; Dierickx & De Beer, 1981; Kraus, 1983; Guthenberg et al., 1983) and show many similarities to the hepatic forms as well as some differences. So far though, little effort has been made to correlate the proposed functions of these enzymes in the different tissues with their observed catalytic properties.

The great number of different subunits found, for instance, in rat liver cytosol poses questions as to the function of the GSH transferases in detoxication. To remove xenobiotics, a complex organism has the choice of a blanket approach (in which it produces a large number of relatively non-specific proteins to deal with the threat) or it uses an approach akin to the immune response (where proteins are produced to deal with specific threats). Although the GSH transferases are induced by treatment with certain compounds, the enzymes themselves are not sufficiently specific for the latter approach. Microsomal cytochrome P_{450} enzymes approximate more closely to this approach, as there is a multiplicity of more specific proteins produced to deal with potential substrates. The induction of the rat GSH transferases will be discussed in greater detail in Section 1.08.

1.07: MICROSOMAL GLUTATHIONE S-TRANSFERASES

The majority of the substrates for GSH transferases can be activated to potentially harmful forms by the microsomal cytochrome P_{450} and hydroxylase enzyme systems. The non-polar electrophilic substrates partition readily into the lipid phase of membranes, the rate-limiting step for their conjugation being their release from the membranes (Boyer et al., 1983). Consequently any transferase activity associated with the membrane fraction is in an ideal position to deal with xenobiotics.

Morgenstern et al. (1979) demonstrated the presence of transferase activity in rat liver microsomes which was stimulated eight-fold by N-ethylmaleimide. This indicated a sulphydryl group in the proximity of the active site. Unlike the cytosolic GSH transferases, the activity was not induced by phenobarbitol, methylcholanthrene and trans-stilbene oxide (Morgenstern et al., 1980). The enzyme(s) had a lower activity, but a wider spectrum of activity than the cytosolic forms according to Krauss & Gross (1979), whereas Morgenstern et al. (1982) found relatively high activities with certain substrates. Sub-cellular fractionation showed that the enzymic activity was associated with the endoplasmic reticulum (Morgenstern et al., 1980) but appeared partially exposed to the cytoplasmic side, positionally therefore in the mainstream of drug metabolism. Initial purification of the enzyme revealed one band on SDS-PAGE (M_r 15,000) which appeared immunologically distinct from GSH transferases A, B and C (Morgenstern et al., 1982).

The activation of the enzyme by N-ethylmaleimide (NEM) was shown to be due to the binding of one molecule of NEM to one cysteine residue on each polypeptide chain of the enzyme. The enzyme is freed from the membrane by solubilization in Triton X-100, with which it forms a micelle

containing 3-4 polypeptide chains (Morgenstern & Depierre, 1983).

In addition to the microsomal form of the enzyme, cytosolic forms are also observed to associate with the endoplasmic reticulum at levels ten-times greater than those predicted for non-specific binding (Morgenstern et al.,(1983). This is in agreement with Lee (1984) who found murine microsomal species had immunological identity with cytosolic forms of the enzyme and were probably the result of specific and non- specific binding to the microsomal membranes.

A distinct particulate GSH transferase has been found by Bach et al. (1984), which is involved in leukotriene interconversion; it is not activated by NEM, nor does it possess GSH S-transferase activity when in a solubilized form.

Is is possible therefore that several forms of microsomal transferase exist along with membrane associated cytoplasmic forms. If the true function of the transferases is detoxication, then the microsomal forms are ideally situated to deal with activated metabolites from other microsomal systems and to prevent lipid peroxidation of membranes (Guthenberg et al., 1983). If this is the case it is suprising that so much of the GSH transferase activity is found in the cytosol, especially if some of the microsomal forms are specific for endogenous substrates (Bach et al., 1984). It is also suprising that the microsomal forms of the enzymes are not induced by compounds that induce other drug metabolizing systems.

1.08: THE INDUCIBILITY OF THE RAT ENZYMES

The fraction known as Y (ligandin) is induced by phenobarbitone (Reyes et al., 1971; Fleischner et al., 1972; Hales & Neims, 1977). Other transferases are induced by butylated hydroxyanisoles and certain polycyclic hydrocarbons (Kaplowitz et al., 1975; Benson et al., 1978) although, earlier Grover & Sims (1964) showed decreased levels in vertebrate species pretreated with phenobarbitone and benzo[a]pyrene. The specificity of induction was investigated by Hayes et al. (1979) who showed induction of the Ya subunit and by Bass et al. (1977) who showed induction of both Ya and Yb subunits. Hayes (1979) also showed differential induction of transferases A and B by phenobarbitone, which indicated a difference in the control of their expression.

As the purification schemes for the transferases became more effective and the different Yb type subunits were separated, so the studies on inducibility were able to become more specific. Di Simplicio et al., (1983) showed the selective induction of GSH transferases containing the Ya and Yb_1 subunits by trans-stilbene oxide. However in rat testis the Yb_2 subunit was induced by phenobarbital (Sheehan et al., 1984), indicating a possible functional difference between the subunits in the different tissues. The rat microsomal transferase (see Section 1.07) however, is not induced by the same agents which induce the cytosolic forms.

On a molecular level, differential mRNA levels for the Ya and Yc subunits following induction by phenobarbital have been observed (Pickett et al., 1982; Kalinyak & Taylor , 1982). This indicates the presence of a different regulatory system for the two subunits as the Ya levels increased markedly, whilst the Yc levels only increased very slightly or not at

all (Daniel et al., 1983) despite extensive sequence homology (Tu et al., 1982) with the Ya type. Such findings must be treated with caution as increased mRNA levels could simply mean a decrease in the rate of mRNA breakdown rather than an increased synthesis. The mRNA's for the Ya and Yb type subunits show little sequence homology (Pickett et al., 1984a; 1984b) indicating that the two groups are the products of separate genes.

Thus although much is now known about the GSH transferases in the rat the overall function of these enzymes is still not clear. The human GSH transferases are even less well characterized, due to the difficulty in obtaining biological material and carrying out experiments on humans.

1.09: HUMAN GLUTATHIONE S-TRANSFERASES

The basic GSH transferases from human liver were first purified and characterized by Kamisaka et al. (1975b). They were named α, β, γ, δ and ε on the basis of increasing pI (7.8 - 8.9) and were found to have very similar amino acid compositions, immunological properties and substrate specificities. On isoelectric focusing gels α, β, and ε appeared as single bands, γ consisted of two bands and δ was made up of three bands, all of which were catalytically active. Differences in amide nitrogen content led to the belief that these proteins were all charge isomers produced by deamidation.

An acidic form ρ was purified from erythrocytes (Marcus et al., 1978) and shown to be distinct from the liver forms. This was not expected, for if deamidation (a protein ageing process) were producing the different basic forms in the liver, cells such as erythrocytes which don't synthesize proteins would be expected to show a greater

degree of protein deamidation. The erythrocyte form showed the acidic pI predicted for extensive deamidation but had a different amino acid composition, was catalytically and immunologically distinct and was therefore probably the product of a distinct gene. The acidic form π from the placenta (Guthenberg et al., 1979) appears to be very closely related or identical to ϱ and has epoxide conjugation activity (Pacifici & Rane, 1981). In addition to the acidic and basic forms, a neutral form μ has also been discovered (Warholm et al., 1980) which will be mentioned in more detail later.

All the neutral/acidic and basic forms are composed of subunit(s) with a M_r of 22,000 except neutral/acidic protein two (Hayes et al., 1983) which has subunit(s) with a M_r of 23,500. Because the neutral and basic forms are immunologically and catalytically distinct it follows that there are likely to be at least two forms of the M_r 22,000 subunit (Hayes et al., 1983).

Acidic GSH transferases Ω and ψ from human liver have also been isolated (Awasthi et al., 1980) and shown to have different amino acid compositions to the basic forms but similar antigenic cross-reactivity. Slight functional differences can be seen between the two groups as the basic forms bind bilirubin but not cholate, whilst these functions are reversed for the acidic forms (Pattinson, 1981). The acidic forms from kidney, spleen, lung and placenta are all closely related (Koskelo, 1983) and are reported to have similar functions. However, in tissues such as these, the relevance of cholate binding seems doubtful.

A particularly interesting transferase is the neutral form μ (Warholm et al., 1980; Guthenberg & Mannervik, 1981), found to be absent in certain individuals. The substrate specificty of this enzyme differs from the other forms as it is particularly active with trans-4-phenyl-3- buten-2-one and benzo[a]pyrene-4,5-oxide (Warholm et al., 1981; 1983).

The activity with the latter substrate indicates its potential importance in the prevention of chemical carcinogenesis and its absence in certain individuals may increase the risk of cancer to such people. A highly sensitive radio-immunoassay for the neutral/acidic enzymes has been developed by Hayes et al. (1983) as a measure of hepatocellular integrity and it is hoped that a similar assay specific for μ can be developed (J. D. Hayes, personal communication).

Thus three groups of transferases have been found so far in human tissues, the basics, the neutrals and the acidics (although the neutral and acidic forms are sometimes classed as one group). The relationships between and within groups is still unclear. The differences and similarities of the basic forms were believed to be due to deamidation (Kamisaka et al, 1975b) or to genetic differences (Marcus et al., 1978; Guthenberg & Mannervik, 1981). Because the basic forms cross-react with antisera raised against the neutral and acidic forms yet have different amino acid compositions, post-translational modification has also been suggested (Awasthi et al., 1980). Guthenberg and Mannervik (1981) however, believe the two forms to be immunologically and genetically distinct.

The genetic relationship and control of the transferases has been investigated (Board, 1980) using starch-gel electrophoresis (Parr et al., 1977). The variation between individuals was considerable (Board, 1981), expressed by three alternative types of staining component. The patterns obtained were characteristic of a heterozygous polymorphic dimeric protein, the components possibly being the products of different loci called GST 1, 2 and 3. The results showed that if individuals with a null phenotype were included as homozygous for the null allele, the observed and expected frequencies for a population achieved Hardy-Weinberg equilibrium (which assumes that in a random breeding population, two alleles a and b will appear in

frequencies a^2, b^2 plus heterozygotes 2ab and remain constant throughout generations). A null allele was demonstrated at the GST 1 locus (probably transferase μ) which might have been the result of gene deletion (Board, 1981). The products of the loci were autosomal and only one form was seen at GST 3 (the erythrocyte type ϱ). Later work (Strange et al., 1984) agrees with these findings after looking at the differences in a wide range of human tissues. What must be remembered though, is that all the human genetic work is carried out after autopsies and that the material used is only from one generation, so the necessary control experiments of back-crosses cannot be carried out to substantiate the evidence.

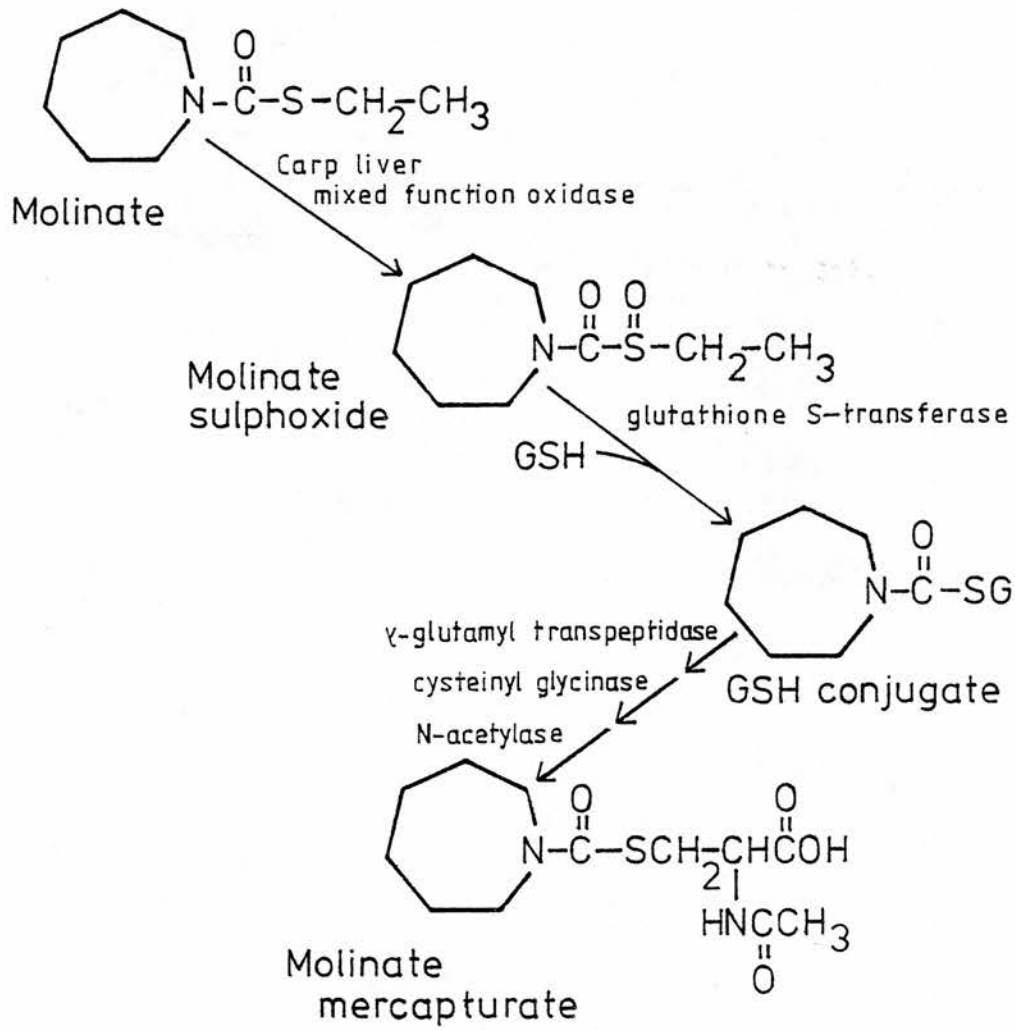
The situation still remains unclear, post-translational modification, deamidation or even mixed-disulphide formation (Ramage & Nimmo, 1983) having still to be disproved. In view of the recent progress in the rat GSH transferase field, the best way to resolve the human problem maybe via the molecular biology approach. The analysis of coding sequencies and differential induction of mRNA's would be able to produce many of the missing answers as regards the genetic control of the enzymes and their genetic relationship, provided the necessary experiments could be carried out.

In other species, little very detailed work on the GSH transferases has been carried out. The GSH transferases in fish will be dealt with in the following Section (1.10) and a brief reveiw of the research carried out on species other than fish follows in Section 1.11.

Fig 1.10

The metabolism of the herbicide molinate by the carp

Lay and Menn (1979)



1.10: GLUTATHIONE S-TRANSFERASES IN FISH

In 1964, Grover and Sims showed the presence of GSH S- transferase activity towards DCNB in goldfish liver cytosol, although this was much lower than that detected in amphibians and mammals. Prior to this, Haywood et al. (1945) reported dye secretion and concentration in the bile of several salmonid species.

Levine et al.,(1971) in a phylogenetic study of BSP binding and uptake failed to demonstrate the presence of ligandin-like proteins in the livers of various elasmobranchs and teleosts. However, selective hepatic uptake and biliary excretion were observed in a study conducted by Boyer et al. (1976) with two elasmobranchs *Squalus acanthias* and *Raja erinacea*. GSH S-transferase activity towards DCNB in these species and several others was shown (Bend & Fouts, 1973). The cytosolic GSH S-transferase activity in several organs of these species was seen to extend to epoxide substrates (James et al., 1979). The fact that elasmobranch fish had GSH S-transferase activity and were able to take up organic anions yet had no ligandin-like proteins led Sugiyama et al. (1981) to carry out a detailed investigation of GSH S-transferase activity in the thorny-backed shark (*Platyrhinoides triseriata*). Enzymic activities were lower than in the rat but binding activity was found, indicating the presence of a "ligandin" in that particular species.

In 1979, a herbicide mercapturate was detected in carp bile (Lay & Menn) following the cleavage of molinate sulphoxide in liver cytosol (see Fig 1.10). Similarly, Roubal et al. (1977) demonstrated the presence of naphthalene mercapturates in liver, flesh and the gall-bladder of young Coho salmon (*Oncorhynchus kitsutch*) exposed to napthalene. The discovery of mercapturates in the bile is surprising as the

enzymes for cleavage and acetylation of the GSH-conjugate are present in greater amounts in the kidney and gut than in the liver (Bray et al., 1959b). If the urine could have been collected, a greater proportion of mercapturates might have been found. An alternative is that kidney and gut metabolites of a GSH conjugate (formed in the liver and secreted in the bile) were reabsorbed in the gut and then removed from the portal blood by the liver prior to release into the bile.

In 1979, Nimmo et al. found substantial transferase activity in several trout tissues, but could detect no lithocholic acid binding activity by the transferases. Gregus et al. (1983) also investigated the trout and showed the conjugation of several substrates with crude tissue extracts. GSH S-transferase activity towards CDNB with a specific activity several times higher than that observed in rat liver was found in the Northern pike (*Esox lucius*) by Balk et al. (1980). However, despite being a rather stationary inhabitant of some of the more polluted European waters, activity towards other substrates was not detected.

As yet, there is little evidence for the induction of transferase activity in fish (Sherwood & Mearns, 1977), although there is plenty of evidence for the induction of other drug metabolising systems (Payne, 1976; Bend et al., 1977; Malins, 1977; Hawkes, 1980; Casterline et al., 1983). Phenobarbitone, whilst being an excellent inducer of rat GSH transferases is ineffective with piscean forms (Overstreet & Howse, 1977), but phenylbutazone and phenacitin are known to induce cytochrome P_{450} systems in fish. Some systems, notably the benzo[a]pyrene hydroxlases, are more inducible in fish than in the rat (Chambers & Yarbrough, 1976).

Sloof et al. (1983) noticed an increase in somatic liver index (the ratio of liver mass to body mass) in fish from polluted waters, accompanied by elevated enzymic activities. Similar results were obtained by exposing fish to municipal

waste-water (Förlin & Hansson, 1982). Chlorinated nitrobenzene residues have been detected in fish tissues (Yurawecz & Puma, 1983), some of which are known GSH S-transferase substrates. It remains to be seen whether or not GSH transferases can be induced in fish exposed to environmental pollutants. The sensitivity of salmonids is already being used to detect pollution by using trout fitted with electrodes (Huve, 1982). Increased neurological activity is observed in response to very low concentrations of pesticides (a response was shown 0.5s after the addition of 10ng/l lindane to the water).

It is well known that members of the *Salmonidae* are very susceptable to environmental pollutants and they clearly react to the presence of these in the water. Fish possess GSH S-transferase activity, but there is no evidence for this activity's being inducible. However, other drug metabolising systems are inducible, which casts doubts as to the role of the transferases in the detoxication of xenobiotics.

The aim of the current study was to investigate the GSH transferases in the salmonids because so little detailed work has been carried out on this enzyme system in fish. Our aims were to compare the system with similar systems in other species and to assess its viabilty as a potential detoxication system. It was also of interest to observe how the proteins in cold-blooded species differed from their counterparts in warm-blooded species. It was hoped that an approach from the comparative point of view might give some clues as to the function of these proteins, particularly as aquatic organisms experience different excretory problems to terrestrial organisms.

1.11: GLUTATHIONE S-TRANSFERASES IN OTHER SPECIES

The GSH transferases have been studied in numerous other vertebrate species, the most detailed work being concerned with murine transferases (Lee et al, 1981; Stockstill & Dauterman, 1982; Lee, 1984). Quite considerable variations of forms and properties were seen in various tissues of strains of inbred mice. In contrast to the rat GSH transferases, all the murine forms are homodimeric proteins and have differing substrate specificities. Hamsters (Smith et al., 1980), guinea-pigs (Di Ilio et al., 1982), sheep (Reddy et al., 1983), cows (Saneto et al., 1982), chickens (Yeung & Gidari, 1980) and pigeons (Wit & Leewangh, 1969) have also been investigated.

As far as invertebrate species go, the most detailed studies have been carried out on insect larvae (Clark & Drake, 1984; Clark et al., 1984) and earthworms (Stenersen et al., 1979; Stenersen & Øien, 1981). Studies have also been carried out with microorganisms such as *Trypanasoma cruzi* (Yawetz & Agosin, 1981) and *Escherishia coli* (Shishido, 1981).

The GSH transferases have also been found in plant species such as corn (Tiermeier & Jaworski, 1983) and in the rubber tree (*Hevea brasiliensis*) (Balabaskaran & Muniandy, 1984).

The overall conclusion is that a large number of transferases have been found in many different species and these show some variation in substrate specificity. Apart from distinct microsomal forms, the GSH transferases work particularly well with CDNB as substrate. Most of the GSH transferases purified seem to be dimeric proteins with M_r's in the region of 30,000 - 50,000 and have pI's varying from acidic to basic. Most of the species investigated in any

detail show several different forms of GSH transferase, which is an indication of their importance, whatever their function might be. The diverse distribution of these proteins throughout the phylogenetic tree may mean that they have functions other than those of detoxication. The activity of the transferases towards artificial substrates is higher than towards naturally-occurring substrates which indicates that the real substrates have still to be discovered.

Because the GSH S-transferases are probably involved in detoxication, because they are found in high concentrations in liver cytosol and because the liver is a prime region of detoxication in the body, a little must now be said on the role of this organ in detoxication and related bodily function.

1.12: SOME ASPECTS OF LIVER AND BILIARY FUNCTION

The liver provides an excretory and digestive function by the formation of bile. The blood supply to this organ is a dual one, composed of oxygen-depleted blood from the hepatic portal vein, which has passed through the capillary bed of the alimentary tract and is therefore rich in nutrients and other absorbed substances, and oxygenated blood from the hepatic artery. The end-organs of hepatic circulation are the liver sinusoids which form the vast capillary network responsible for the exchange of substances between flowing blood and the epithelial cells lining the sinusoids. Behind these lie the hepatocytes responsible for most liver functions; they are divided by numerous bile-canaliculi, leading ultimately towards the gall-bladder and excretory processes.

Compounds absorbed in the gastro-intestinal tract pass through the liver before entering the general circulation,

and a high proportion of these are removed from the circulation in a single pass through the liver (known as the "First-Pass" effect, Klaassen & Watkins, 1984). Compounds that are removed in the sinusoids must pass through spaces in the sinusoidal epithelium (fenestrations, which are impermeable to erythrocytes) and enter the Space of Disse, to be taken up by the hepatocytes by carrier mediated systems (non-polar compounds can pass through the membranes by diffusion alone). Organic anions are then metabolised or conjugated, usually with glucuronic acid or GSH. The GSH transferase activity is not uniformly distributed in the liver (Redick et al., 1982), but is associated more with the cytochrome P_{450}-rich regions of the centrilobular parenchymal cells, thus being ideally situated to deal with secondary metabolites.

One of the most important substances removed is bilirubin, a primary haem degradation product from senescent erythrocytes which is produced in phagocytes; it is toxic and can lead to neurological dysfunction. Bilirubin is released into the circulation, reversibly bound by albumin and removed from the circulation by the liver. The rate of bilirubin uptake may be dependent on binding to GSH transferases within the hepatocyte, preventing back-diffusion into the plasma (Wolkoff, 1980). Unconjugated bilirubin is non-polar so it diffuses rapidly across biological membranes and therefore cannot be excreted effectively due to reabsorption in the gut lumen. In the course of intrahepatic circulation, bilirubin is conjugated (mainly with glucuronic acid) to form more polar derivatives which are non-toxic and readily excreted via the bile and removed through the gastro-intestinal tract. In cases of intestinal stasis, the reabsorption of conjugated bilirubin in the gall-bladder and intestine can occur leading to increased formation of unconjugated bilirubin and hence toxicity. In non-mammalian vertebrates the major bile pigment is unconjugated biliverdin which is readily excreted. This leads to the question of why in mammals biliverdin is further

metabolized to the toxic non-polar bilirubin.

The bile-salts are an important group of organic anions which are recycled by the liver through the enterohepatic circulation. They are removed from the bloodstream by a saturable energy- and Na-dependent system (Klaassen & Watkins, 1984). Under conditions of cholestasis, the biliary removal of these substances is slowed and they accumulate in the liver and circulatory system, leading to tissue damage. The rate-limiting step of their removal is active-excretion across the bile-canaliculus membrane into the bile. The gall-bladder then concentrates the bile five- to ten-fold, contracting due to hormonal stimuli. The function of the gall-bladder is to store and concentrate the bile on fasting, then to coordinate the release of bile on feeding. In "nibblers" such as the rat, the constant influx of food would mean constant gall-bladder contractions; probably as a consequence of this the rat does not have a gall-bladder. The concentration of bile-salts in bile of the rat is in the millimolar range, whilst in the bloodstream the concentration is in the micromolar range (although the concentration in hepatic portal blood sometimes approaches millimolar). Reabsorption of the bile-salts in the gastro-intestinal tract depends on their states of ionization. Ionized forms depend on sodium-coupled active transport systems whilst others are taken up passively; they are then transported back to the liver bound to albumin, HDL and LDL.

The role of the GSH transferases may be to prevent diffusion of organic anions back into the plasma, but as yet there is no conclusive evidence for their involvement. Organic anion binding studies are usually carried out in dilute solution with purified binding proteins. The results fail to take into account the effects of other ligands present in the hepatocyte. Although binding constants for a number of ligands can be obtained, the system used is strictly artificial.

The physiology above applies to many species, but in the case of the salmonid fish, there are other seasonally dependent factors which must be taken into account.

1.13: CERTAIN ASPECTS OF THE PHYSIOLOGY OF SALMONIDS

The *Salmonidae* are poikilothermic teleosts which normally inhabit the cleaner fast-flowing freshwater rivers of the world. However, *Salmo salar* the Atlantic salmon and other salmon species are anadromous, migrating from the sea to fresh-water to spawn and in the process negotiating natural obstacles in their long journey (their name is derived from the French verb *salire*, meaning to jump).

Because they are poikilothermic, their behaviour is directly affected by changes in water temperature, with the time taken for food to pass through the gut being temperature-dependent (Sedgwick, 1982). At low temperatures the somatic liver index is increased (Egaas & Varanasi, 1982; Sloof et al., 1982) along with various enzymic activities including GSH transferase. These changes are probably connected with steroidogenesis and hormonal alterations (Koivusaari et al., 1981; Kime & Manning, 1982). However, the maxima of GSH transferase activity did not coincide with the maxima of activity for the other drug-metabolizing enzymes. The cold water-temperatures of the winter mean that fish are likely to be semi-starved and may show other metabolic differences. Fasting reduces hepatic GSH levels in the rat (Lauterburg et al., 1982) by as much as 30% and changes in the levels of GSH in the Baltic salmon have been shown to parallel changes in water temperature (Härdig & Höglund, 1983).

An important factor in salmonid physiology is the change that is undergone when these fish prepare to migrate upstream to spawn. There is a complete biochemical

re-organization of the tissues (Hoar, 1957), most pronounced in salmon species. These fish return to the rivers to spawn after a winter (grilse) or more (salmon) in the sea; the term grilse coming from the Norse term *gralax* meaning grey salmon or spring fish. These migrating fish store fat in their muscle tissue which decreases as the gonads develop. Plasma cortisol levels increase in the migrating salmon (Love, 1970; 1982), which may cease to feed as much as eight weeks before entering the river. The cock fish undergoes a greater degree of starvation than the hen, by ceasing to feed earlier. This factor which may explain the greater percentage mortality in the male spawning population.

Associated with increased plasma cortisol levels and starvation are physical changes in the gut which possibly becomes occluded and will not accept food. The stomach contents of migrating salmon change visibly as the fish move further upstream; river mouth fish may contain some recognisable food (mostly marine in origin) whereas further upstream the stomachs are completely empty (Sedgwick, 1982). This leads to one of the mysteries surrounding the behaviour of these fish, which is how they are caught on baited lines and lures when they will not accept food; the answer is still unknown.

Once salmon cease to feed, degeneration of the gastro-intestinal tract occurs which is likely to be associated with high levels of corticosteroids. The intestine and pyloric caeca all degenerate and microvilli become shortened and reduced in number. There is also an 85% loss of fat in the journey from the sea to the spawning grounds, which is replaced by water, causing an alteration in the muscle texture of the fish. Liver glycogen falls from 2% w/v to 0.2% w/v in the first week (Love, 1982), leading to the mobilization of the amino acid pool (as free amino acid levels do not appear to change) and of body lipids for energy.

The coloration of the flesh of salmon is due to carotenoids stored in muscle; as the fish matures and fat is removed to the gonads the carotenoids become more apparent (Love, 1982) and cause the red appearance of the skin later in the season. These pigments are also in active movement during the developement of the gonads, entering the egg to give colour to the developing larvae and to stimulate chemotaxis of the spermatazoa. Other changes include a mobilization of calcium causing the humped head in some species and an increased retention of the bile salt cholate (Love, 1982). The main constituents of salmon bile are cholate and chenodeoxycholate which are conjugated mainly with taurine (Denton & Yousef, 1974). So extreme are these changes that in certain species of salmon (*Oncorhyncus*) death occurs soon after spawning and cannot be prevented by any hormonal treatments.

As well as hormonally-induced changes, other indirect effects are noticed in these fish. During periods of starvation the enterohepatic circulation of bile-salts is affected. The gall-bladders of feeding fish are virtually empty (Talbot & Higgins, 1982); bile-salts flow directly from the liver to the hind-gut without storage and concentration in the gall-bladder. The appearance of the bile also changes, being pale-amber in fed fish, but darker in starved fish; a change possibly due to the oxidation of bilirubin glucuronide to biliverdin glucuronide (Love, 1982). It is likely that differences in the enterohepatic circulation occur between wild and farmed fish. This is because contraction of the gall-bladder is triggered by hormonal stimuli from the feeding response. Farmed fish have regular feeding times, hence gall-bladder contractions will occur in the same regular manner as would occur in humans, but in wild fish (particularly in the summer when the warm water temperature is not affecting the feeding response), feeding is more random, approximating closely to the "nibbling" behaviour of rats. As a result bile trickles continuously from the gall-bladder, which appears empty.

So in this group of fish, massive physiological changes occur as fish mature and spawn, coupled to the differences associated with feeding behaviour. These effects must be considered in any work involving the biochemistry of the salmonids. In addition to these, there are also genetic variations within and between species. Species of fish (salmon and trout) from different rivers of the same country show variations in the electrophoretic behaviour of their proteins (Sedgewick, 1982). These include serum proteins, haemoglobins, erythrocyte antigens and cytosolic proteins (Wilkins, 1972) and considerable differences between different salmonids have been observed (Love, 1970; Laird et al., 1982). Sex differences are not easily determined before dissection, so unless a limitless supply of material is available, work sometimes has to be carried out with a mixture of material.

As far as genetic differences are concerned, material from wild populations used in this work was obtained from the same river to minimize genetic variation. The origins of commercially-obtained materials were carefully recorded to try to distinguish between "real" biochemical differences and genetic variation. Unfortunately, the scale of salmon farming means that the progeny in any one set of pens are the products of many parents and therefore pure-bred fish are very difficult to obtain, which is clearly very different to the situation which occurs with laboratory animals.

In the current study, our initial source of rainbow-trout (Penicuik Trout Farm) closed for economic reasons. As a result we had to change to another source (Beecraigs Country Park) and cope with any resultant variation. Salt-water salmon were obtained approximately twice a year from our source (Marine Harvest Ltd.) and seasonal differences had to be allowed for. In addition, when differences were observed, it proved difficult to obtain fish of the same age and from the same salmon-pens due to the constant turnover of stock. Seasonal differences also had

to be accounted for, as changes in hormonal levels and nutritional status may have influenced the results obtained. Fresh-water salmon were difficult to obtain and as a result one could not afford to be choosy (ie: rejecting livers because of unusual appearance etc.) in using such material.

In summary there are a number of factors which have had to be allowed for in this work. The only true solution is to work with inbred lines of fish or to obtain enormous ammounts of material. As neither of these was feasible, numerous control experiments had to be carried out to try and quantify differences due to seasonal and genetic variation.

SECTION 2

MATERIALS

SECTION 2: MATERIALS

All common reagents were supplied by either Sigma Chemical Co., Poole, Dorset, U.K. or BDH Chemicals Ltd., Poole, Dorset, U.K.

2.00: Dialysis

Visking tubing was supplied by the Scottish Scientific Instrument Centre, Edinburgh, U.K.

2.01: Cytosol preparation and column chromatography

Sodium azide, ethanolamine, Triton X-100, sodium borohydride, hydroiodic acid and 2-mercaptoethanol were from BDH Chemicals Ltd., Poole, Dorset, U.K. Epoxy-activated sepharose 6B, CL-sepharose 6B, 1,4-butanediol diglycidyl ether, reduced and oxidized glutathione, DL-dithiothreitol and triethylamine were supplied by Sigma Chemical Co., Poole, Dorset, U.K. N-Acetyl cysteine was from Boehringer Corporation, Lewes, East Sussex, U.K. and iodohexane from K and K Laboratories Inc., New York. Sephadex (G-25, G-75, G-100, CM-C50, DEAE A50), DEAE-Sepharose 6B, polybuffer exchangers PBE 118 and 94, polybuffers 96 and 74 and Pharmalyte 10.5-8 were supplied by Pharmacia Fine Chemicals Ltd., Hounslow, Middlesex, U.K. Bio-rad HT grade hydroxylapatite was from Bio-Rad Laboratories, Richmond, California, U.S.A.

2.02: Glutathione S-transferase assays

p-Nitrobenzyl chloride, p-nitrophenethyl bromide, 1,2-epoxy-3-(p-nitrophenoxy)propane, 1,2-dichloro-4-nitrobenzene, ethacrynic acid, p-nitrophenyl acetate, bromosulphophthalein and dehydroisoandrosterone were from Sigma. 1-Chloro-2,4-dinitrobenzene, perchloric acid, acetonitrile and chromium trioxide were from BDH

Chemicals Ltd. Trans-4-phenyl-3-buten-2-one was supplied by Aldrich Chemical Co., Gillingham, Dorset, U.K. and monobromobimane by Calbiochem Behring, La Jolla, California, U.S.A. DC-alufolien keiselgel precoated silica-gel plates were a gift from Mr A. Gray, the solvent system used to run them, the phosphomolybdic acid developing stain and the dry chloroform were a gift from Mr R. P. Ford and the alumina was given by Dr G. L. Atkins; all from the Department of Biochemistry, University of Edinburgh. The disposable chromatographic column was a gift from Mr P. Newman, Dept. of Haematology, Edinburgh Royal Infirmary.

2.03: Glutathione peroxidase assays

Cumene hydroperoxide and hydrogen peroxide were from BDH Chemicals Ltd. Glutathione reductase and nicotinamide adenine dinucleotide phosphate (reduced form) were supplied by Sigma Chemical Co.

2.04: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and staining

Acrylamide (Electran grade), sodium dodecyl sulphate (specially pure), ammonium persulphate, trichloracetic acid, bromophenol blue, Coomassie blue (Page blue 83, for electrophoresis), n-butanol, ammonia, trisodium citrate, cupric sulphate, formaldehyde, sodium fluoride and sodium thiosulphate were all from BDH Chemicals Ltd. Polyacrylamide was from Aldrich Chemical Co. and urea (specially pure) was from Serva Feinbiochemica, Heidelberg, West Germany. Pyronin Y, MW-SDS-70L molecular weight markers, silver nitrate and glycerol were from Sigma Chemical Co. N,N´-methylene diacrylamide and N,N,N´,N´-tetramethylethylenediamine were from Koch-Light laboratories Ltd., Colnbrook, Bucks, U.K. Chymotrypsin and *Staphylococcus aureus* V8 protease were a gift from Miss L. A. Kilpatrick, Department of Biochemistry, University of Edinburgh.

2.05: Electroelution, photography, antibody production and autoradiography

Benzamidine, phenylmethylsulphonyl fluoride, sodium sulphite and bovine serum albumin (fraction 5) were all from Sigma Chemical Co. Nitrocellulose sheets were from Schleicher & Schuell GmbH, Dassel, West Germany and Eppendorf tubes were supplied by Mackay & Lynn Ltd., Edinburgh, U.K. Polaroid 665 black and white film was bought from Edinburgh Cameras Ltd, Edinburgh, U.K. *Staphylococcus aureus* cells, radio-iodinated protein A, Freunds complete and incomplete adjuvents, horse serum, Nonidet P-40 (NP-40), Agfa Curix RP1 X-ray film, G334 fixer, Aditan and Ilford PQ universal developer were all gifts from Dr D. K. Apps and Dr J. H. Phillips, Department of Biochemistry, University of Edinburgh.

2.06: Preparation of microsomal glutathione S-transferases

N-Ethylmaleimide was supplied by Sigma Chemical Co.

2.07: Bradford protein assay and fluorimetry

Coomassie blue G (Serva blau G) was from Serva Feinbiochemica. Orthophosphoric acid was from BDH Chemicals Ltd, and Rose Bengal, lithocholic acid, cholic acid, and 8-anilino-1-napthalenesulphonic acid (hemimagnesium salt) were from Sigma Chemical Co.

2.08: Amino acid and N-terminal analyses

The chemicals for amino acid analysis were very kindly provided by Dr M. L. G. Gardner and those for N-terminal analysis by Miss L. A. Kilpatrick, both of the Department of Biochemistry, University of Edinburgh and solvents for HPLC analysis were from Fisons plc, Loughborough, U.K.

SECTION 3

METHODS

SECTION 3: METHODS

3.01: Preparation of dialysis tubing

Visking tubing of diameters 18mm, 27mm and 32mm was cut into strips of appropriate length and boiled in water containing sodium bicarbonate and EDTA (di-sodium salt); about a spatula full of each was added to 250ml of water. The bicarbonate was present to keep the pH favourable for the action of EDTA in removing metal ions. The tubing was removed, rinsed in distilled water and triple-knotted at the lower end, the sample poured in and the tubing sealed by three further knots. Unless otherwise stated the water used in all experiments was double distilled.

3.02: PREPARATION OF CYTOSOL

3.02.01: Fish

Rainbow trout (*Salmo gairdnerii*) 100-600g were supplied by Penicuik Trout Farm, Penicuik, Midlothian, U.K. and also by Beecraigs Fish Farm, Beecraigs Country Park, Linlithgow, West Lothian, U.K. Brown trout (*Salmo trutta*) 100-400g were line-caught from Arkle beck, Arkengarthdale, North Yorkshire, U.K. Livers of salt-water salmon (*Salmo salar*) were supplied by Marine Harvest Ltd., Loch Sunart, Nr. Fort William, Highland, U.K. Fresh-water salmon (*Salmo salar*) 3-6kg were caught by fly-fishing in the River Oykel, Highland, U.K.

3.02.02: Transport of fish and storage of livers

Rainbow trout were transported alive in well-oxygenated water to the laboratory, where their livers were perfused and used immediately. Brown trout and fresh-water salmon livers were removed on capture (and sometimes perfused), then kept on ice until they could be frozen. Salt-water salmon livers were perfused or left unperfused for comparison with fresh-water salmon, removed

and then stored on ice until they could either be used or frozen. Livers were stored at -70°C, then thawed at room temperature.

3.02.03: Perfusion of livers and preparation of cytosol

Livers were perfused *in situ* with ice-cold 154mM-NaCl except those from line-caught fish which were perfused after removal. Livers were minced and then homogenized in 4 vol. ice-cold 22mM-potassium phosphate buffer, pH 7.4, (containing 1mM-DL-dithiothreitol and 250mM-sucrose) using a motor-driven glass/teflon homogenizer. The homogenate was centrifuged (10,000 rpm; r_{av} 7.0cm, producing 9000g) for 25 min at 4°C, the supernatant decanted and centrifuged (35,000 rpm; r_{av} 8.12cm, producing 111,000g) for 80min at 4°C. The supernatant (cytosol) was decanted and filtered through glass wool to remove particulate and lipid matter.

3.03: Column chromatography

All columns were operated and packed in a cold room at 4°C, and while not in use sodium azide (0.02%w/v) was added to the column buffer to prevent microbial contamination.

3.03.01: Packing of columns

Gel suspensions to be packed into columns were degassed under vacuum to prevent air bubbles forming in the columns while packing. The columns were partly filled with their respective packing buffers which were syringed in from the lower end to remove air bubbles from their sintered end-pieces and connected tubing. This prevented air bubbles from forming at the glass/liquid interface as the suspensions were poured down glass rods (to minimize turbulence) into the columns. Commercially-produced columns (Wright Scientific Ltd., Kenley, Surrey, U.K.) were filled utilizing a packing extension, and because of the extra volume provided, these columns were completely filled with buffer before the packing slurry was poured in. Once these columns were packed, adjuster assemblies were inserted and the columns equilibrated as necessary. With chromatofocusing and other columns where high resolution was required the adjuster assemblies were positioned so that they touched the gel bed to eliminate any dead space where mixing might occur. With all other columns (those having no facility for adjustment), end pieces were inserted and the columns equilibrated and run with a layer of buffer above the gel at all times to prevent drying out and to lessen mechanical disturbance from the material being pumped on.

3.03.02: Gel-filtration chromatography

Sephadex beads (G-25, G-75 and G-100) supplied in dry powder form were swollen overnight in 20mM-potassium phosphate (pH 7.4) running buffer. Suspensions of G-75 and G-100 were packed into a column of dimensions 1.6cm x 50cm. In both cases a thin layer of G-25 was included between the gel matrix and support mesh, to prevent gel-fines (caused by the mechanical fracture of the beads) from blocking the support mesh. The columns were equilibrated with several volumes of running buffer and sample was then layered carefully on top of the gel. Running buffer was layered above this and the columns run at 19.2ml/h. Fractions (2.5ml) were collected using an ISCO model 1220 fraction collector (Fisons Scientific Apparatus Ltd., Loughborough, U.K.).

3.03.03: Cation-exchange chromatography

CM-C50 sephadex was swollen overnight in several volumes of CM-buffer (10mM-sodium phosphate, pH 7.5, containing 1mM-N-acetylcysteine as a thiol protectant) and packed into a 2.2cm x 15cm column. Cytosol (dialysed overnight at 4°C against 3 changes of 50 vol. of CM-buffer) and partially-purified enzyme from affinity chromatography (dialysed in an identical manner, but against CM-buffer containing in addition, 10mM-2-mercaptoethanol to reduce any disulphides present) were applied to the column and eluted at 38.4ml/h. After the collection of 35 fractions (2.5ml) a linear 0-200mM-NaCl gradient was applied. This was produced by pumping 100ml of CM-buffer containing 200mM-NaCl at 19.2ml/h into a mixing vessel containing 120ml of CM-buffer, from which the column was fed at a flow-rate of 38.4ml/h.

3.03.04: Anion-exchange chromatography

DEAE A-50 Sephadex

DEAE A-50 sephadex was swollen overnight in several volumes of DE-buffer (50mM-TRIS/HCl, pH 7.6) and packed into a 2.6cm x 26cm glass column. Cytosol (dialysed overnight at 4°C against 3 changes of 50 vol. DE-buffer) was applied to the column and then eluted with DE-buffer at 19.2ml/h. After the collection of 28 fractions (2.5ml) a 0-200mM gradient of KCl was applied in an identical manner to that used for CM-C50 sephadex, with the exception of the inflow and outflow rates from the mixing vessel which were 11.2ml/h and 19.2ml/h respectively, producing a gradient that was slightly concave as the inflow/outflow ratio was not exactly 2:1.

DEAE Sepharose 6B

DEAE sepharose (supplied as a pre-swollen gel) was washed and equilibrated with column running buffer (25mM-ethanolamine/acetate, pH 9.4) and packed into a 1cm x 30cm adjustable chromatography column. Several column volumes of this buffer were pumped through (48ml/h), followed by the sample which had been dialysed overnight at 4°C against 3 changes of 50 vol. running buffer. The column was developed with several volumes of running buffer, followed by a 0-200mM-NaCl linear linear salt gradient in column buffer and 2.5ml fractions were collected.

3.03.05: Affinity chromatography

Epoxy-activation of CL-Sepharose 6B

The method of Sindberg and Porath (1974) was used to activate 100ml of CL-sepharose 6B (supplied as pre-swollen gel), which was washed on a Buchner funnel, suction-dried and weighed. To every gram of gel was added 1ml of 1,4-butanediol diglycidyl ether and 1ml of 600mM-NaOH containing 2mg/ml sodium borohydride. The coupling reaction was allowed to proceed overnight at room temperature, then stopped by washing with copious volumes of distilled water on a Buchner funnel until no traces of the oily diglycidyl ether could be seen. This method allows the coupling of ligands to insoluble gel matrices via bifunctional oxiranes such as 1,4-butanediol diglycidyl ether, which has epoxide groups at both end of its structure. These are reacted with hydroxyl groups from the agarose polymer and then again with amino or suphydryl groups on the ligand. A dual function is also served by the spacer, which may react with the polymer using both of its epoxy groups, forming a cross-link between strands of the gel.

Coupling of GSH to epoxy-activated Sepharose 6B

The method of Simons and Vander Jagt (1977) was used in which the reaction mixture was kept at pH 7.0 to ensure coupling to the sulphydryl group of GSH. At pH values greater than 7 coupling occurs with an amino group and the resulting ligand has a lower affinity for the glutathione S-transferases.

Epoxy-activated Sepharose (prepared commercially or as described above) was washed in several volumes of buffer A (22mM-phosphate buffer, pH 7.0, prepared by adding 80ml of 20mM-potassium dihydrogen orthophosphate to 280ml of 100mM-disodium hydrogen phosphate and diluting to 1 litre

with distilled water). The gel was suspended in 200ml of this buffer to which was added 3.2g of GSH in 32ml of distilled water (pH adjusted to 7.0 with 2M-KOH). Coupling was allowed to proceed for 24h at 37°C in a shaking water bath, after which the gel was washed and then resuspended in distilled water containing ethanolamine to 1M, and allowed to stand for 4h to block any remaining active groups. The gel was then washed with 1 litre of 100mM-sodium acetate pH 4 , 1 litre of 100mM-sodium borate pH 8 (both containing 500mM-KCl and with pH values adjusted with glacial acetic acid) and finally with 1 litre of buffer A.

GSH-affinity chromatography

The GSH-coupled gel was packed into a 1cm x 30cm adjustable column and washed (24ml/h) with several column volumes of affi-buffer. Cytosol was then pumped onto the column, which was eluted with affi-buffer until the eluent appeared colourless. The column was developed with 50ml of 100mM-TRIS, pH 9.6 followed by 200ml of the same buffer containing 10mM-GSH (final pH 8.85) and 5ml fractions were collected. The column was repacked periodically to remove gel aggregates.

Synthesis of S-hexylGSH

Using the method of Vince and Wadd (1969), iodohexane was coupled to GSH by reaction with the cysteine sulphydryl group.

GSH (3.0g) was dissolved in 10ml of distilled water, to which 10ml of 2M-NaOH was then added. Absolute ethanol was added until the cloud point was reached, at which stage 2.0g of iodohexane was added slowly with vigorous stirring. The mixture was left standing overnight at room temperature before the pH was adjusted to pH 3.5 by the

dropwise addition of hydroiodic acid. The flask was chilled on ice until a white precipitate formed, which was collected and washed with copious volumes of distilled water. If the precipitate was left for too long in the reaction mixture, it became coloured by the iodine present, but remained functional for affinity chromatography. After washing, the precipitate was dried under vacuum overnight and then stored desiccated at 4^0C until needed.

Coupling of S-hexylGSH to epoxy-activated sepharose 6B

This coupling procedure follows the method of Mannervik and Guthenberg (1981) in which S-hexylGSH was coupled to the active epoxy groups by the glutamic acid amino group.

Epoxy-activated sepharose 6B (15g dry-powder form, pre-swollen for 15min in distilled water) was washed with 1.5 litres of distilled water. S-HexylGSH (1.5g) dissolved in 70ml distilled water (adjusted to pH 12 with 2M-NaOH) was stirred in and allowed to couple with the gel suspension for 16h at 30°C in a shaking water bath. Excess ligand was then removed by washing with distilled water and 50ml of 2M-ethanolamine/acetate (pH 9) was added and the suspension left for 4h at 30°C to block any unreacted epoxy groups. The gel was then washed with two 500ml volumes of 100mM-sodium acetate (pH 4, containing 500mM-NaCl), interspaced by one wash with 500ml of 100mM-TRIS/HCl, pH 8, containing 500mM-NaCl.

S-HexylGSH affinity chromatography

S-HexylGSH-sepharose 6B was suspended in column running buffer (10mM-TRIS/HCl pH 7.8, containing 0.02%w/v sodium azide) and packed into a 1cm x 30 cm adjustable chromatography column. After washing with several column volumes of running buffer (48ml/h), cytosol (50-400ml) was applied. The column was developed with 2 column volumes of running

buffer which was layered above the cytosol in the vessel which fed the column thus removing a manual buffer change. Non-specifically adsorbed material was removed by eluting with 2 column volumes of running buffer containing 200mM-NaCl. Specifically adsorbed material was then eluted with 2 column volumes of running buffer (containing 200mM-NaCl and 5mM-S-hexylGSH) and fractions (5ml) were collected. Because of the hydrophobicity of the S-hexylGSH, the powdered form had to be vigorously stirred into the running buffer with 2M-NaOH until it had dissolved. The pH was then readjusted to 7.8 very carefully with 2M-HCl. After use, both GSH and S-hexylGSH affinity columns were washed with affi-buffer and column buffer respectively (containing 0.2%v/v Triton X-100) to remove any hydrophobically bound material from them.

3.03.06: Chromatofocusing

Preparation of samples for chromatofocusing

A variety of samples were applied to the PBE 94 and PBE 118 chromatofocusing columns and it was vital that these samples were fully equilibrated with the running buffer and thoroughly desalted before application. This was achieved by dialysis (48hr; 6 changes; 100 vol.) against the respective running buffer. Prior to dialysis, material purified by affinity chromatography was concentrated by ultrafiltration to a volume less than 10ml, whilst less pure material was lyophilized and then suspended in a minimal volume of running buffer.

Chromatofocusing using Polybuffer exchanger 94 (PBE 94)

After equilibration on a Buchner funnel with column running buffer (25mM-ethanolamine/acetate; pH 9.4), PBE 94 was degassed and packed into a 1cm x 60cm adjustable column. After the column had been washed with this buffer

at 100ml/h until it was fully equilibrated and packed firmly (a pre-requisite for chromatofocusing) the flow-rate was reduced to 24ml/h and a few ml of eluting buffer applied to the column. This was applied to ensure that the sample when applied to the column was not subjected to pH shock resulting in yields of enzyme activity reduced by up to 20%. The eluting buffer used for pH gradients of 9-6 was Polybuffer 96 diluted 10x and titrated to pH 6 with glacial acetic acid. When a gradient of pH 9-7 was required identical buffer pH 7 was used. For chromatofocusing pH 8-5, the running buffer was 25mM-TRIS/acetate pH 8.3 and the eluting buffer, Polybuffers 96 and 94 (15ml and 35ml) diluted 10x and titrated to pH 5 with glacial acetic acid before degassing under vacuum to remove carbon dioxide. The sample was then applied to the column and eluted with 500ml of the respective eluting buffer. The column was finally developed with either a pulse of 1M-NaCl or a 0-1M linear gradient of NaCl (both made up in water), 2.5ml fractions being collected throughout.

Chromatofocusing using Polybuffer exchanger 118 (PBE 118)

Using 25mM-triethylamine/HCl (pH 11) as running buffer, PBE 118 was prepared and packed into a 1cm x 30cm adjustable column in exactly the same way as PBE 94. After equilibration of the column, material excluded from a PBE 94 column (equilibrated and desalted by dialysis overnight against 3x 100 vol. changes of running buffer) was pumped on to the column and eluted with 300ml Pharmalyte pH 8-10.5 (diluted 80x and titrated to pH 7 with concentrated HCl). The column was finally purged with 1M-NaCl and 2.5ml fractions collected.

3.03.07: Hydroxylapatite chromatography

Bio-Rad HT grade hydroxylapatite was packed into a 1.6cm x 30cm adjustable chromatography column and washed at 24ml/h with 10mM-sodium phosphate, pH 6.7. The column was considered equilibrated when the Na^+ concentration of the eluant had reached that of the buffer applied (14.24mM). The concentrations were determined using an Instrumentation Laboratory Systems 501 Na^+/K^+ Analyser (Instrumentation Laboratory Systems, Milan, Italy). Samples (dialysed overnight against 3x 100 vol. changes of equilibration buffer) were applied and eluted with this buffer until 40 fractions (2.5ml) had been collected. The column was then developed with a 0-250mM concave sodium phosphate gradient, pH constant at 6.7. This was produced in a mixing vessel with inflow and outflow values of 11.2ml/h and 24ml/h respectively and the Na^+ concentration was recorded in the fractions.

3.04: CONCENTRATION OF SAMPLES

Ultrafiltration

Samples were ultrafiltered under 70 psi (nitrogen) pressure using an Amicon 65 ultrafiltration cell fitted with a YM30 membrane (molecular weight cut-off, 30,000) supplied by Amicon Ltd., Woking, Surrey, U.K. To prepare new membranes for use, 50ml water was ultrafiltered through them removing anti-microbial and storage agents. New membranes were marked on the lower outside edges with an indelible marker to ensure insertion the correct way up when wet. After use membranes were soaked overnight in 1M-NaCl and then stored in 10%v/v ethanol which was removed before use by ultrafiltering 50ml water.

Lyophilization

Samples to be freeze-dried were placed in round-bottomed flasks and frozen as a uniform thin coat around the inside edges by immersion and rotation in -40°C methanol. They were then left on the freeze-drier overnight under vacuum.

3.05: ASSAY OF GLUTATHIONE S-TRANSFERASE ACTIVITY

3.05.01: Synthesis and additional purification of substrates

Δ-5-Androstene-3,17-dione

Δ-5-Androstene-3,17-dione was synthesized from dehydroepiandrosterone by the method of Djerassi et al., (1956). All the reagents had nitrogen bubbled through them for 5 min before the reactions commenced to prevent hydroperoxide formation. To 400ml of acetone at 10-15°C (distilled over potassium permanganate), 3g of steroid was added. Then 2.75ml chromium trioxide reagent (26.72g chromium trioxide in 23ml conc. sulphuric acid, diluted to 100ml with water) was added rapidly with vigorous stirring. Nitrogen was bubbled through the reaction mixture for 5 min which was then diluted to two litres with water and left until a precipitate had formed. The precipitate was filtered and washed using a Buchner funnel and dried overnight under vacuum. A microspatula full of dried product was dissolved in 500µl methanol and a similar quantity of starting steroid dissolved in 500µl isopropyl alcohol. Both were spotted onto a 5cm x 7.5cm precoated silica-gel plate and given two lanes each. The plate was run using a petroleum ether (60-80% v/v): diisopropyl ether: glacial acetic acid (30:70:2 by vol.) system and dried in an oven. The plate was photographed under ultra-violet light and then sprayed (2% w/v phospomolybdic acid in methanol)

Table 3·05·02

Substrate	[GSH]	[Substrate]	pH	λ_{max}	$\Delta\varepsilon$
	mM	mM		nM	$mM^{-1} cm^{-1}$
1,2-Dichloro-4-nitrobenzene	5·0	1·0	7·5	345	8·5
1-Chloro-2,4-dinitrobenzene	1·0	1·0	6·5	340	9·6
p-Nitrobenzyl chloride	5·0	0·5	6·5	310	1·9
1,2-Epoxy-3-(p-nitrophenoxy)propane	10·0	0·5	6·5	360	0·5
Bromosulphophthalein	1·0	0·03	7·5	330	4·5
trans-4-Phenyl-3-buten-2-one	0·25	0·05	6·5	290	-24·8
Ethacrynic acid	1·0	0·2	6·5	270	5·0
p-Nitrophenyl acetate	1·0	0·1	7·0	400	8·79
p-Nitrophenethyl bromide	5·0	0·1	6·5	310	1·2
Δ^5-Androstene-3,17-dione	0·1	0·07	6·5	248	16·3

to visualize any steroids present. Finally, a small amount of product was dissolved in dry chloroform and placed in the NaCl crystal cell of a Perkin-Elmer 257 Grating Infra-Red Spectrophotometer (Perkin-Elmer Ltd., Beaconsfield, Bucks, U.K.) which was used to scan the sample (using normal slit width) at medium scan speed.

1-Chloro-2,4-dinitrobenzene

About 15g 'Analar' CDNB was dissolved in a minimum volume of 95%v/v ethanol and passed through a disposable chromatography column containing alumina. The solution was placed in a Pyrex beaker on a hot plate, concentrated three-fold and placed on ice. Any crystals that were formed were redissolved in ethanol and recrystallized the same way twice more before being dried over silica-gel in a vacuum desiccator.

3.05.02: Glutathione S-transferase activity assays

The assay conditions used are shown in Table 3.05.02 and follow published methods, with minor modifications. Activity with Δ-5-androstene-3,17-dione was measured using the method of Benson and Talalay (1976). p-Nitrophenyl acetate and monobromobimane activities were assayed by the methods of Keen and Jakoby (1978) and Hulbert and Yakubu (1983) respectively. The remaining assays were carried out using the methods of Habig et al. (1974). The Δ-5-androstene-3,17-dione assay measured the absorbance change due to the isomerization of a double bond from the Δ-4 position to the Δ-5 position, whilst the p-nitrophenyl acetate assay recorded the formation of p-nitrophenol. The remaining assays measured the change in absorbance or fluorescence due to the formation of a GSH-conjugate.

$$\frac{a.x}{\frac{25.S}{60}} \times \frac{1}{\varepsilon} \times V \times \frac{1000}{v} = \text{Factor}$$

$$\text{Factor} \times \text{Tan}\theta = \text{Reaction rate } (\mu\text{mol/ml.min}^{-1})$$

$a.x$ = Combined sensitivities of chart recorder and spectrophotometer

25 = The width in cm. of the chart paper spanned by 1 absorbance unit

S = Chart speed (cm/sec)

ε = Millimolar extinction coefficient of the product

V = Reaction volume

$\frac{1000}{v}$ = Volume (v) of enzyme added in µl, corrected to ml.

Table 3·05·03

Reaction rate calculation

3.05.03: Spectrophotometric assays

All assays were carried out in 4ml cuvettes and run against a reagent blank (of identical composition, but minus protein sample), with the exception of column fractions assayed with 1-chloro-2,4-dinitrobenzene to obtain an elution profile. In all cases the assay buffer used was 120mM-potassium phosphate at the respective pH for the different assays. Cuvettes were made up to a final volume of 3.1ml by the addition of 250µl GSH solution dissolved in water and 100µl substrate dissolved in 95% v/v ethanol (except Δ-5-androstene-3,17-dione which was dissolved in 95% v/v methanol). After mixing the contents of the cuvette with a stirring rod, 50µl of protein solution was added and after mixing again the absorbance change was recorded at the correct fixed wavelength. When column fractions were being assayed and when kinetic studies were being carried out, the assay buffer was made up containing GSH to the correct concentration and 2.95ml added to each cuvette. This not only saved time but increased the accuracy of the assays by removing a pipetting step. The resulting absorbance change was visualized on a chart recorder running at a known speed, calibrated so that one absorbance unit represented a known number of divisions on the chart paper. The gradient of the line recorded (measured as the tangent of the angle produced) was then multiplied by a correction factor to give the rate of reaction as shown in Table 3.05.03. Because of doubts as to the validity of some of the results obtained with 1,2-epoxy-3-(p-nitrophenoxy)propane as substrate using fixed wavelength, the reaction was studied using a scanning spectrophotometer to determine whether or not the absorbance change which occured was taking place at the correct wavelength to be caused by the formation of a GSH-conjugate. In addition, the reaction was repeated using as a control, deproteinized material (produced by perchloric acid precipitation, centrifugation and neutralization of the

supernatant with 2M-KOH).

3.05.04: Centrifugal analysis

Enzyme activity with 1-chloro-2,4-dinitrobenzene and the absorbance of protein solutions at 285nm were measured using a <<Cobas Bio>> centrifugal analyser, Roche Diagnostica, Basel, Switzerland. The machine was programmed following the manufacturers instructions. Details of the parameters used can be found in the appendix.

3.05.05: Fluorimetric assay of enzyme activity

The formation of a GSH-monobromobimane conjugate was measured using a Perkin-Elmer 3000 fluorescence spectrophotometer. The excitation wavelength was set to 400nm and the emission wavelength to 475nm. The excitation and emission slits were set to 15nm and 10nm respectively. The fluorimeter cuvettes were made up to contain 3.0ml 50mM-potassium phosphate (pH 6.5), 100µl GSH (in 10mM-disodium EDTA, pH 4.5 to give a final concentration of 100µM) and 5µl protein solution. The reaction was started by the addition of 10µl monobromobimane (in acetonitrile), to give a final concentration of 10µM. The rate of reaction was only measured as a gradient produced on a chart recorder to look for differences in substrate specificity between enzymes.

3.05.06: Assay of glutathione peroxidase activity

Glutathione peroxidase activity towards cumene hydroperoxide and hydrogen peroxide (1.2mM and 0.25mM final concentrations respectively) was measured using a modification of the coupled assay system of Reddy et al. (1981). The assay measures the decrease in absorbance at 340nm caused by the oxidation of reduced $NADP^+$ which acts as a cofactor during the reduction of GSSG by glutathione reductase. The GSSG was initially formed by the oxidation

of GSH by glutathione peroxidase to produce reducing power for the reduction of the peroxide substrates.

The cuvettes (1.5ml) were made up with 700µl reaction buffer (100mM-TRIS/HCl, pH 7.2; 3mM-disodium EDTA; 1mM-sodium azide; 1.2mM- or 0.25mM- peroxide substrate), 50µl glutathione reductase (1 I.U. enzyme activity), 100µl GSH and 100µl $NADPH_2$ (both to give final concentrations of 1mM). The contents were mixed and the reaction started by the addition of 150µl protein solution. The blank rate of reaction (obtained when the protein solution was replaced by water) was then subtracted and the final rate calculated using a millimolar extinction coefficient for $NADPH_2$ of 6.22.

3.06: ENZYME KINETICS

3.06.01: Determination of K_m values for GSH and CDNB

Using a variation of the standard assay procedure, initial velocities were determined at varying concentrations of CDNB and 1mM-GSH. The procedure was repeated varying GSH (from 1-10mM) at 1mM-CDNB. All assays were carried out in duplicate and run against reagent blanks of the corresponding substrate concentration. The results were expressed as double-reciprocal plots of initial velocity against substrate concentration.

3.06.02: Empirical half-saturation concentration determinations

Because of the non-linearity of the double-reciprocal plots, an empirical method was used to obtain values for the half-saturation concentrations of GSH and CDNB for different proteins. Initial velocities (V_1, V_2) were determined in quadruplicate at two concentrations (S_1, S_2) of the substrate concerned and at a fixed concentration of the other substrate. The empirical half-saturation concentration (K_{emp}) was obtained from the Michaelis-Menten equation using the method of Atkins and Nimmo (1981) and its standard error calculated using the jacknife technique (Miller, 1974). The equation derived was:

$$K_{emp} = \frac{V_2 - V_1}{\frac{V_1}{S_1} - \frac{V_2}{S_2}}$$

The derivation of this equation from the Michaelis-Menten equation can be found in the appendix.

The substrate concentrations used were 0.2mM-GSH with 0.2mM and 1mM-CDNB, and 0.2mM-CDNB with 0.2mM and 5mM-GSH.

3.06.03: Inhibition of the conjugation of CDNB with GSH

The effect of other second substrates on CDNB conjugation was tested using standard assay cuvettes containing in addition, 100µl of second substrate. These were run against reagent blanks which contained 100µl of the solvent used to dissolve the second substrate . The contents of the cuvettes were however, added in a different order. The reaction was started by the addition of the CDNB (to give final concentrations of 1.0, 0.5, 0.33 and 0.25mM), the protein having been added with the assay buffer, GSH and the other second substrate. The concentrations of second substrate used were the same as for substrate specificity assays (except ethacrynic acid which was used at 0.1, 0.05, 0.02 and 0.01mM). The initial velocities obtained were expressed as a percentage of the rate of reaction in the absence of the inhibitor and were also plotted against the CDNB concentration used. The aim of these experiments was to establish differences between proteins rather than to quantify their kinetic behaviour.

3.06.04: The effect of S-hexylGSH on CDNB conjugation activity

Because all the proteins used in the kinetics experiments had been purified using S-hexylGSH affinity chromatography, the effect of this ligand on enzyme activity was investigated. Under conditions identical to the second substrate inhibition experiments, reaction cuvettes containing assay buffer, GSH, S-HexylGSH (0.25, 0.10, 0.025 and 0.01mM final concentration) and protein were mixed and the reaction started by the addition of CDNB to 1mM. The initial velocities obtained were plotted as reciprocals against the concentration of S-hexylGSH used.

3.07: SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-PAGE (modified from the method of Laemmli, 1970) was carried out using 12% or 15% w/v acrylamide gels.

3.07.01: Gel solutions

Solution A, separating gel buffer: 1.5M-TRIS/HCl, 8mM-disodium EDTA, 0.4% w/v SDS, pH 8.8. The pH was always adjusted before addition of SDS which damages pH electrodes.

Solution B, acrylamide stock: Acrylamide:N,N'-methylenediacrylamide (bis-acrylamide), 30:0.8% w/v.

Solution C, stacking gel buffer: 0.5M-TRIS/HCl, 8mM-disodium EDTA, 0.4% w/v SDS, pH 6.8.

Solution D, polyacrylamide stock: 3% w/v polyacrylamide, 1mM-sodium azide, 1mM-sodium fluoride.

Solution E, sample incubation buffer 4x concentrated: 0.2M-TRIS/HCl pH 6.8, 8% w/v SDS, 8mM-disodium EDTA, 40% w/v glycerol, 4% v/v 2-mercaptoethanol, 0.0025% w/v bromophenol blue.

Solution F, electrode buffer 5x concentrated: 0.4M-TRIS base, 1.92M-glycine, 0.5% w/v SDS, 10mM-disodium EDTA.

Gel fixing solution: 10% v/v glacial acetic acid, 20% v/v methanol.

Gel staining solution: 7.5% v/v glacial acetic acid, 50% v/v methanol, 0.25% w/v Coomassie blue R.

Gel destaining solution: 7% v/v glacial acetic acid, 10% v/v methanol.

3.07.02: Preparation of gel samples

Protein was precipitated from samples by adding an equal volume of 20% w/v trichloracetic acid and the mixture left on ice for 30min then centrifuged (1000g; 10min). The precipitated protein was dissolved in sample incubation buffer (4x diluted), neutralized by the addition of 1M-TRIS, heated at 70°C for an hour and then loaded onto gels or frozen for later use. This precipitation technique was used for samples containing potassium (which precipitates out SDS) and those containing polybuffer (which leaves blue stains on the lower half of gels). All other samples (and polybuffer samples which were run without delay) were added to 0.33 vol. of solution E and heated as before.

3.07.03: Assembly of gel cassettes and the pouring of gels

Two glass plates (19cm x 16.5cm one with a 2cm x 14cm notch removed centrally from the top edge, giving the plate two lugs) were washed with methanol, dried and clamped together separated by two lightly greased teflon spacers. The cassette so formed was clamped vertically with the bottom edge resting in a trough (1.3cm x 19cm x 0.6cm) cut out of a perspex block.

To pour two separating gels (12% w/v acrylamide) the following solutions were mixed together in a 100ml beaker; A: 15ml, B: 24ml, D: 10g and water: 10.4ml. If 15% w/v acrylamide gels were required, B was increased to 30ml and the water decreased to 4.4ml. Then 10ml were removed into a 20ml plastic container with 500µl of 10% w/v ammonium persuphate (APS) and 30µl of TEMED (N,N,N′,N′-tetramethylethylenediamine). After mixing, the solution was quickly divided between the perspex wells of two gel cassettes and allowed to polymerize. Capillary action caused the solution to rise up 5mm up inside the gel

cassette, effectively sealing the bottom of the cassette. Following this, 300µl APS and 30µl TEMED were added to the remaining separating gel which was poured into the gel cassettes to a level about 4cm below the cut out section of the lugged plate. Water-saturated n-butanol was layered on top with a pasteur pipette so that on polymerization the top of the gel remained level. Once the gel had set, the solvent was poured off and the top of the gel washed with water until all traces of solvent had been removed. Traces of water were then removed from the gel surface by inserting a piece of blotting paper between the plates.

The stacking gel solutions were mixed by inversion in a 20ml plastic container (B 2.4ml, C 4.0ml, D 2.6g, water 6.8ml, TEMED 10µl and APS 150µl) and poured into the gel cassettes, filling them. Quickly Teflon combs were inserted into the gaps provided by the removed notches of the lugged plates and were removed as soon as the gel had polymerized. The wells which were formed were washed out with diluted electrode buffer and any liquid remaining removed with a syringe. It should be noted that stacking gels were only poured an hour or so before the gels were run to prevent ions from the separating and stacking gels from mixing.

The gel cassettes were removed from the perspex blocks (any trailing strands of gel were trimmed off) and clipped onto the gel tanks with the lugged plate facing the tank so that the notch on the plate matched the one on the tank. A layer of grease was applied to the tanks beforehand to lie between cassette and tank effecting a seal so that buffer from the top reservoir did not leak out to the lower one later. The top tank was filled with electrode buffer (diluted 5x) and the gel samples underlayered (using a microsyringe) into the now buffer-filled wells. After pausing for a minute or so to detect any leaks from top to lower reservoirs, the lower reservoir was filled with diluted electrode buffer. The

buffer from the top reservoir was reused in the lower reservoir in following runs. The lid was placed on the gel tank, the power packs connected and turned on. Typically for an overnight run, the gels were run at 60V and for daytime runs 150V for 6 hours. Conditions were varied to suit individual needs and were also dependent on temperature as gels in the summer tended to run faster than gels in the winter. Once the bromophenol blue dye-front had reached the base of the gel (or run off the gel, depending on requirements), the power supply was disconnected, the gel cassette removed, taken apart and the gel removed and placed in gel fixing solution for at least half an hour.

After fixing, the gel was stained for an hour at 50°C, rinsed briefly in distilled water and then destained (50°C)in several changes of gel destaining solution as required. Destaining was helped by including pieces of foam rubber with the gels to adsorb the stain that was leached from the gels by the destaining process.

3.07.04: Cleveland gels

Proteins were applied to SDS-PAGE gels by a modification of the method used by Cleveland et al. (1977). Gels were prepared as before but using a single toothed comb to provide a single well. Alternatively the gel cassettes were laid on their sides after the stacking gel had been poured and polymerized to about 2cm from the cut-out section and two further volumes of stacking gel poured in and polymerized sequentially to produce the sides of a well. Gels were fixed (15min), stained (15min) and destained (1h) and the band of protein seen was cut out and soaked in buffer C for 2h.

SDS-PAGE gels (15% acrylamide w/v) were poured using wider perspex spacers and with a stacking gel 2-3cm longer.

The protein strip was cut into lengths so as to fit into the wells of the gels just poured. These wells were filled with buffer C and the top reservoir then filled with diluted electrode buffer. The protein strips were quickly inserted (with forceps) into each of the wells and tamped down with a spatula. Sample buffer 1 (50mM-TRIS/HCl pH 6.8, 20mM-disodium EDTA, 1% w/v SDS and 20% v/v glycerol) was then underlayed into each well to lie halfway up each gel slice. Protease (5µl) at chosen concentrations in sample buffer 2 (sample buffer 1 but with 10% v/v glycerol) was then layered on top of the sample buffer 1. To prevent the contamination of the microsyringes and their needles with proteases, a small section of small bore rubber tubing was attached to the end of the needle to contain any protease drawn up for injection into the wells. The gels were then run, fixed, stained and destained as for normal SDS-PAGE gels.

3.07.05: Preparative gels

SDS-PAGE gels were run using single wells (see Section 3.07.04); however the spacers used were made by fixing two perspex spacers together with vacuum grease to produce a gel of double thickness. The gels were run as normal but instead of fixing were stained for 2mins, placed on a light box and the protein band required cut out and kept for electroelution.

3.08: UREA GELS

SDS-PAGE Urea gels were prepared following the method of Anderson et al. (1983) and using the same methods as before for cassette assembly and pouring of gels. However, the gel solutions used differed as follows:

3.08.01: Gel solutions

Solution A, stock separating gel buffer and lower tank electrode buffer 5x concentrated: 1M-TRIS/H_2SO_4 pH 7.8, 0.2% w/v SDS.

Solution B, upper tank electrode buffer: 0.074M-TRIS/HCl pH 7.8, 0.1% w/v SDS.

Solution C, separating gel acrylamide stock: 34.2% w/v acrylamide, 1.8% w/v N,N´-methylenediacrylamide (bis-acrylamide).

Solution D, stacking gel acrylamide stock: 5% w/v acrylamide, 1.25% w/v bis-acrylamide. This solution made stacking gels that were mechanically unmanageable so was usually replaced with Solution B from SDS-PAGE gels.

Solution E, ammonium persulphate solution: ammonium persulphate 2.4% w/v.

Solution G, sample application buffer: 0.139M-TRIS/ glacial acetic acid pH 7.8, 0.5% w/v SDS, 20% w/v sucrose.

Solution H, well filling solution: solution G minus sucrose.

Solution I, SDS reducing solution: 25mM-DL-dithiothreitol, 10% w/v SDS, 5mM-disodium EDTA,

20% w/v sucrose.

Solution J, tracker dye: 2% w/v pyronin Y, 0.01% w/v sodium azide; in 50% w/v sucrose.

3.08.02: Preparation of separating gel

Separating gel solution was prepared by mixing 9.6ml A with 10.7ml C and 23g of urea was then dissolved in the mixture. The volume was then adjusted to 47ml with water. For sealing the bottom of the gel cassettes, 10ml of this solution was taken and mixed with 500µl E and 12µl TEMED and divided between two cassettes. After polymerization a further 500µl E and 12µl TEMED were added to the remaining gel solution which was poured to form two separating gels as before.

3.08.03: Preparation of stacking gel

Stacking gel solution was made up (A 2.0ml, D 5.0ml, water 2.8ml, E 100µl and TEMED 5µl) and poured into the washed, rinsed and dried gel cassettes. Combs were inserted and after polymerization removed, after which the wells were washed and prepared as before.

3.08.04: Sample preparation and application

Pure protein samples were digested at room temperature for varying lengths of time with *Staphylococcus aureus* V8 protease and chymotrypsin at a number of different protease concentrations. To stop the reaction 0.2 vol. solution I was added and the samples placed in a boiling water bath for 5min.

The wells were filled to a depth of about 5mm with solution H. The samples were then underlayed, 2µl of tracker dye solution was added to each well which were then filled with solution B. The upper reservoir was then

filled very carefully with B so as not to disturb the wells and the lower reservoir filled with solution A at a five-fold dilution. Gels were run overnight at constant voltage (100V) and fixed as for SDS-PAGE gels before being silver-stained.

3.09: SILVER STAINING OF GELS

This staining technique uses the method of Wray et al.(1981) and is more sensitive than the Coomassie method.

Fixed gels were washed in three changes of 50% v/v methanol to remove glycine originating from the electrode buffers (this obviously applies to the SDS-PAGE technique; Section 3.07). Solution A (0.8g $AgNO_3$ in 4ml water) was added dropwise with constant stirring to solution B (1.4ml 88% v/v ammonia in 21ml of 0.1M-NaOH) to form solution C in which the washed gels were stained for 15min with constant agitation. The stain was discarded and the gels washed in at least 3 changes of water and developed in solution D (2.5ml 1% w/v trisodium citrate; 250µul 38% v/v formaldehyde, made up to 500ml with water) with constant shaking at room temperature. The staining was then terminated when the desired degree of staining had been produced by bathing the gels in 50% v/v methanol/ 10% v/v glacial acetic acid. Overstained gels were destained in solution E (made by mixing equal volumes of a 43.6% w/v sodium thiosulphate solution and of a solution consisting of 3.7g NaCl and 3.7g cupric sulphate dissolved in water) and the reaction terminated with 10% v/v acetic acid solution.

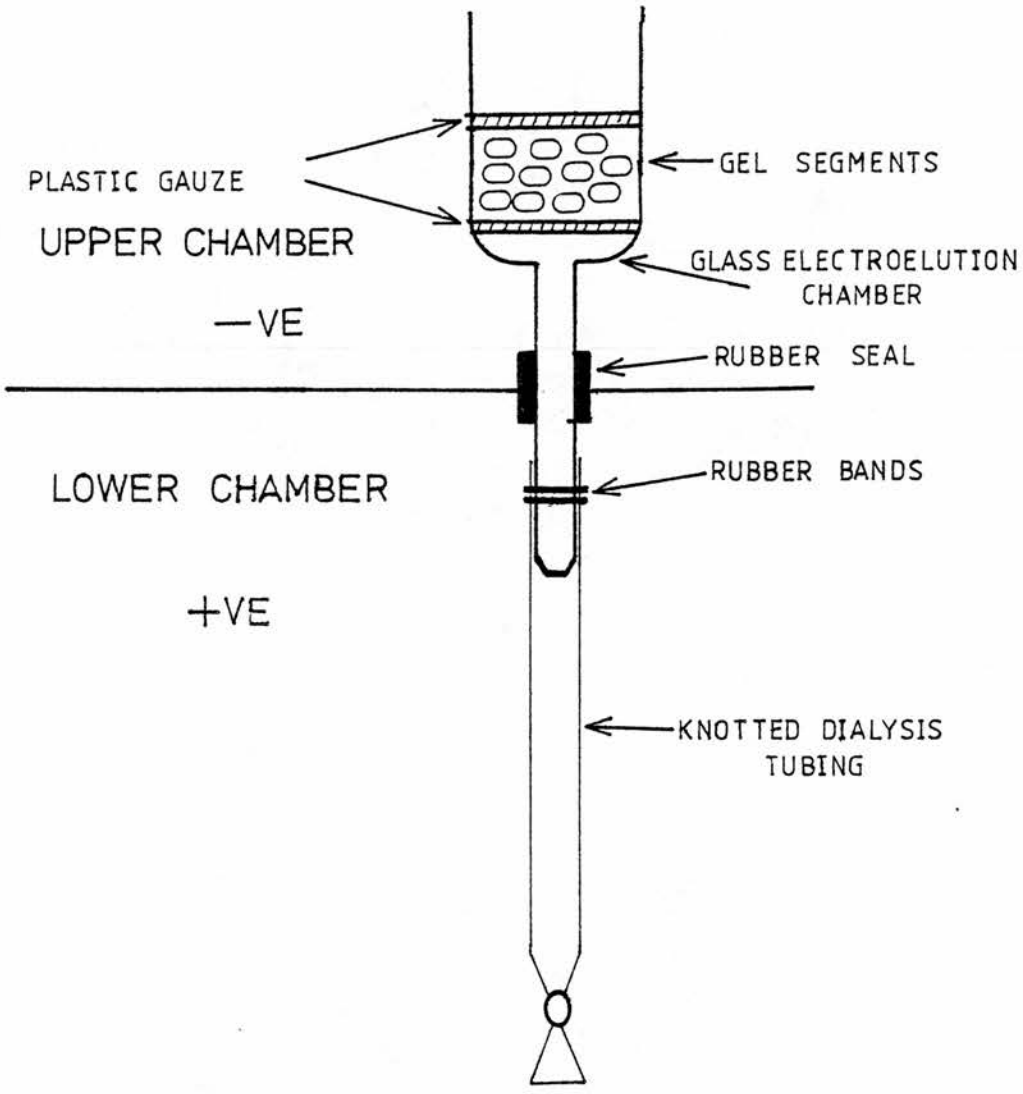


FIG 3.10 ELECTROELUTION

SIX GLASS ELECTROELUTION CHAMBERS COULD BE ACCOMMODATED INBETWEEN THE UPPER AND LOWER CHAMBERS (RESERVOIRS).

3.10: ELECTROELUTION

Electroelution was used to produce pure proteins from gel samples containing mixtures of proteins. Bands cut out from preparative gels were cut into sections of about 1cm in length and placed in the wide neck of an electroelution tank chamber (see Fig 3.10) and bracketed between two plastic gauze inserts to prevent the sections from leaving the chamber. The reservoirs of the electroelution apparatus were filled with electrode buffer which was the same buffer as used previously for SDS-PAGE (Section 3.07), but containing in addition, two protease inhibitors benzamidine and phenylmethylsulphonyl fluoride (PMSF). Each protease inhibitor (5ml of 100mM stock solutions of each in water and 95% ethanol respectively) was added to every litre of electrode buffer. Fresh PMSF was made up each time because of its instability in solution. The upper reservoir was filled so that the electroelution chamber was submerged, care being taken to exclude air bubbles. The sample was electrophoresed at 70V for about 7h until all the sample had migrated to the bottom of the dialysis tubing. The length of the dialysis tubing attached underneath the chamber was chosen so that the bottom of the tubing was slightly above the electrode in the lower reservoir. The dialysis tubing was then removed and tied off at the upper end before dialysis (18h) against 3 changes of 1 litre of 100mM-TRIS/HCl pH 7.5 (containing 50mM-NaCl). The sample was then freeze-dried overnight, resuspended in 500µl of water and divided into 10 equal fractions. Fractions were incubated with *Staphylococcus aureus* V8 protease and chymotrypsin at different concentrations of each (0.000005, 0.0005, 0.005, 0.05 and 0.5mg/ml) for 1h at 25°C. The samples were boiled with SDS-reducing solution (see Section 3.08.04) to stop the reaction and were then applied to SDS-PAGE urea gels.

3.11: PHOTOGRAPHY OF GELS AND AUTORADIOGRAPHS

A vertically-mounted Polaroid MP4 land camera with Polaroid 665 black and white film (Polaroid Corporation, Cambridge, Massachusetts, U.S.A.) was used for photography.

Gels were placed on a glass plate on a light box in a darkened room. Focusing was carried out at full aperture and a ruler placed next to the gel proved invaluable for fine focusing. Coomassie stained gels (using an orange filter), silver stained gels and autoradiographs were all photographed at f22/0.5s, but these conditions were altered for gels with different intensities of staining (usually by one stop in either direction). Fluorescent gels were photographed typically at f4.5 for 3min whilst illuminated under ultra-violet light.

After exposure the print was removed carefully and after 45s the backing paper removed, the print varnished and the negative fixed (5min in 15% w/v sodium sulphite). The fixed negatives were washed in running water for 15min then hung up to dry and stored in special envelopes to prevent scratching. Prints were then made from these on request by Medical Illustration, University of Edinburgh Medical School.

3.12: PRODUCTION OF ANTIBODIES

Pure protein solution (1ml) was mixed with an equal volume of Freunds Complete Adjuvant using a Sorvall Omnimixer model 17106 (DuPont Instruments, Newtown, Connecticutt, U.S.A.) and sufficient of the mixture (not more than one ml) injected into female New Zealand White rabbits to give each rabbit a dose of approximately 200µg protein. After 5 weeks a booster injection of 100µg protein was given (suspended in an equal volume of Freunds Incomplete Adjuvant) and after a fortnight the rabbits were bled (50-30ml being taken depending on the size of the rabbit). The blood was collected in 100ml beakers and allowed to clot at room temperature for 1-2h. The beakers were then rimmed with a glass rod and the clot left to contract overnight at 4°C. The supernatant (serum) was decanted and centrifuged (15min; 2500g; 4°C) then divided into 1ml portions which were then stored at -70°C.

3.12.01: Immune replicas

This method (modified from Towbin et al., 1979)) relies on the electrophoretic transfer of proteins from a gel onto a sheet of nitrocellulose paper alongside the gel (referred to as Western blotting). The paper is then washed with bovine serum albumin (BSA) to block non-specific binding sites and incubated with antisera, any antibodies present binding to proteins they recognise on the paper. After removing non-specifically bound material by washing, the blots are incubated with radiolabelled Protein A which binds specifically to the bound antibodies. After further washing the blots are dried and autoradiographed so that any reaction of the antibodies with specific proteins can be clearly visualized. The following solutions were made up:

Solution A, Transfer buffer: 20mM-disodium hydrogen phosphate, 20% v/v methanol and 0.02% w/v SDS made up to 10 litres and stored at 4°C.

Solution B, Horse serum buffer: 2.5% w/v bovine serum albumin (fraction 5), 0.05% w/v sodium azide, 20mM-TRIS, 0.9% w/v NaCl and 5% v/v horse serum (inactivated by incubating at 56°C for 30min), final pH 7.2 (adjusted with 2M-HCl) made up to 100ml and stored at -20°C.

Solution C, bovine serum albumin buffer: solution B minus horse serum, stored identically.

Solution D, TRIS-salt buffer (10x concentrated): 100mM-TRIS/HCl, 9% w/v NaCl, pH 7.2, 5 litres made up and discarded after use.

Two SDS-PAGE gels (12% w/v acrylamide) were run (see Section 3.07.03), removed from the cassettes (stacking gels were cut off on removal) and one corner snipped for identification. The electroblot electrophoretic transfer tank (E-C Apparatus Corporation, St.Petersburg, Florida, U.S.A.) was half-filled with transfer buffer and 4 sheets of blotting paper plus 2 sheets of nitrocellulose paper (all 14.5cm x 16cm) and 3 scouring pads (supplied with the apparatus) were all left to soak in the buffer. The transfer cassette was assembled in a film-developing tank filled with transfer buffer (to exclude air bubbles) by firstly laying the cassette opened and with the holey-grid side facing downwards. A scouring pad was laid on the holey-grid followed sequentially (one item at a time) by blotting paper, nitrocellulose, gel, blotting paper, scouring pad, blotting paper, nitrocellulose, gel, blotting paper and finally the third scouring pad. The cassette was then closed and fastened (with a fastner which slipped over the power lead connections on either side of the cassette and by three

elastic bands on each of the non-hinged edges of the cassette) and placed in the transfer tank (now filled with transfer buffer) and run for 75min at 0.8mA.

After blotting transfer the cassette was opened and the nitrocellulose sheets were removed. Each sheet had the corner facing the snipped corner of the adjacent gel removed for identification and was then placed in a plastic box. During this procedure the side of the sheet that had faced the adjacent gel was kept facing upwards at all times. The sheets were washed (1h, 37°C in a shaking water bath) in 50ml buffer C to swamp non-specific binding sites on the nitrocellulose followed by a wash with 50ml buffer B. This buffer contained 1ml of antiserum and was poured off (retained for repeated use) after 90min. The sheets were washed for 35min (5 changes of buffer) in diluted TRIS-salt buffer and incubated for an hour with diluted TRIS-salt buffer containing 5-10µl of ^{125}iodine labelled Protein A (3.4 x 10^4 cps/µl). They were then washed with diluted TRIS-salt buffer (5 washes), the middle wash contained the detergent Nonidet P-40 (0.1% v/v). The incubation buffer and the first wash were kept for radioactive disposal, whilst the other washes were discarded. The sheets were air-dried overnight and autoradiographed.

3.12.02: Autoradiography

Under conditions of total darkness, nitrocellulose sheets were sandwiched between glass plates with a sheet of X-ray film enclosed next to the side which remained uppermost during the preceeding incubations. After 3 days exposure the film (AGFA-Gevaert Curix RP1) was developed for 10min in Ilford PQ universal developer (diluted 10x), stopped by immersion (10s) in 5% v/v glacial acetic acid and fixed for 5min in film-fixer (AGFA-Gevaert G334 diluted 5x with water and containing 25ml Aditan print hardener per litre of working solution). Film sheets were then hung up to dry and photographed as mentioned previously. If bands failed to develop properly or overdeveloped the exposure time was varied accordingly.

3.12.03: Immunoprecipitation

Aliquots (5 x 1ml) of cytosol were incubated with 5µl of a 10% v/v suspension of *Staphlococcus aureus* cells for 10min on ice and centrifuged (5min, 4000rpm) in a microfuge (MSE Microcentaur, Mackay and Lynn Ltd., Edinburgh,U.K.). The supernatant was incubated overnight at 4°C with antiserum (50µl, 100µl, 250µl, 500µl and 1ml) on a constantly rotating mixing device. After overnight incubation, more cells (1ml of 10% v/v suspension) were washed sequentially with 900µl (to keep 10% v/v suspension) of 0.5% v/v, 0.1% v/v and 0.05% v/v Triton X-100 before addition to each incubation which was then left for a further 1h on ice. After centrifugation (5min, 4000rpm) and two washings with the Triton X-100 solutions (same order) to remove non-specifically bound material, the pellets were resuspended in 6.25mM-TRIS/HCl, pH 6.8 (containing 2% w/v SDS, 6M-urea and 5% v/v 2-mercaptoethanol) and boiled for 5min to release bound proteins from the cells. After centrifugation (5min, 4000rpm), the supernatant (1ml) was added

to 50µl of SDS-PAGE sample incubation buffer and run on SDS-PAGE against serum and cytosol controls.

3.13: BRADFORD PROTEIN ASSAY

The method used was modified from that published by Bradford (1976).

Serva blau G dye (25mg) was dissolved in 25ml 95% v/v ethanol, to which 50ml of orthophosphoric acid and 25ml of water were then added. This stock solution was kept in a dark bottle at 4°C to be diluted 5-fold and allowed to reach room temperature before use. Proteins to be assayed were used undiluted, diluted 10-fold and diluted 100-fold in 0.9% w/v NaCl. Protein (100µl) was added to dye reagent (1ml) in a 1.5ml disposable plastic cuvette and mixed. After 30min incubation at room temperature the absorbance at 595nm was measured (against a blank of dye reagent plus saline). A standard curve was produced using bovine serum albumin in saline (0.01, 0.02, 0.05, 0.1, 0.2, and 0.5 mg/ml). For really accurate protein assays a fresh standard curve was prepared every time the dye reagent was diluted for use, however for less critical assays, the original standard curve produced when each batch of dye was dissolved, was used. Assays were carried out in triplicate or quadruplicate and particular attention was paid to inserting the cuvettes vertically into the spectrophotometer as the 1ml cuvettes could be inserted at an angle giving a false absorbance reading.

3.14: PREPARATION OF MICROSOMAL GLUTATHIONE S-TRANSFERASES

Cytosol (70ml) was prepared (see Section 3.02.03) using a modification of the method used by Morgenstern et al. (1982). The 111,000g pellet was kept, resuspended in 150mM-TRIS/HCl (pH 8.0) and ultracentrifuged twice more (111,000g) before resuspension in 35ml 0.25M-sucrose. N-Ethyl maleimide (10ml of a 10mM solution in 10mM-potassium phosphate, pH 7.0) was added dropwise (with gentle agitation) and after 2min, GSH (2ml of 0.1M solution) was added to prevent inactivation of glutathione S-transferases by N-ethylmaleimide. The solution was stirred for 5min and Triton X-100 (10ml of a 10% v/v solution) was added dropwise to solublize the membranes. The mixture was stirred for 20min then assayed for glutathione S-transferase activity with 1-chloro-2,4-dinitrobenzene as substrate.

3.15: PREPARATION AND FRACTIONATION OF MICROSOMAL MEMBRANES TO INVESTIGATE 1-CHLORO-2.4-DINITROBENZENE PARTITIONING

Microsomal pellets were prepared as mentioned previously (see Section 3.02.03), resuspended in 150mM-TRIS/HCl pH 7.5 (containing 25mM-potassium chloride, 5mM-magnesium chloride and 250mM-sucrose) and centrifuged (111,000g; 80min; 4°C). This process was repeated until the supernatant contained no detectable protein (using the Bradford protein assay).

The pellet was then homogenized in the same buffer and 1-chloro-2,4-dinitrobenzene (CDNB) added to 2mM. After incubation overnight (at 4°C on a constantly revolving platform), the mixture was centrifuged and the high speed supernatant scanned between 280 and 500nm in a spectrophotometer. The results were compared with those obtained for the original buffer (plus 2mM-CDNB). Successive washes and spins followed (using the same buffer, minus

CDNB) and the supernatants were scanned as before. The preparation was repeated but the washed microsomes were centrifuged in sucrose gradients (using a method devised by J. G. Pryde, 1984, personal communication). Stepwise sucrose gradients were prepared by pouring 5ml of resuspension buffer (150mM-TRIS/HCl pH 7.5; 25mM-KCl; 5mM-$MgCl_2$ containing 0.5M-sucrose) into 30ml ultracentrifuge tubes. Then 10ml of the same buffer (but containing 0.85M-sucrose) was very carefully underlayered into the tubes using a 25ml plastic syringe fitted with a long needle. Successive underlayers containing 1.15M-sucrose (10ml) and 1.4M-sucrose (5ml) were added and finally an equal ammount of microsomal suspension (homogenized in buffer containing 1.4M-sucrose using a glass-teflon homogenizer) was underlayed into each tube. The tubes were then centrifuged (111,000g; 60min; 4°C) and the bands formed between the sucrose layers removed by aspiration. The sucrose concentrations were made up and corrected (using concentrated sucrose or water) by refractive index measurement using an Abbe Refractometer (Bellington and Starky Ltd, London, U.K.).

Each of the fractions were resuspended in 30ml resuspension buffer (using a glass/teflon homogenizer) and the total protein measured for each fraction using the Bradford assay. The tubes were re-centrifuged and the pellets resuspended in buffer (containing 2mM-CDNB) and incubated overnight on a constantly revolving platform at 4°C. Then the tubes were centrifuged and the supernatant discarded. The pellets were resuspended in buffer (minus CDNB) and centrifuged as before, the process was repeated several times and each supernatant was scanned spectrophotometrically and compared with buffer standards containing known concentrations of CDNB.

3.16: FLUORIMETRIC ASSAY OF BINDING TO GLUTATHIONE S-TRANSFERASES

The increase in fluorescence when proteins bind to the dye 8-anilino-1-napthalenesulphonic acid (ANS) was measured using the technique of (Sugiyama et al., 1978).

To begin with (following the method of Nimmo and Cramb, 1984), bovine serum albumin (100µl of a 4.4µM solution in 10mM-potassium phosphate pH 7.0) was added to ANS solution (2.95ml of 10µM in 10mM-potassium phosphate pH 7.0). The excitation wavelength was scanned (at a fixed emission wavelength of 520nm; slit widths 2.5nm/15nm excitation/emission) until a fluorescence maximum was seen. This was repeated at fixed excitation (400nm; slit widths 15nm/2.5nm) scanning the emission wavelength until the optimum wavelengths were obtained (those at which maximal fluorescence occurred) for both. Then scans were repeated at increasing concentrations of bovine serum albumin to show any shift in fluorescence maxima as binding occurred. This was then repeated with purified glutathione S-transferases eluted from an affinity column. From these results the optimum wavelengths (emission 410nm/excitation 480nm) were chosen to measure fluorescence due to the binding of glutathione S-transferases to ANS.

3.16.01: Fluorescence dilution titrations

For these assays purified, filtered enzymes were used which were of known protein concentration (assayed accurately by the Bradford method see Section 3.13). ANS solution was added to a fluorescence cell (containing 3ml protein solution) to give a final concentration of 1.29µM. After mixing, the fluorescence (at 410nm emission/480nm excitation; slit widths 15nm emission/20nm excitation) was measured. Small aliquots (10µl-1000µl) were removed from

the cell and replaced by equal volumes of 1.29μM ANS, thus diluting the protein but keeping the ANS concentration constant. A control experiment (using protein solution and the phosphate buffer) was run simultaneously so that the intrinsic fluorescence due to protein could be subtracted from each of the readings. The results were expressed as a double reciprocal plot of fluorescence against protein concentration to give a value for the protein concentration which would produce maximal enhancement of fluorescence.

3.16.02: Fluorescence quenching

Purified glutathione S-transferases (100μl) were added to 2.9ml of 600μM-ANS in 10mM-potassium phosphate pH 7.0 and the protein concentration was altered to give fluorescence readings of sufficient magnitude. Various ligands were added in 100μl of ethanol and the resultant fluorescence measured. The ligands were; 1-chloro-2,4-dinitrobenzene 1mM (final concentration), ethacrynic acid 0.2mM, p-nitrobenzyl chloride 0.5mM, 1,2-epoxy-3-(p-nitrophenoxy)propane 0.5mM, lithocholic acid 5mM, cholic acid 5mM, bromosulphophthalein 0.03mM, rose bengal 4μM and Δ-5-androstene-3,17-dione 0.07mM (but dissolved in methanol). The change in fluorescence was compared with the change due to dilution by the addition of 100μl methanol or ethanol.

3.17: AMINO ACID ANALYSIS

Glutathione S-transferases were purified to homogeneity (verified by SDS-PAGE) and freeze-dried in acid-washed round bottomed flasks. They were resuspended in water to give concentrations sufficient to allow 10-15nmol of each amino acid to be applied to the analyser. For this 500µl of resuspended sample was added to 500µl of 12M-HCl (kept in a sealed dispenser) in a Pyrex glass test tube (12.5cm x 1.4cm) labelled accordingly with a diamond pencil. English Pyrex was used because French made tubes were inadequately annealed. The sample mixtures were frozen in a dry-ice and ethanol mixture and allowed to thaw (to remove oxygen). The tubes were then heated in an oxygen/propane flame and the necks drawn out to form a narrow mid-section of about 6cm length but leaving the neck of the tube intact (care was taken to avoid spilling the tube contents during this operation). The samples were re-frozen and connected to a vacuum pump (fitted with a dry-ice/ethanol cold trap to prevent HCl vapour from entering the pump) and allowed to thaw. With the vacuum still connected, the tubes were returned to the flame and the narrow mid-piece sealed (care being taken not to flex the narrow section). Once the mid-piece had melted through, the two ends drifted apart and the sealed lower section of the tube (containing acid and sample) was left for 24h in a heating block at 110°C.

Tubes were then cooled and centrifuged in a bench top centrifuge (1min, 5000rpm) to bring the liquid down from the narrow drawn out necks. Each tube was then scored 2.5cm from the base with a glass cutter to produce a line around about half the circumference of the tube. A glass rod was placed in the flame and heated to white heat (goggles being worn to prevent eye damage from the strong ultraviolet light given off). It was quickly placed on the score line of

a tube causing the tube to crack all the way round. The tops were then eased off all the tubes which were desiccated *in-vacuo* over solid NaOH and phosphorous pentoxide overnight. Sodium borate buffer (0.1N, pH 10, 250µl) was added to each tube in order to oxidize cysteine residues to cystine as cysteine co-elutes with proline (Gardner, 1984) and the tubes were then sealed with several layers of parafilm. The tubes were left for 3-4h and mixed several times to dissolve solids. Sodium citrate buffer (0.2N, pH 2.2, 250µl) was injected through the parafilm to bring the volume back to 500µl and the pH to 2.2. This equilibrated the sample to the correct pH for application to the column which was packed with an ion-exchange resin ("Ultropac 8", LKB Biochrom Ltd, Cambridge, U.K). If neccessary, particulate matter was removed at this stage with a 0.22µm filter (Amicon Ltd, Woking, Surrey, U.K.).

Samples (50µl) were injected into automatic sample loading capsules (porous) which had been washed to pH 2.2 with the sodium citrate buffer (2ml injected in until it ran out of the other end, at which stage the capsule was dried with a good quality tissue wipe). Internal standard (nor-leucine, 20µl containing 10nmol) was injected followed by a further 20µl sodium citrate buffer, pH 2.2. Capsules were dried and loaded into the amino acid analyser (LKB 4400 amino acid analyser fitted with a 2220 recording integrator, LKB Biochrom Ltd, Cambridge, U.K.) followed finally by a dummy capsule to act as a weight to load the last capsule in. The samples at pH 2.2 bound tightly to the top of the column and were eluted by a series of stepwise buffer changes (Protein Chemistry Notes 10, LKB Biochrom). The buffers were 0.2N-sodium citrate and borate buffers leading to pH steps of pH 3.2, pH 4.25 and pH 10. Results were printed out automatically, corrected to the internal standard, samples being reloaded if neccessary to get correct loadings for detection. The results obtained were compared by the technique of Cornish-Bowden (1983).

3.18: N-TERMINAL AMINO ACID MICROSEQUENCING

The method used was that of Chang (1983a).

3.18.01: The solutions used

Dimethylaminoazobenzene isothiocyanate (DABITC) was prepared by dissolving DABITC (1g) in 150ml boiling acetone (99.8% v/v) and passing it through a sintered glass funnel to remove insoluble matter. DABITC crystals were precipitated by cooling to -20°C overnight, and kept (in darkness) for later use.

3.18.02: Preparation of DABITC derivatives of protein N-terminal amino acids

Pure proteins were freeze-dried in acid washed stoppered tubes (each tube containing at least 22µg of polypeptide for homodimeric proteins and 44µg for heterodimers) and redissolved in 50% v/v pyridine (triple-distilled over KOH 10g/l; ninhydrin 1g/l and KOH again 10g/l). DABITC was then added (10µl of a 10nmol/µl solution in pyridine), the tubes flushed with nitrogen, sealed and then heated for 50min at 54°C. After the addition of 5µl phenylisothiocyanate (PITC) the tubes were flushed, resealed and incubated for a further 20min at the same temperature. The contents were extracted 3x with heptane:ethyl acetate (2:1 v/v, 250µl) and the lower (aqueous) layer dried for 1-2h under vacuum. The heptane had been distilled with methanol (which was then washed out with water), dried on a molecular-sieve (molecular sieve 4, BDH Chemicals Ltd) and redistilled, whilst the ethyl acetate was washed with 5% w/v sodium carbonate followed by saturated calcium chloride and finally dried on a molecular sieve. The dried residue was dissolved in 50µl trifluoracetic acid (TFA, distilled over anhydrous calcium sulphate; 10g/l), flushed and heated at 54°C for 10min. The TFA was removed by desiccation (30min)

and the cleaved N-terminal derivative extracted with water (50µl) and butyl acetate (150µl, prepared in the same way as the n-heptane) before being dried under a stream of nitrogen. The remaining aqueous layer was dried under vacuum and stored at -20°C for sequential cleavage. The labelled N-terminal amino acid was dissolved in 50% v/v TFA (40µl), heated for 45min at 54°C and dried under nitrogen.

3.18.03: TLC analysis

The dried residues were resuspended in 20µl 95% v/v ethanol and 1-2µl applied by capillary tube to polyamide sheets (BDH Chemicals Ltd). The sheets were then chromatographed using acetic acid (HPLC grade):water (2:1 v/v), then dried before running in the second dimension using toluene:acetic acid:hexane (2:1:1 by vol.). The sheets were dried and developed with HCl vapour. The spots which occurred were compared with standard DABITC derivatives (Chang, 1983b).

3.18.04: Preparation of standard amino acid DABITC derivatives

This work was carried out by L.Kilpatrick, Department of Biochemistry, University of Edinburgh, who kindly allowed me to use the samples which she prepared.

Amino acids (50mg) were dissolved in 10µl triethylamine solution (made up with 5ml 0.2M-acetic acid, 0.5ml triethylamine, 50ml acetone and 50ml water) and treated with DABITC (50µl of a 4nmol/µl solution in acetone). They were heated for 1h (54°C), dried under vacuum and redissolved in 40µl water. TFA (100µl of a 50% v/v solution) was added and the mixture heated for 45min at the same temperature. After vacuum desiccation the DABITC derivatives (about 200nmol) were dissolved in a suitable volume of 95% v/v ethanol for TLC analysis. Histidine, glutamate and aspartate derivatives were adjusted to pH 10 with 1M-NaOH.

3.18.05: Separation of DABITC-derivatized N-terminal Amino Acids by High Performance Liquid Chromatography (HPLC)

For this work an ALTEX HPLC system was used which comprised two pumps (model 110 A), a programme controller (model 421) and a Hitachi 100-10 spectrophotometer fitted with an ALTEX flow cell (all from ALTEX Scientific, Berkeley, California, U.S.A.).

Before starting a series of runs, the pumps (one for each solvent) were primed with their respective degassed solvents to remove trapped gas bubbles. A running programme was set up so that at the beginning of a run, acetic acid (35mM, pH 5, referred to as acetate) flowed through the system, but over five minutes the solvent concentrations were changed to acetate 55%: acetonitrile 45% (v/v), the flow rate at all times being 1ml/min. These concentrations were held for five minutes and then altered over the course of 5 min to 30% acetate: 70% acetonitrile (v/v) and held at these levels for eight minutes. Over the following five minutes the acetonitrile concentration was raised a further 10% and held at 80% v/v for four minutes. The concentrations were reduced over the next five minutes back to acetate 55% v/v: acetonitrile 45% (v/v) and a programme loop was added taking the programme back automatically to the end of the section where the acetonitrile concentration was 45% (v/v). Samples were injected (10-25µl) onto into the system one minute after loop-back had occurred; the column that was used was a µBondapack C18 reverse phase chromatography column of dimensions 7.8mm x 30cm (Waters Associates, Northwich, Cheshire, U.K.) fitted with a CO-PELL ODS pre-column (Castle Laboratory Systems, Stirling, U.K.). The DABITC derivatives that were eluted from the column were detected in the flow-cell of the spectrophotometer at 420nm with the scale set to 0-0.02 absorbance units for a full scale deflection. The peaks were visualized using a Servoscribe 1S chart

recorder (Kelvin Electronics Ltd, Glasgow, U.K.) and the retention times on the column carefully recorded for comparison with standard DABITC derivatives.

SECTION 4

RESULTS AND DISCUSSION

SECTION 4: RESULTS AND DISCUSSION.

4.1: THE ACTIVITIES OF THE GLUTATHIONE S-TRANSFERASES OF *Salmo gairdneri* TOWARDS EPOXIDE AND PEROXIDE SUBSTRATES

Because of uncertainty as to the role played by the GSH S-transferases in detoxication it was thought important to find out which substrates the transferases showed activity towards in relation to their proposed functions. Initially two kinds of activity were investigated; GSH epoxide transferase activity and GSH peroxidase activity. Activity towards an epoxide substrate, 1,2-epoxy-3-p-(nitrophenoxy)propane (ENPP) was investigated because of the susceptibility of hatchery reared trout to hepatomas induced by aflatoxins in their feed (Adamson, 1967). Aflatoxins are metabolized by microsomal enzyme systems to form highly carcinogenic diol-epoxides which are the ultimate carcinogens. It is of interest to see whether or not trout have the capacity to detoxify harmful epoxides. No such activity was detected in the liver of the Northern pike (Balk et al., 1980). As a model substrate, ENPP is not ideal because it is an alkyl epoxide and aflatoxin epoxides are polyaromatic. Assays using aromatic epoxide substrates such as styrene oxide are complex. Radio-labelled substrates are used and the products are separated chromatographically (James et al., 1976); the substrates require custom synthesis and are therefore extremely costly. ENPP was therefore chosen because it was readily available and relatively inexpensive.

GSH peroxidase activity was investigated because of the vulnerability of fish to lipid peroxidation. The higher the degree of unsaturation in a lipid, then the greater the vulnerability to lipid peroxidation. Fish lipids are in general more unsaturated than lipids in warm blooded

Fig. 4.1.01

Elution profile from G−100 sephadex of trout liver cytosol.

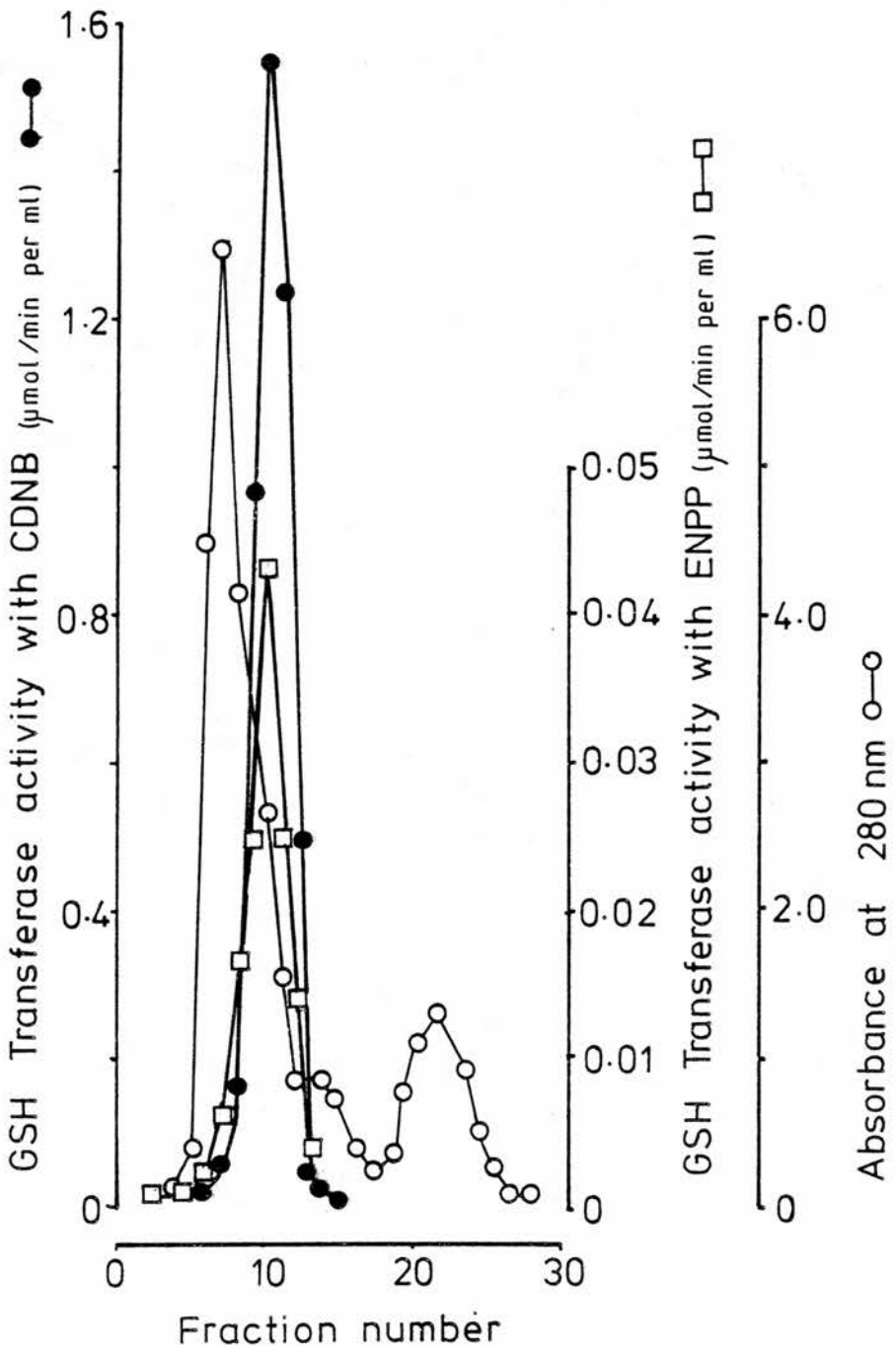
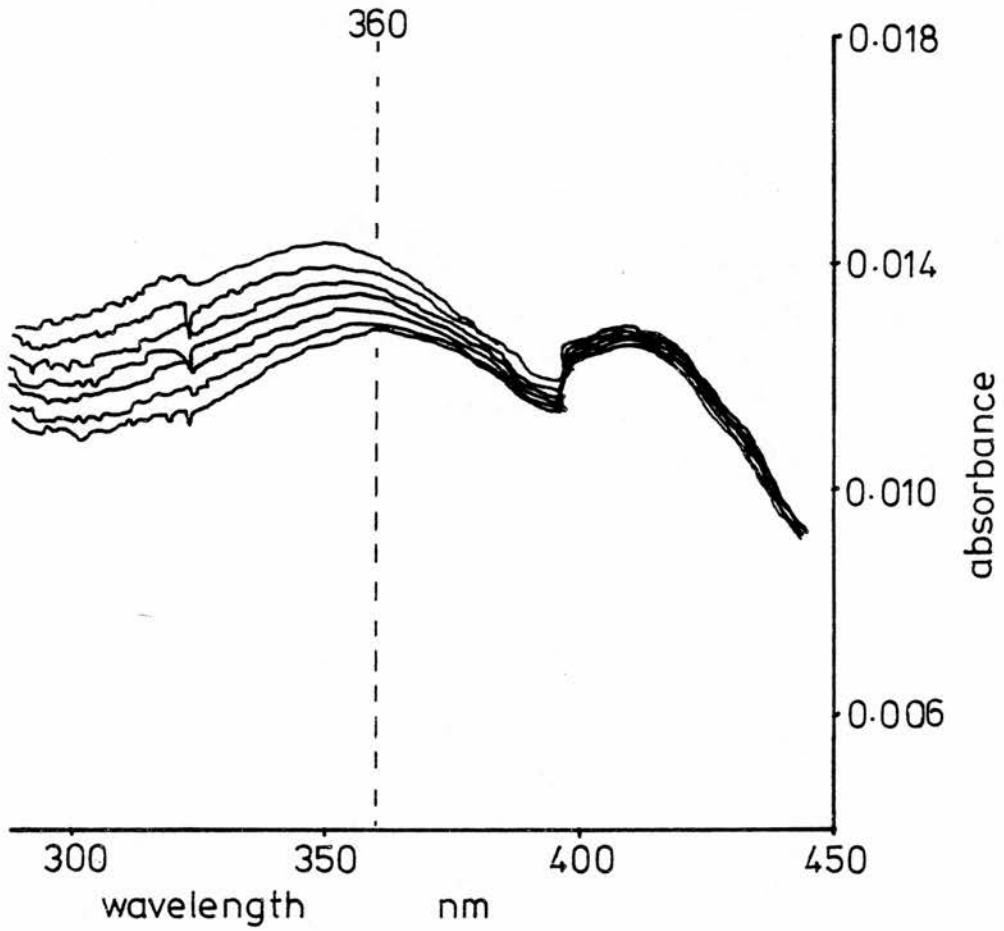


Fig. 4.1.02

The increase in absorbance due to the formation of a 1,2-epoxy-3-(p-nitrophenoxy)propane (ENPP)—GSH conjugate.

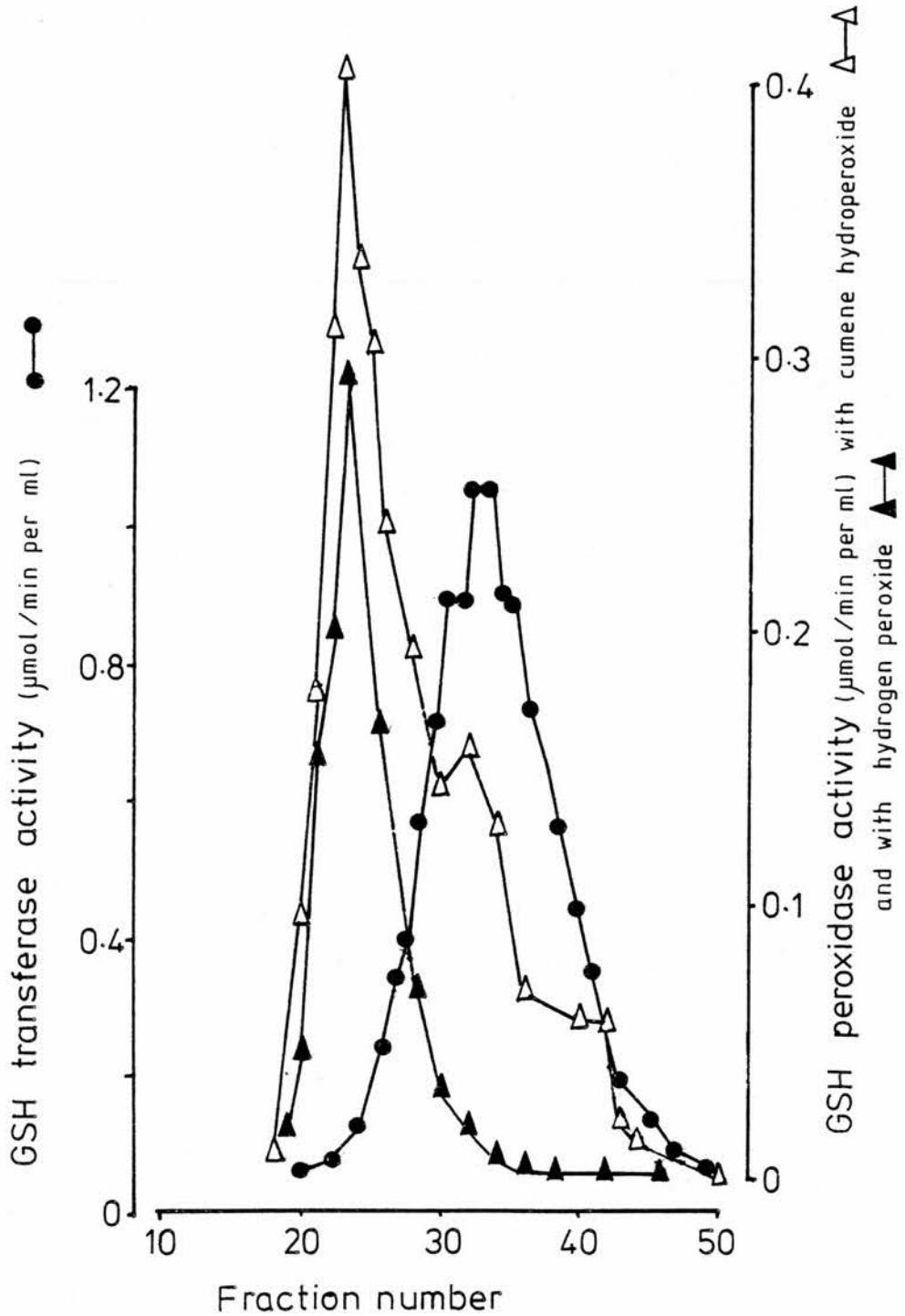


400µl trout liver cytosol added to assay mixture containing 5mM-GSH + 0·5mM-ENPP read against a reagent blank.

Scan interval = 52 seconds.

Fig. 4.1.03

Elution profile from G-75 sephadex of trout liver cytosol.

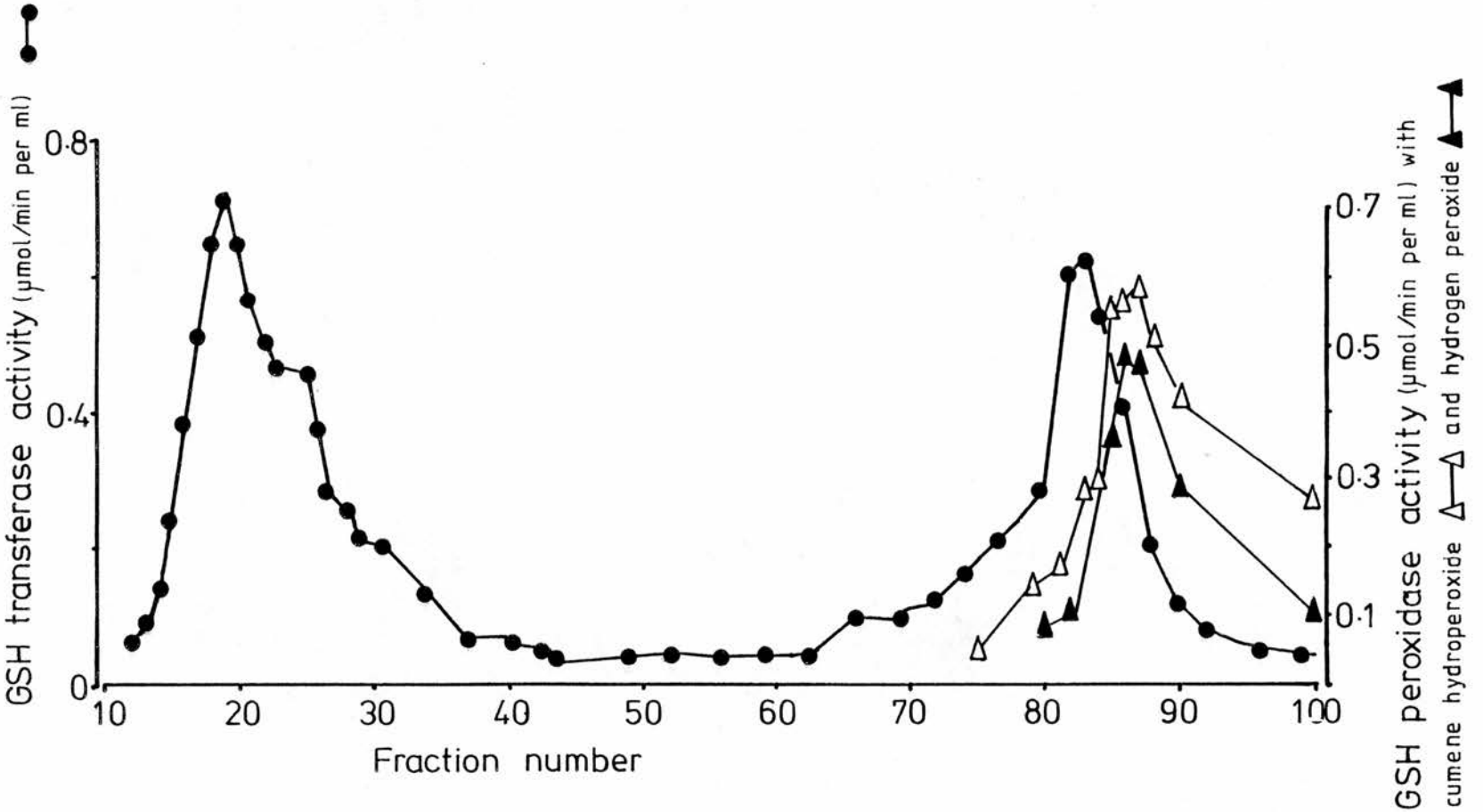


animals due to the requirement for flexibility at lower temperatures (Hazel, 1979). Two forms of GSH peroxidase are known to exist; a selenium dependent form, GSH peroxidase A (Mills, 1957) and GSH peroxidase B (Awasthi et al., 1975) which is associated with GSH transferase activity (Prohaska & Ganther, 1977). The two can be distinguished on the basis of their catalytic activities; GSH peroxidase B will work only with organic hydroperoxides whereas GSH peroxidase A also works with hydrogen peroxide as a substrate. Clearly such vulnerabilty to lipid peroxidation might be reflected in increased levels of GSH peroxidase activity. If a high level of GSH peroxidase B was found this might explain the high concentration of GSH transferases in the liver of the trout (Nimmo et al., 1981).

When trout liver cytosol was applied to sephadex G-100 and fractions collected were assayed for activity towards CDNB and ENPP (Fig. 4.1.01), the two activities co-eluted separate from the bulk of the material absorbing light at 280nm. However, some problems were encountered with the ENPP assay making it difficult to distinguish the enzymic rate from the non-enzymic rate. A spectral scan was carried out (Fig. 4.1.02) to see if the measured absorbance change was due to the formation of a GSH-conjugate or due to increased turbidity. The results showed that the absorbance increase was at the correct wavelength for it to be due to the formation of a conjugate, although the rate of reaction itself was very low.

When trout liver cytosol was applied to sephadex G-75 and the collected fractions assayed for activity towards CDNB and two GSH peroxidase substrates (cumene hydroperoxide and hydrogen peroxide; Fig.4.1.03), the bulk of the GSH peroxidase activity eluted in a peak prior to the GSH transferase activity. This is almost certainly GSH peroxidase A-type which has an M_r of 90,000 and shows activity towards both GSH peroxidase substrates. The remaining

Fig. 4.1.04 Elution profile from DEAE-A-50 sephadex of trout liver cytosol.



peroxidase activity appears in a second, less well defined peak, eluting with GSH transferase activity. This peak showed activity towards cumene hydroperoxide but not towards hydrogen peroxide a characteristic of GSH peroxidase B. The fact that two peaks of GSH peroxidase with different activities towards cumene hydroperoxide and hydrogen peroxide are separated by gel-filtration chromatography agrees well with the findings of Lawrence & Burk (1976) and suggests the presence of GSH peroxidase B in trout liver.

Clearly activity towards epoxide and peroxide substrates is found in trout liver, which warrants further investigation to determine which individual transferases such activities are associated with.

When dialysed trout liver cytosol was applied to a CM-C50 cation-exchange column (elution profile not shown), the GSH peroxidase activity was not bound to the column as it was associated with more acidic proteins. To try to obtain some resolution of activity, dialysed cytosol was reapplied to a DEAE-A50 anion-exchange column (Methods; 3.03.04) and the fractions collected assayed for GSH transferase and GSH peroxidase activities (Fig. 4.1.04). The GSH peroxidase activities towards both peroxide substrates eluted just behind the most acidic transferase peak as a broad peak. This indicated the presence of GSH peroxidase A; better resolution was required to see if GSH peroxidase B was also present. DEAE-A50 was not suitable as application of a salt-gradient to a column onto which cytosol had been pumped caused the gel to break up, presumably due to the presence of lipids in the cytosol. To achieve higher resolution, the technique of chromatofocusing was chosen (Sluyterman & Elgersma, 1978) as this method was known to separate the rat GSH transferases particularly well (Mannervik & Jensson, 1982).

When cytosol (partially purified by GSH affinity

chromatography; Simons & Vander Jagt, 1977) was applied to a PBE 94 chromatofocusing column (section 3.03.06) and eluted, the resultant profile (not shown) revealed that GSH peroxidase activity was eluted as a sharp peak in fractions following the last peak of GSH transferase activity. Furthermore, activities with both peroxide substrates co-eluted, indicating that the GSH peroxidase activity in trout liver is due to GSH peroxidase A. Earlier results (Fig. 4.1.03) had indicated the presence of GSH peroxidase B, but at this stage assay conditions had not yet been optimised and the results may have been artefactual. Alternatively, GSH peroxidase may have a low affinity for GSH-sepharose and be lost in the flow-through volume. However, no peroxidase B type of activity was observed to bind to S-hexylGSH sepharose either (results not shown) probably indicating the absence of this enzyme in trout liver cytosol. The very high GSH peroxidase A activity that was detected suggests that this enzyme would probably cope with any peroxides that were formed.

Further study of enzymes with GSH epoxide transferase activities were left until a purification scheme for the enzymes had been established so that overall substrate specificites could be determined.

Before chromatofocusing was chosen, in an attempt to improve resolution, the cation exchange step was scaled up and several hundred ml of dialysed cytosol were applied. The GSH transferase activity was subsequently separated into two groups called acidics (eluted with column running buffer) and basics (eluted with a salt-gradient). Both peaks were then partly purified using GSH affinity chromatography and applied to anion and cation-exchange columns respectively (using a shallower salt-gradient for the basic material). However, the basic material subsequently re-eluted where the acidic material would have eluted and it was decided to investigate the change as an affinity purification step was very important for the rapid

Fig. 4.2.01 Elution profile from CM C-50 sephadex of trout liver cytosol

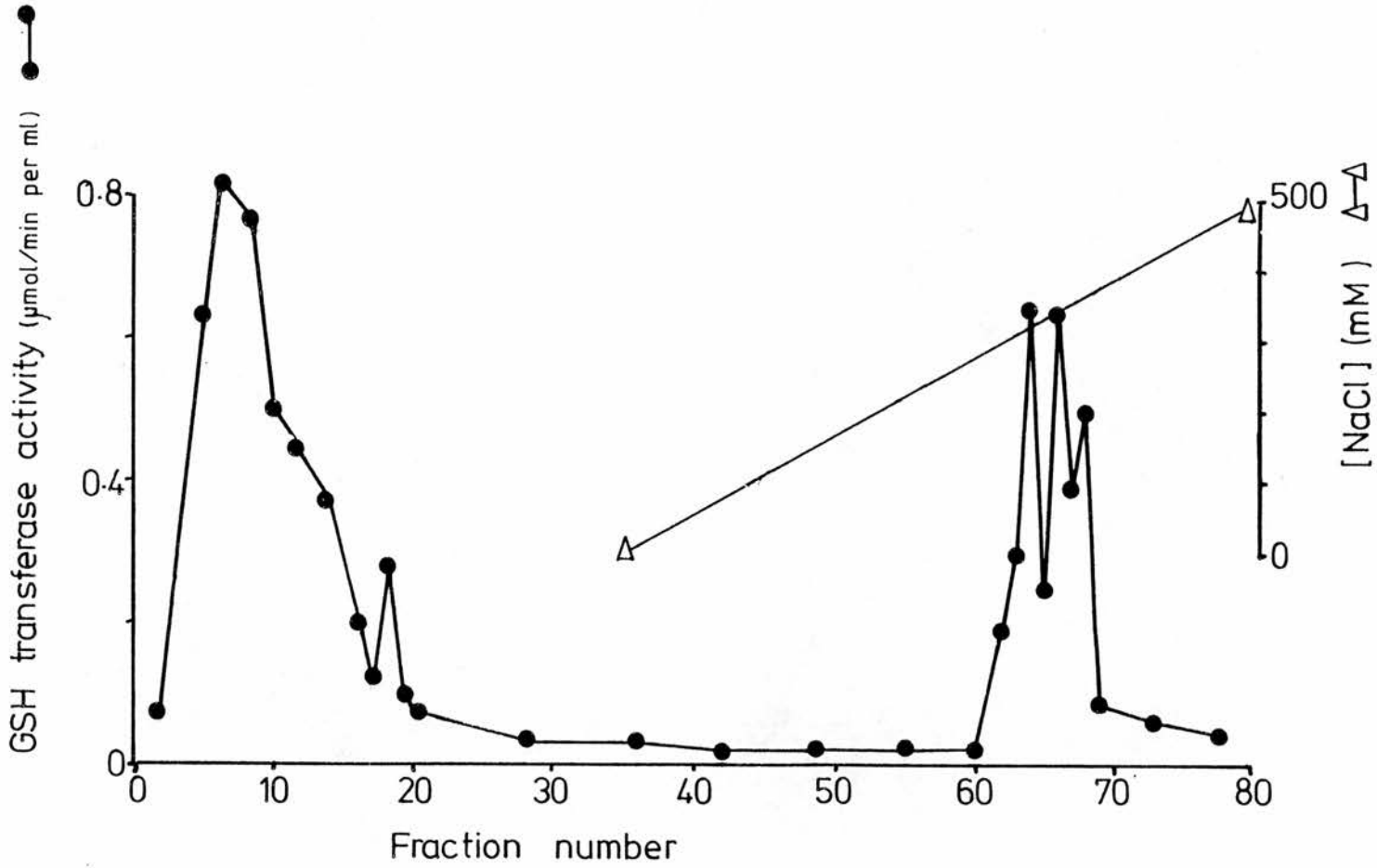


Fig.4.2.02 Elution profile from DEAE A-50 sephadex of the acidic GSH transferase fraction from CM C-50 sephadex, partially purified beforehand by GSH affinity chromatography.

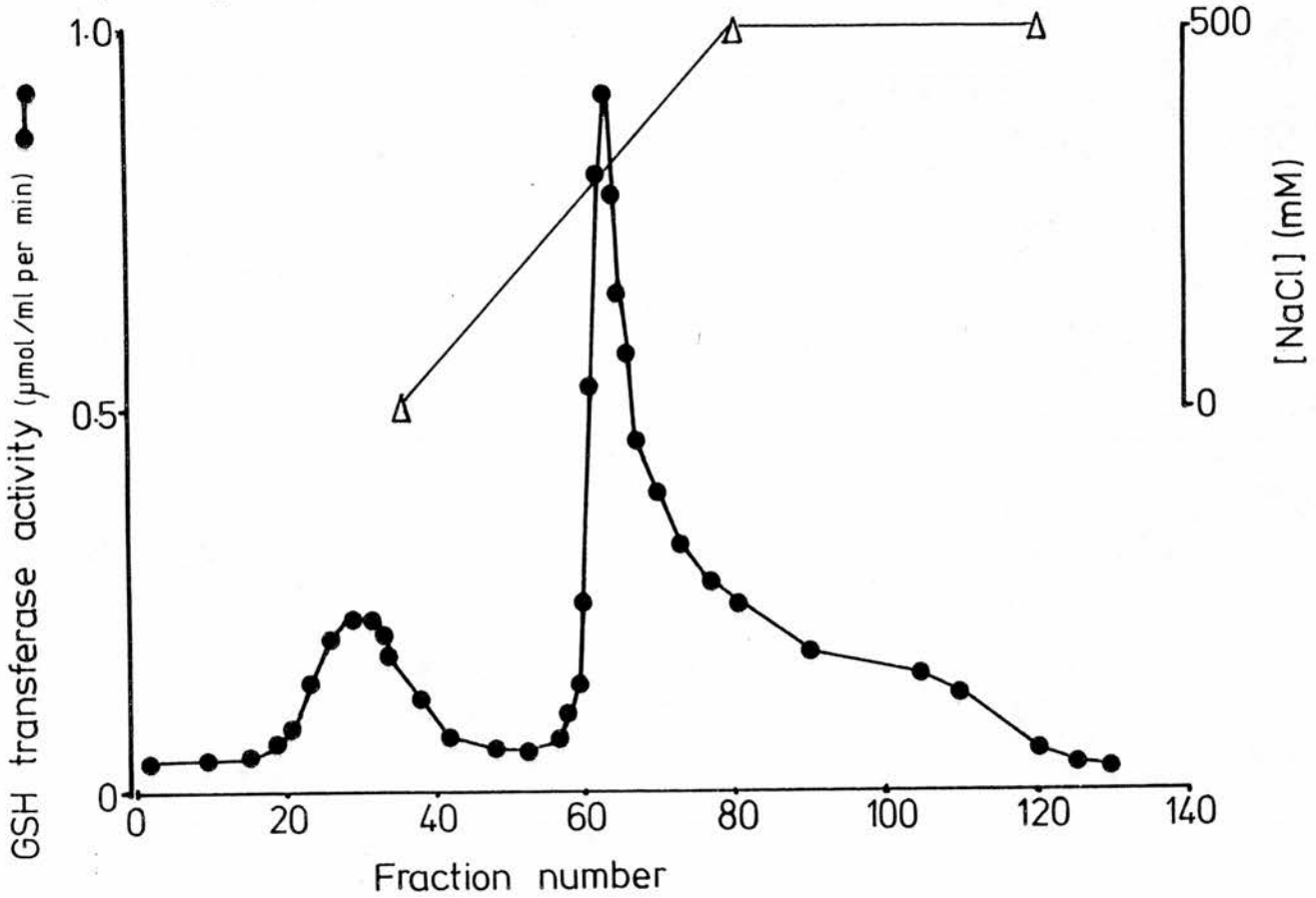


Fig.4.2.03 Elution profile from CM C-50 of the basic GSH transferase fraction from CM C-50 sephadex, partially purified by GSH affinity chromatography before reapplication to the cation-exchange resin.

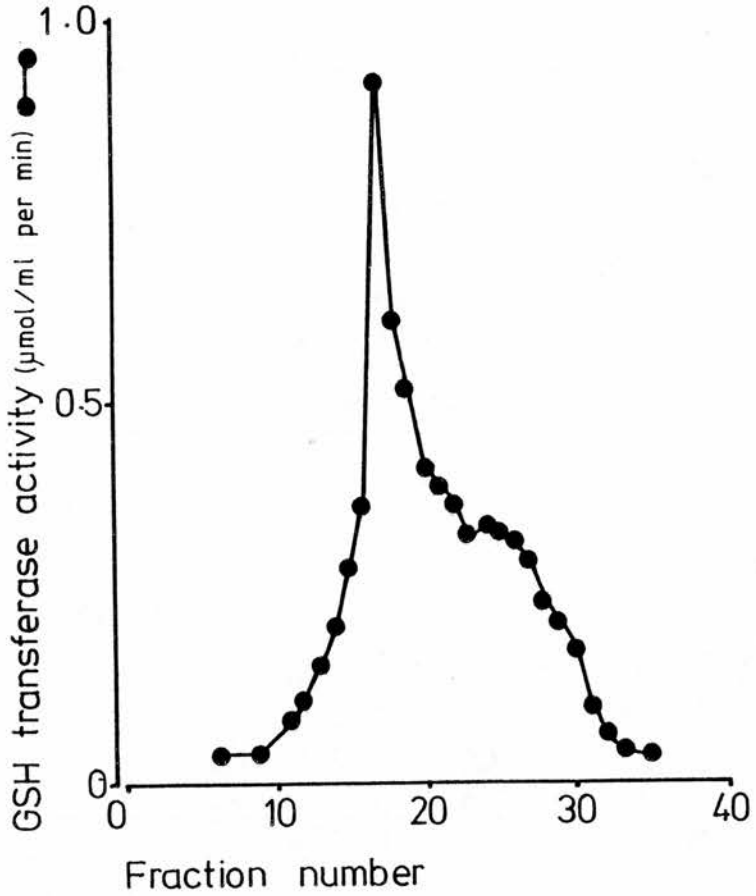


Fig. 4. 2.04 Elution profile from CM C-50 sephadex of trout liver cytosol (15ml) dialysed overnight.

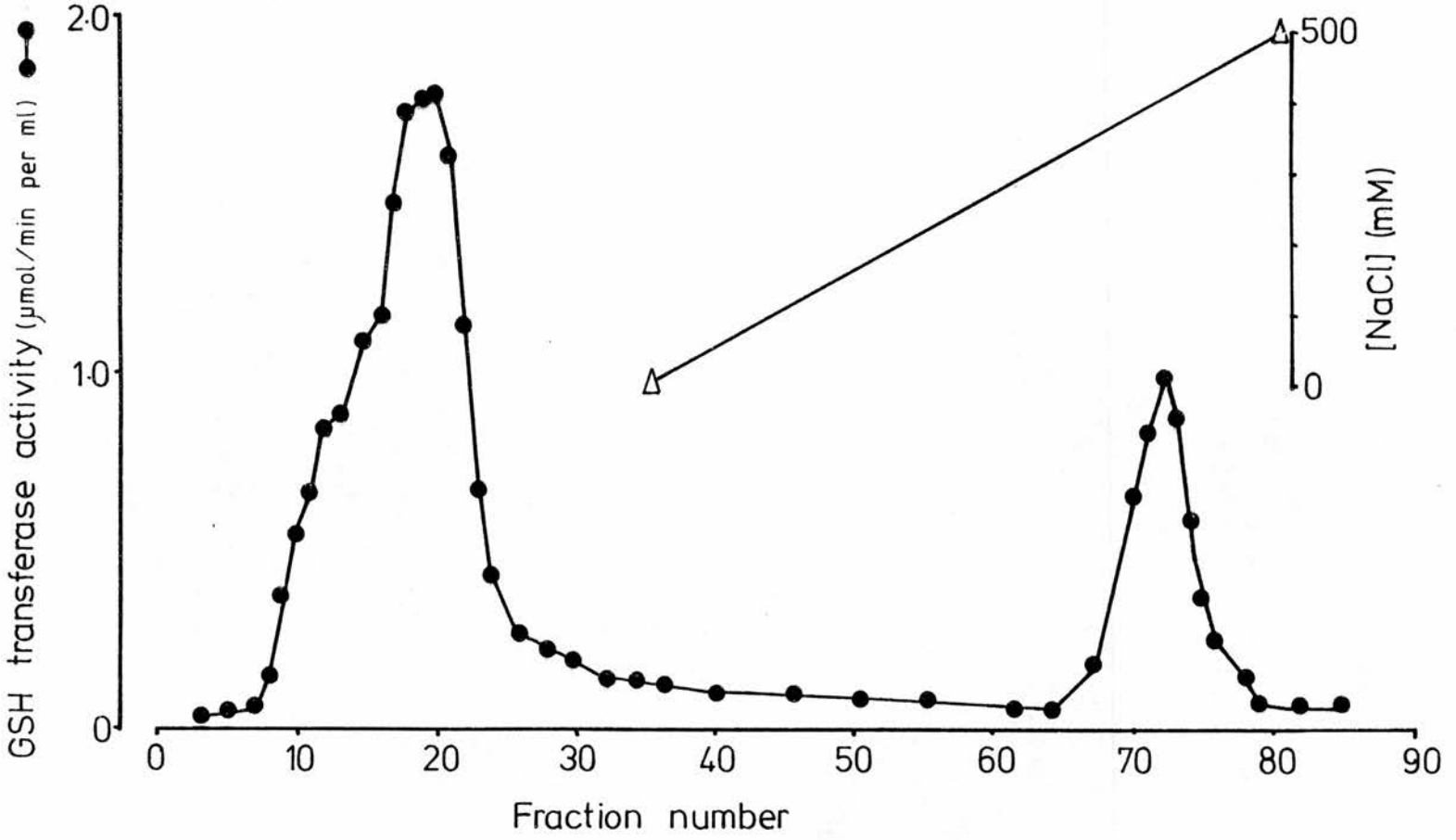
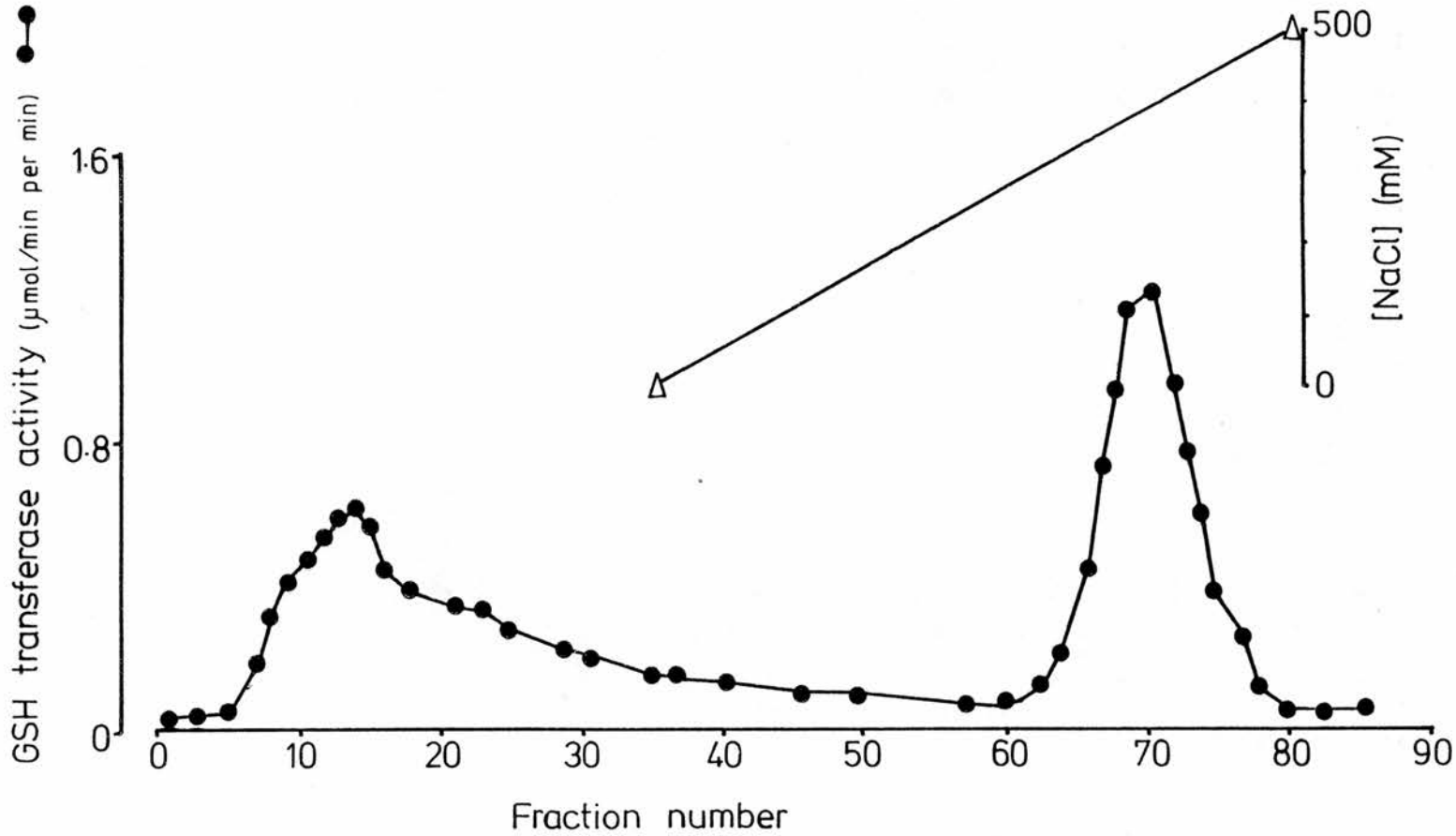


Fig. 4.2.05 Elution profile from CM C-50 sephadex of trout liver cytosol (15 ml) dialysed 80 h.



purification of these enzymes. The change implied that the pI's of the basic transferases were being lowered by the chromatographic step.

4.2: THE EFFECT OF GSH AFFINITY CHROMATOGRAPHY ON THE ISOELECTRIC BEHAVIOUR OF THE GSH TRANSFERASES OF RAINBOW TROUT

When dialysed cytosol is applied to CM-C50 sephadex (methods section 3.03.03) the GSH transferase activity is eluted in two fractions. An acidic fraction is eluted first followed by a more basic fraction which is eluted using a 0-500mM-NaCl gradient (Fig. 4.2.01). Both fractions appear to contain several GSH transferases as the acidic peak shows a shoulder of partially retarded material followed by a peak of activity and the basic fraction contains several peaks. To resolve these constituents, both fractions were partially purified by GSH-affinity chromatography and then applied to DEAE-A50 and CM-C50 ion-exchangers respectively.

The acidic fraction was resolved into at least two forms (Fig. 4.2.02), but the basic material was eluted from the cation-exchanger before the salt-gradient was applied (Fig. 4.2.03). This suggests that the basic material had undergone a change in isoelectric behaviour (as both samples were extensively dialysed before chromatography to ensure they were both equilibrated and desalted). The effect was either due to the affinity chromatography step itself or due to the time factor in reapplying the protein to the cation- exchanger. Deamidation, an ageing process in proteins might conceivably cause this to occur.

To investigate this, cytosol was divided into two equal portions; one portion was dialysed overnight and applied to the cation-exchanger (Fig.4.2.04) whilst the other portion was dialysed for 80h before chromatography (Fig.4.2.05). The

Fig. 4.2.06 Elution profile from CM C-50 of dialysed GSH transferase activity after GSH affinity chromatography.

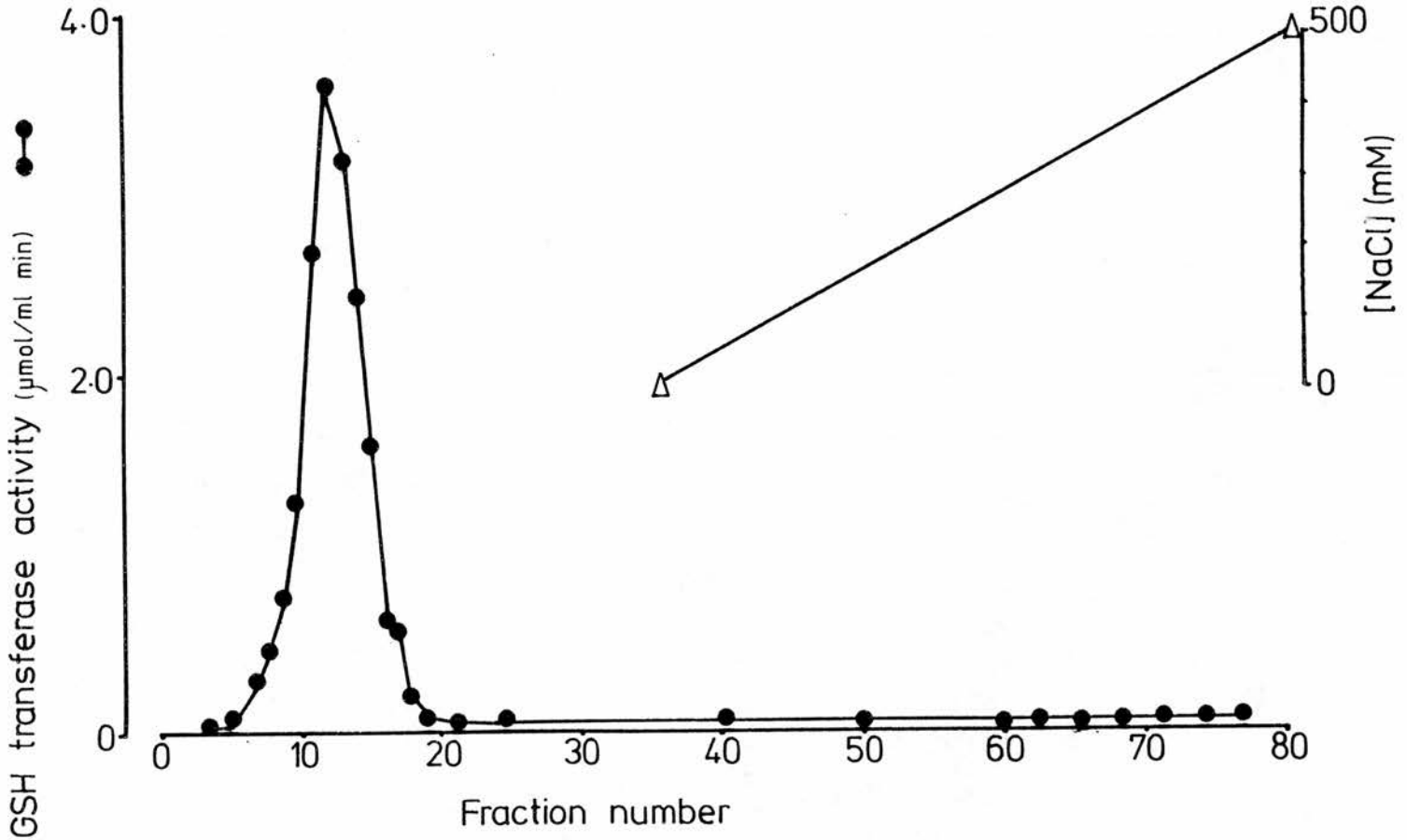
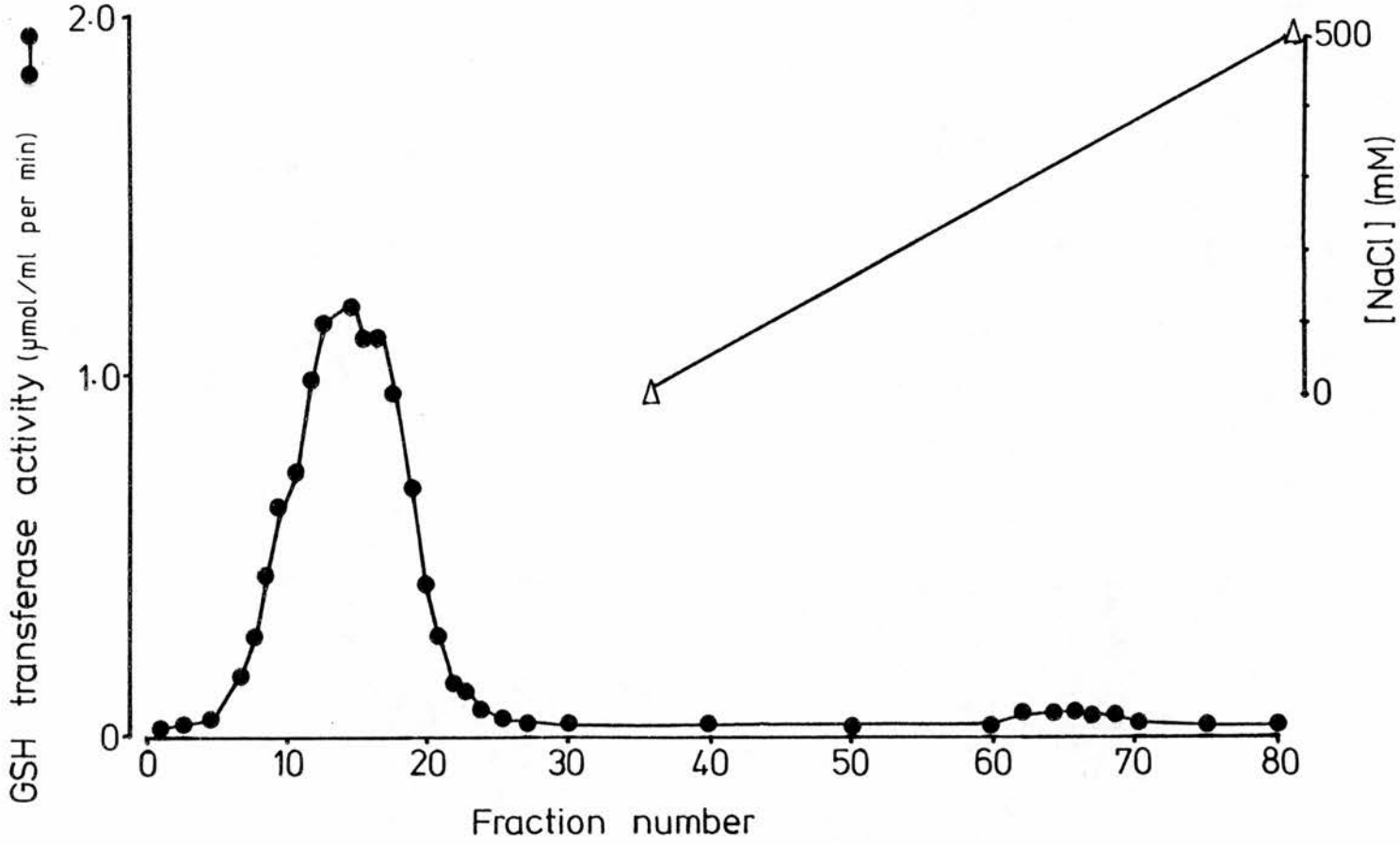


Fig.4.2.07. Elution profile from CM C-50 of GSH transferase activity after GSH affinity chromatography and dialysis (36h) against buffer containing in addition, 10mM-2-mercaptoethanol.



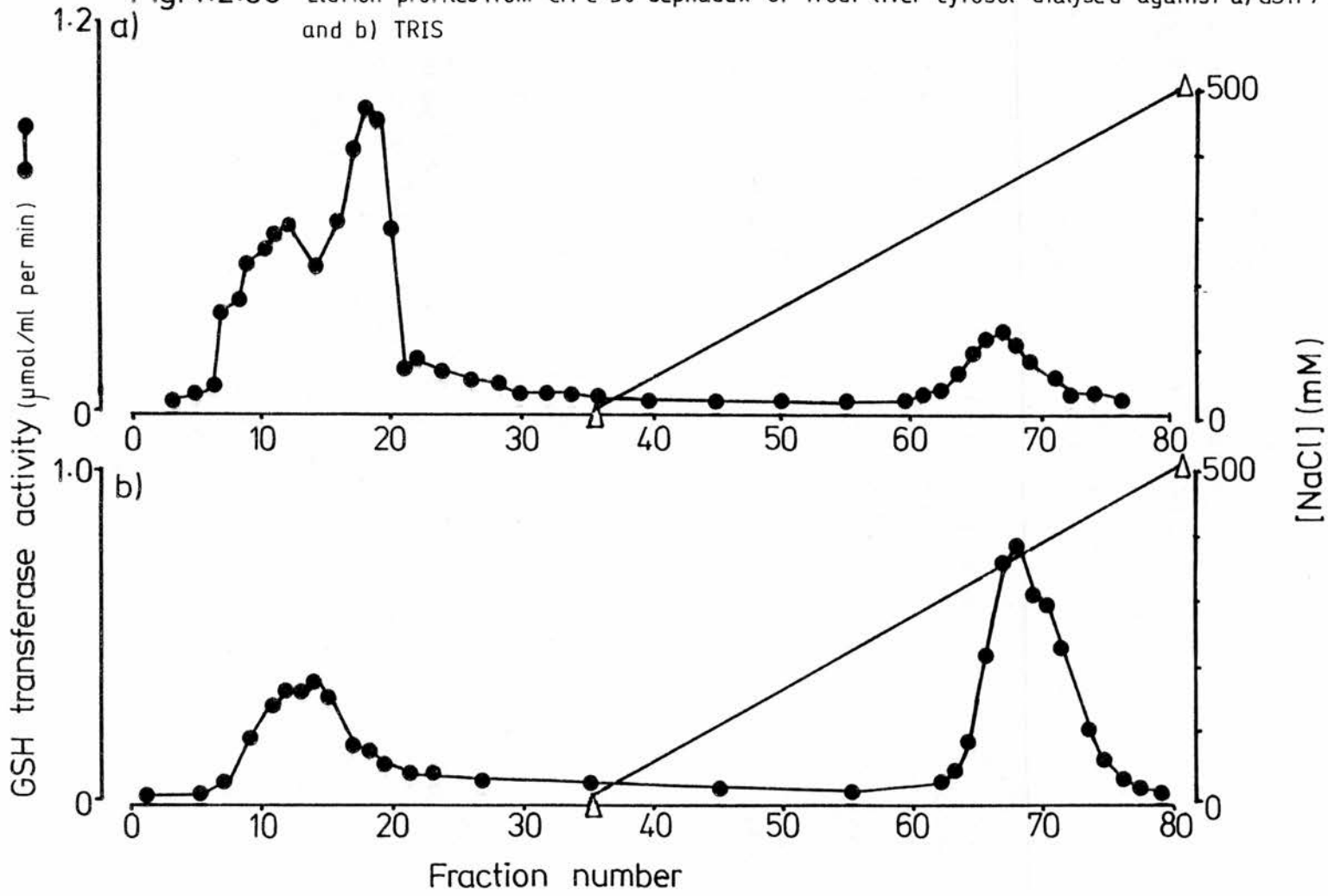
results show clearly that the effect is not time-dependent, and if anything, is diminished by the greater stability of the basic GSH transferase activity. The absorbances at 280nm for the two figures, although not included, show that the decrease in the activity of the acidic fraction is not mirrored by an absorbance decrease and is therefore due to instability. These results indicate that affinity chromatography is responsible for the alteration of the isoelectric behaviour of the proteins.

As the enzymes are eluted from the GSH affinity column with 100mM-TRIS buffer pH 9.6 containing 10mM-GSH, oxidized GSH (GSSG) will be formed and may participate with protein thiol groups in thiol-exchange reactions to form mixed- disulphides (Jocelyn, 1972) leading to a lowering of the isoelectric points of the proteins. However, if this is the case it should be possible to re-reduce the disulphides by treatment with strong reducing agents.

To test this theory, cytosol was partially purified by affinity chromatography and divided into two portions. One portion was dialysed overnight before chromatography (Fig. 4.2.06) and the other was dialysed with buffer containing in addition, 10mM-2-mercaptoethanol before chromatography (Fig. 4.2.07). Dialysis against mercaptoethanol produced a slight change manifested as a broadening of the acidic peak (meaning greater retention) and a miniscule basic fraction. These results were followed up by treating one portion of activity from the affinity column with GSH reductase (50 U) and 1mM-reduced NADP and by dialysing another portion for 36h against buffer containing 10mM-DL-dithiothreitol. Both treatments produced small changes (results not shown).

The next stage of the investigation involved checking the conditions required for elution from the affinity matrix. If oxidation of GSH were being increased by the high pH of elution, then a reduction of that pH might alleviate the effect. However, activity could not be eluted from the

Fig. 4.2.08 Elution profiles from CM C-50 sephadex of trout liver cytosol dialysed against a) GSH / TRIS and b) TRIS



column with 10mM-GSH in 22mM-phosphate buffer pH 7.0 or by GSSG at either pH 7.0 or 9.6 but only by GSH in TRIS buffer pH 9.6 (results not shown). As this was the case it was decided to find out if the alteration of pI's was due to GSH, the pH or both.

Cytosol was divided into two portions; one portion being dialysed overnight against 100mM-TRIS pH 9.6 and the other against 5mM-GSH in 100mM-TRIS. Both portions were subsequently dialysed overnight against column buffer before chromatography (Figs. 4.2.08a and 4.2.08b). In Fig. 4.2.08a, the basic fraction is reduced, whereas that of the acidic fraction is increased. This implies that some of the basic fraction elutes with the acidic fraction. In 4.2.08b the elution profile is unchanged apart from the decrease in enzyme activity of the acidic peak. The results showed very clearly that GSH was required for the effect and that pH alone was not responsible. In a further experiment, the affinity column was developed with buffers which had all been degassed under vacuum, saturated with nitrogen and contained 10mM-DL-dithiothreitol. The eluted material was then chromatographed on the cation-exchanger as before (elution profile not shown). An increase in the height of the basic peak was observed along with a broadening of the acidic peak suggesting that the oxidation of GSH to GSSG was involved in the effect. By expressing the elution profiles as a ratio of the acidic to the basic fractions, comparisons can be quickly made. The normal ratio expected is in the range 1 - 3. After purification using GSH affinity chromatography this increased to greater than 50 but reduced to about 6 by the use of degassed buffers containing dithiothreitol. However a ratio of 6 was still too large to be acceptable.

SDS-polyacrylamide electrophoresis (results not shown) gave no indication of any alteration of the mobilities of the enzyme subunits, but small changes caused by the addition of a small molecule such as GSH were unlikely to

Fig. 4.2.09 Elution profiles from GSH and S-hexyl GSH sepharose 6B of trout liver cytosol.

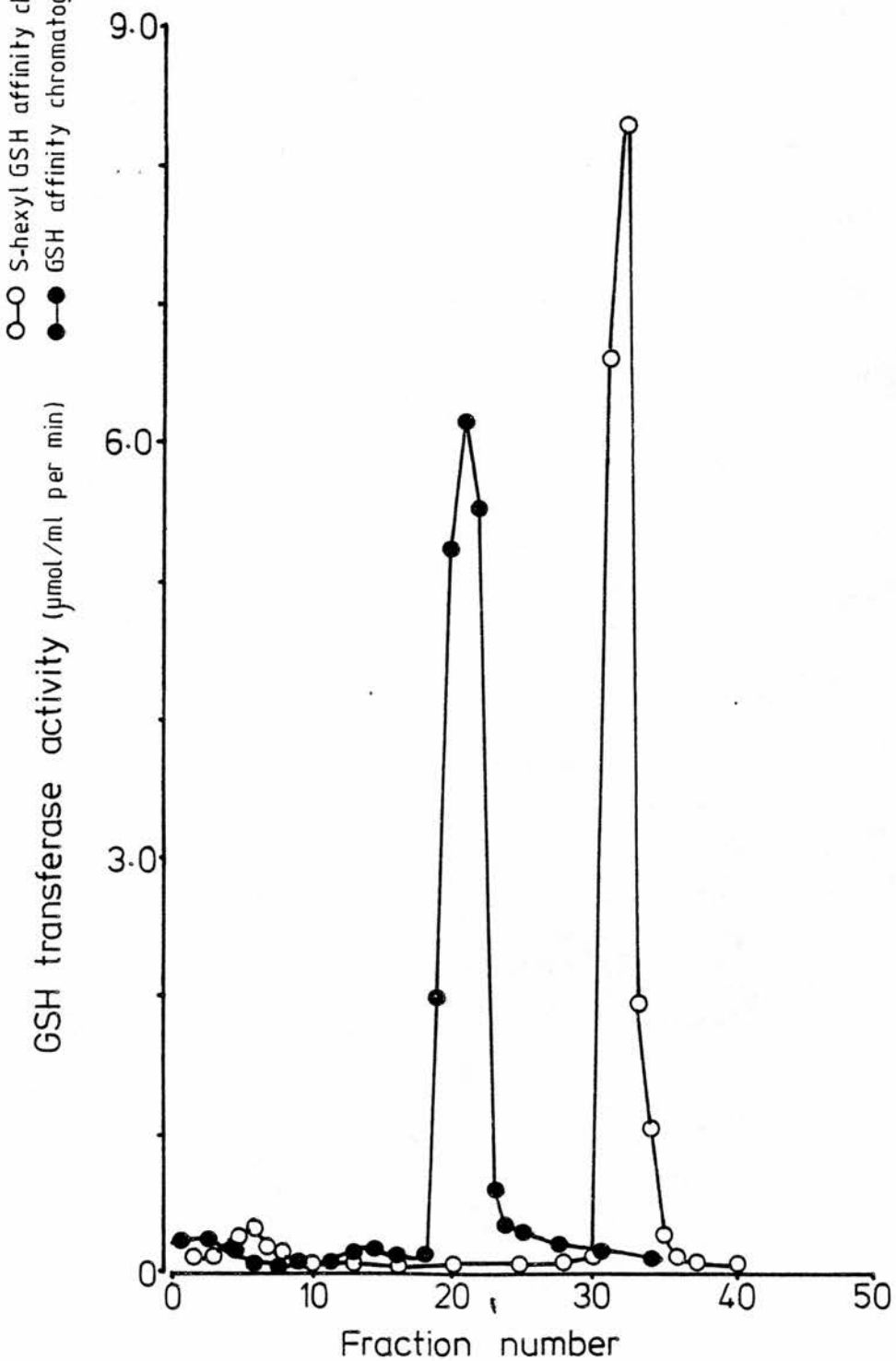
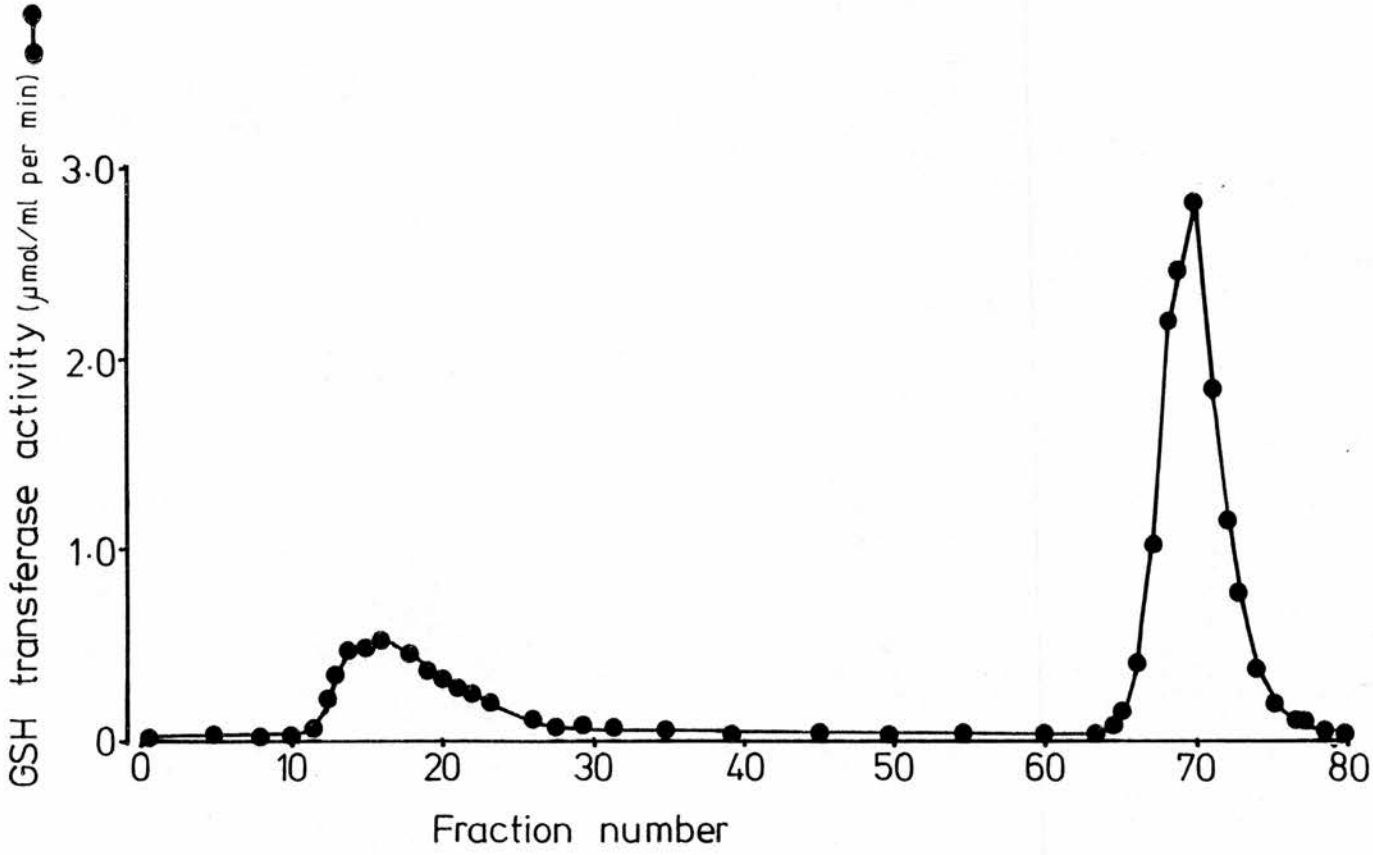


Fig.4.2.10 Elution profile from CM C-50 sephadex of GSH transferase activity after S-hexyl GSH affinity chromatography.



have been detected by the gel system used at that time.

Modifying the conditions of elution to prevent the effect had proved ineffective and as an alteration of the proteins was unacceptable a different form of affinity chromatography was chosen. This was because in order to achieve maximum resolution of isoenzymes using chromatofocusing, the amount of protein applied to the column should be minimized (Sluyterman & Wijdenes, 1978). S-Hexyl GSH affinity chromatography had been used very effectively by Mannervik & Jensson (1982), so this type of matrix was then made and its effectiveness compared with the GSH affinity column (Fig. 4.2.09).

The results show that the S-hexylGSH matrix is more efficient, producing a sharper peak of GSH transferase activity, which was subsequently applied to CM-C50 sephadex. The resulting elution profile (Fig. 4.2.10) is very similar to Fig. 4.2.05 in that the basic fraction is unaltered and larger than the less stable acidic fraction (ratio of acidic to basic = 0.5); this increase may also have been due to the basic enzymes having a higher affinity for the matrix as about 5% of the total activity passed straight through the column. Clearly S-hexylGSH affinity chromatography is very suitable for use with the GSH transferases from rainbow trout.

The effect caused by GSH affinity chromatography appears to be due to covalent linkage of GSH to the proteins to form mixed disulphides. An ideal final experiment would have been to have eluted the GSH affinity column with radiolabelled GSH, to have applied the GSH transferase activity to a gel- filtration column and assayed the fractions for transferase activity and radioactivity. Unfortunately the cost of radiolabel was to prove prohibitive. S-hexylGSH cannot affect the proteins in the same way as it cannot form a disulphide as its cysteinyl thiol group is blocked by the hexyl residue.

Several authors report using GSH affinity chromatography prior to pI determinations (Awasthi et al., 1980; Irwin et al., 1980; Nishiya et al., 1981) and ion-exchange chromatography (Saneto et al., 1980; Simons & Vander Jagt, 1980). If the effect is seen to occur to the same extent in other species as it does in rainbow trout, then several pI values and elution profiles may have to be re-evaluated.

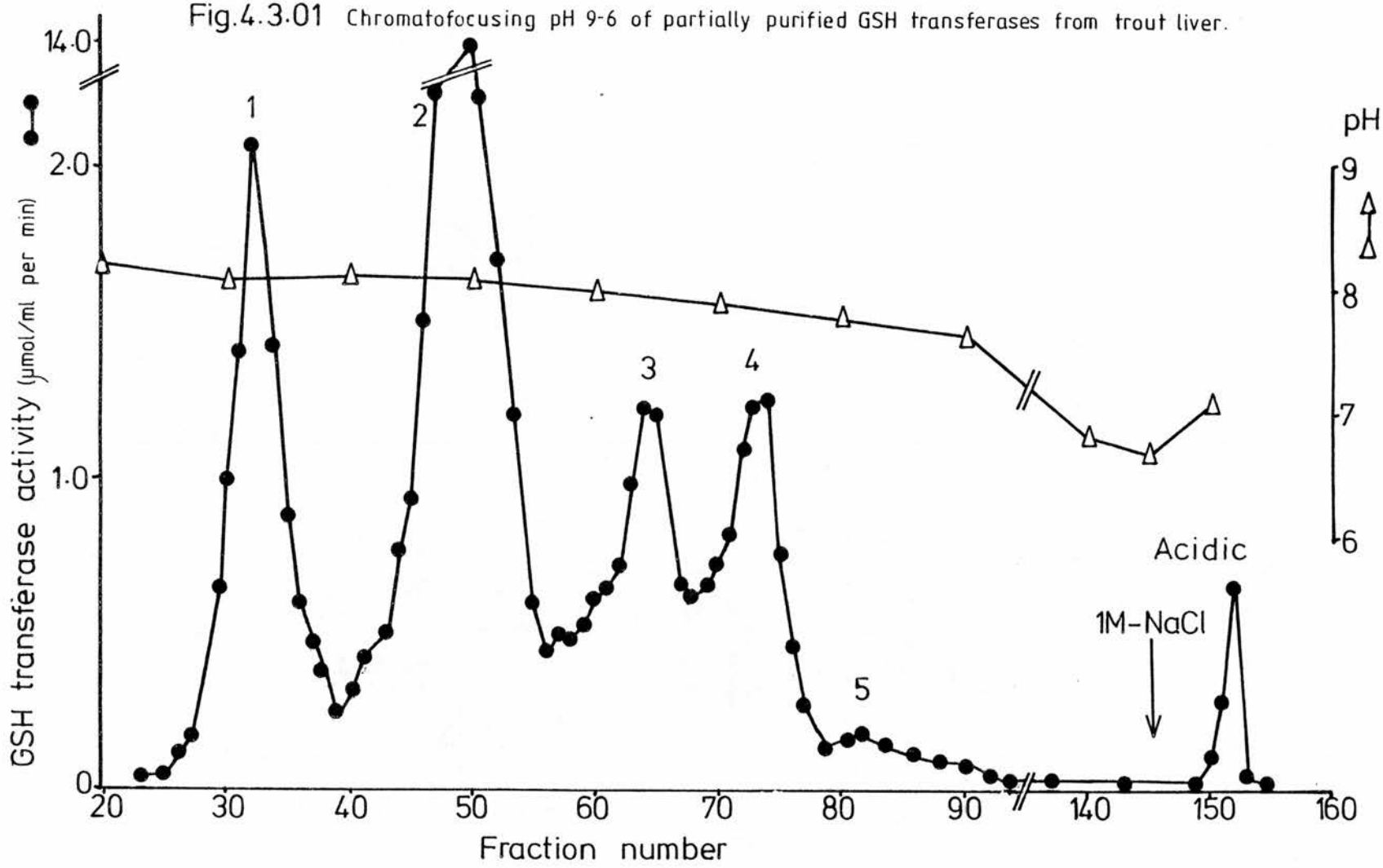
The suitability of S-hexylGSH affinity chromatography meant that chromatofocusing of trout liver GSH transferases with a view to characterizing individual enzymes could now proceed.

4.3: CHROMATOFOCUSING OF THE GSH S-TRANSFERASES OF RAINBOW TROUT

Chromatofocusing (Sluyterman & Elgersma, 1978) is a high resolution technique based on ion-exchange chromatography and isoelectric focusing. Samples are usually eluted from conventional ion-exchange resins by changing the pH, by increasing the ionic strength or by applying a counter-ion. Chromatofocusing uses the pH change method, but instead of applying a pH gradient formed by mixing two buffers externally, a gradient is formed on the column itself. This is achieved by using the buffering action of the charged group on the ion-exchanger and eluting the column (equilibrated at a higher pH) with buffer of a lower pH.

To discuss the theory a number of assumptions have to be made. These are that, if all the proteins are positively charged at the starting pH they will run with the buffer, that if they are negatively charged they will be completely retarded and that the buffer flowing through the column is divided into an infinite number of theoretical plates. Therefore, a protein with a pI of 9.0 will run down the column with the buffer until a point is reached where the

Fig.4.3.01 Chromatofocusing pH 9-6 of partially purified GSH transferases from trout liver.



pH of the buffer aliquot is slightly greater than 9.0. The protein is now negatively charged and binds to the column. When a second buffer aliquot passes, the pH is lowered and the protein moves down the column until retarded by a pH greater than 9.0 again. If the second aliquot had contained some of the same protein a focusing effect would have been achieved at the second point of retardation.

The process continues and proteins with different pI's can overtake each other on the column until finally the proteins are eluted in order of decreasing pI, the pH at which they are eluted is known as the apparant pI, this may or may not equal the true pI of the protein. The performance of such columns is measured as the band-width of eluted peaks. Peaks eluted in the mid-range of the gradient are optimally resolved; the band width of peaks increases with both ionic strength (which increases during the run) and with protein concentration. Consequently, the buffers used with such columns are very dilute. The conditions laid down in section 3.03.06 for sample preparation and column equilibration are essential for the maximal performance of this method.

The first section of work deals with rainbow trout obtained from the Penicuik trout farm before its closure in the summmer of 1983. Preparations were based on the livers from several individual trout to provide more material and to limit the differences between preparations caused by the variation of individuals.

Initially cytosol that had been purified using affinity chromatography was chromatofocused in the pH range 9-6 to find out roughly where the transferases eluted and to obtain approximate values for their apparant isoelectric points. The elution profile (Fig. 4.3.01) obtained showed at least five basic and one acidic transferase although several of the peaks had prominent shoulders. One of the basic forms (2) comprised 65% of the total activity. The run was

Fig.4.3.02 Chromatofocusing pH 9-7 of partially purified GSH transferases from trout liver.

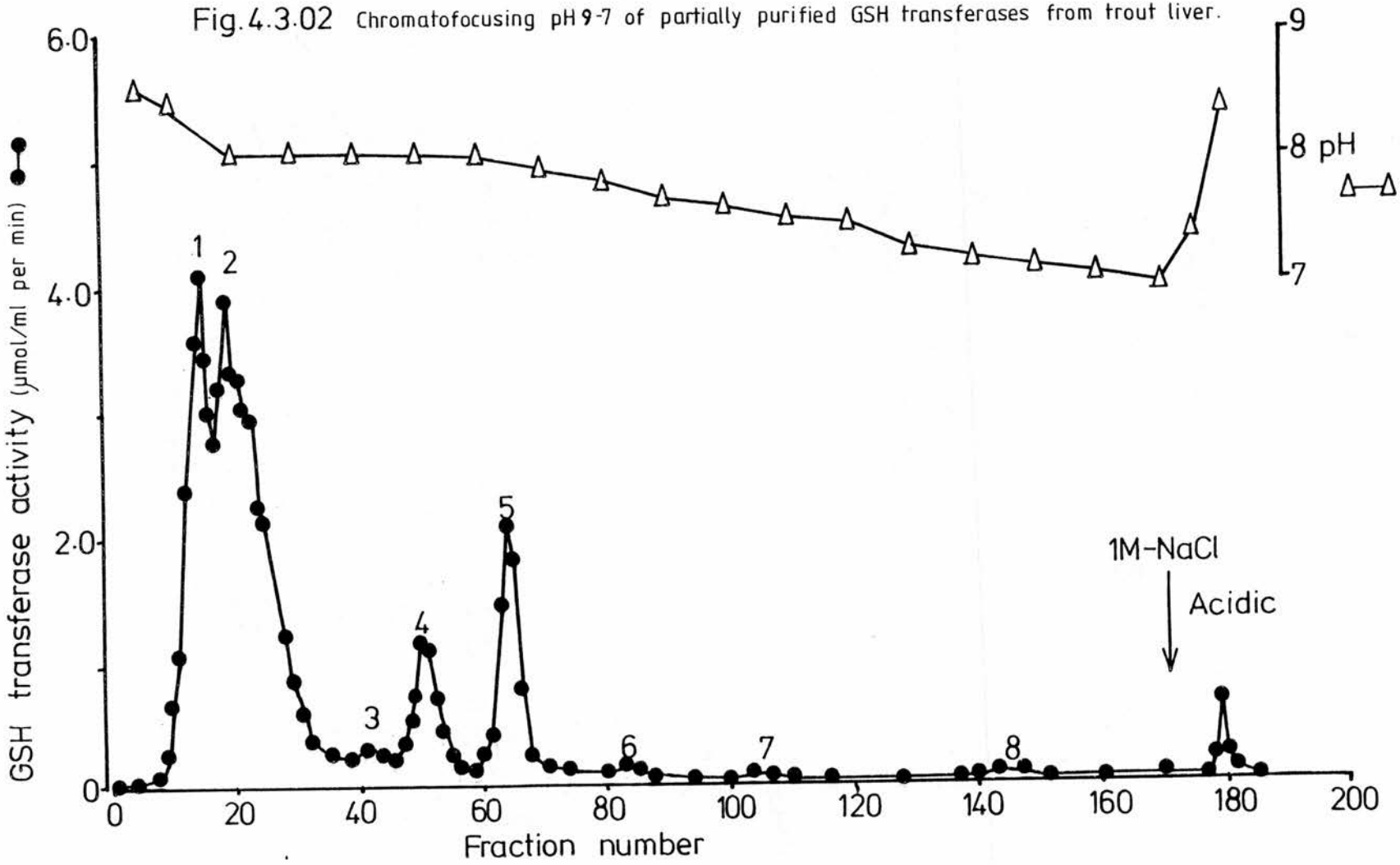


Fig.4.3-03 Chromatofocusing pH 9-7 of partially purified GSH transferases from trout liver.

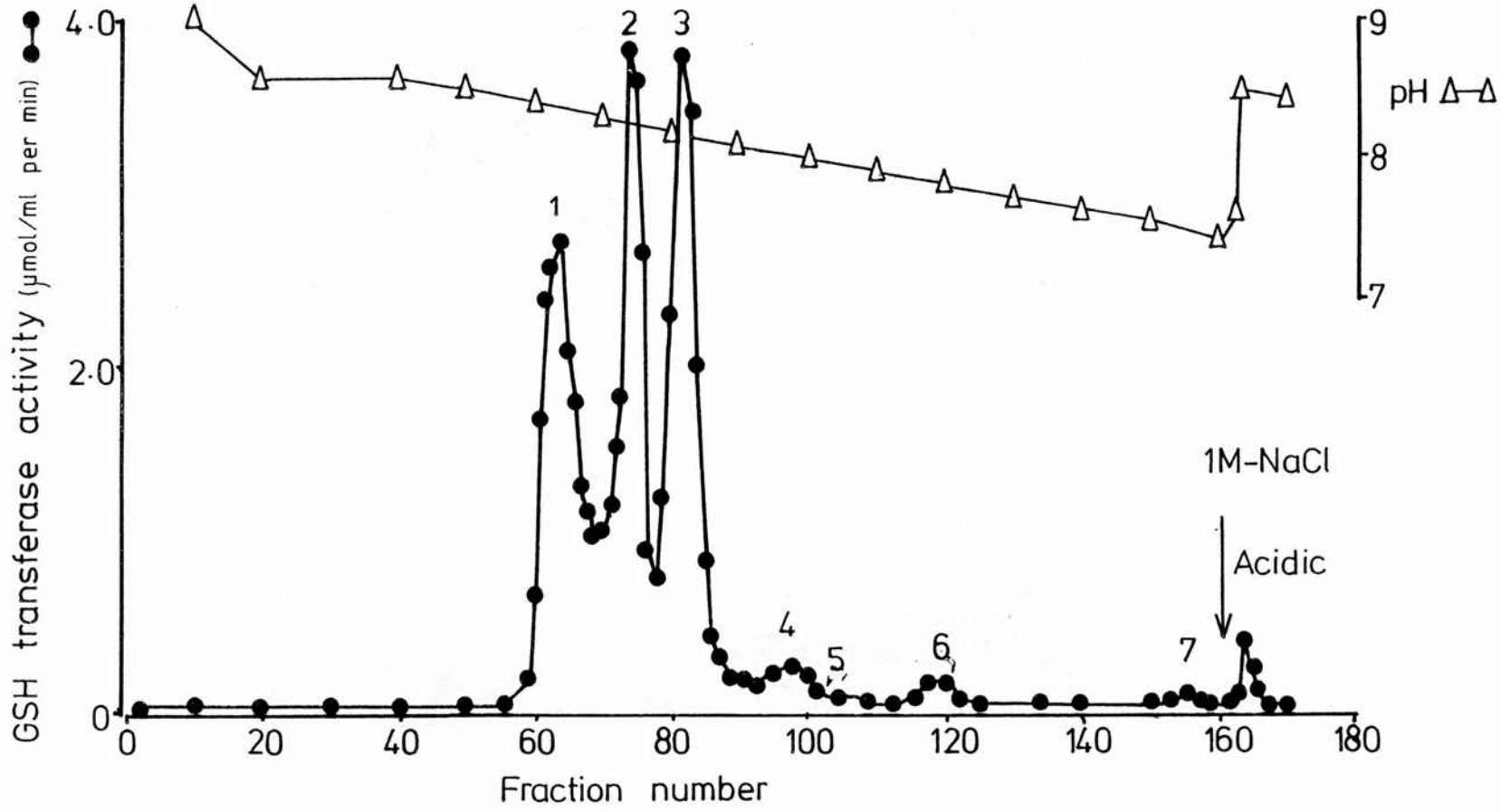


Fig. 4.3.04 Chromatofocusing pH 9-7 of basic GSH transferases from trout liver, partially purified by chromatofocusing pH 8-5 and affinity chromatography.

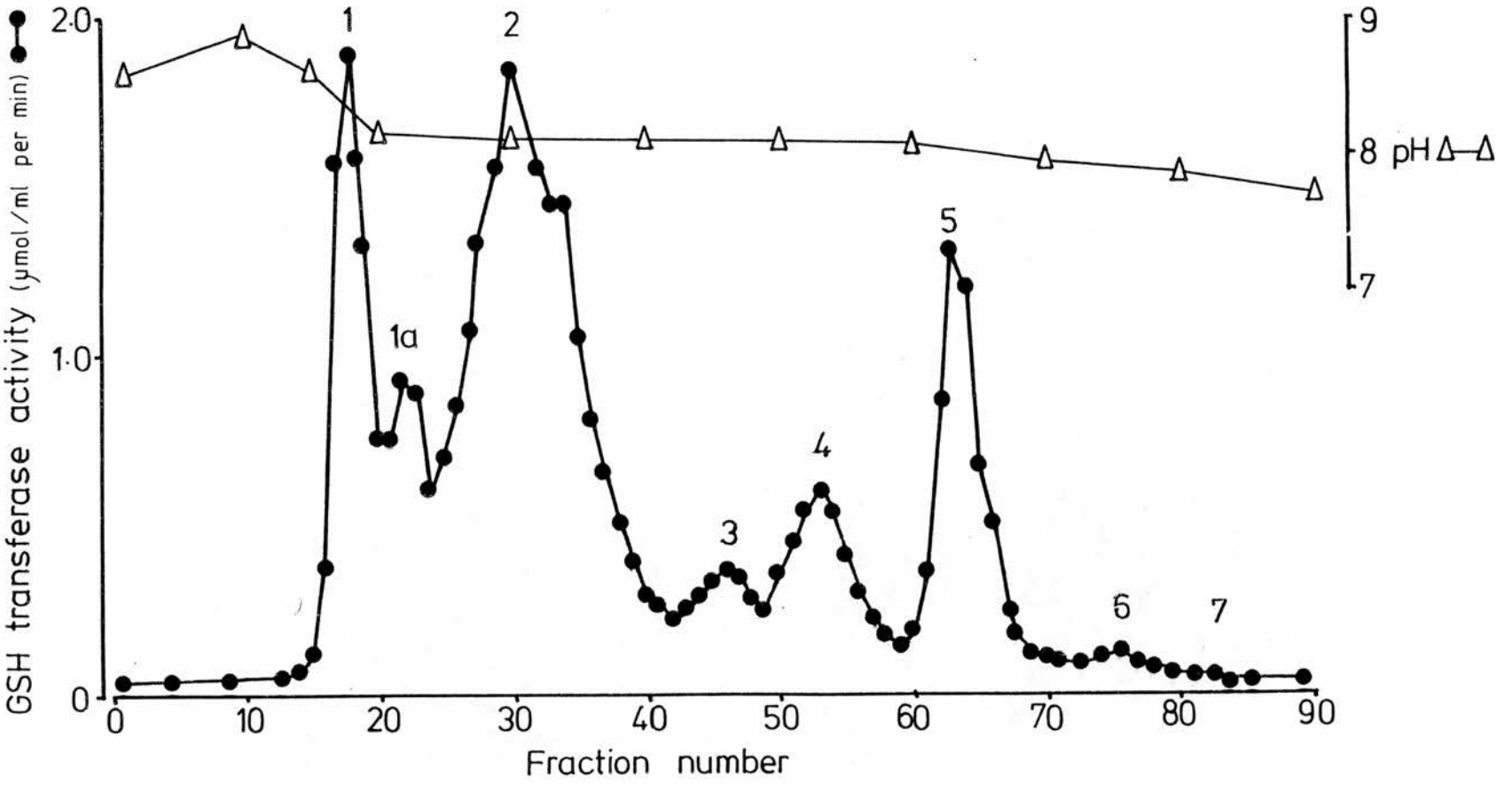


Table 4.3.01. A comparison of the apparent pI values of GSH transferases from rainbow trout from Penicuik and Beecraigs trout farms.

	Penicuik				Beecraigs	
	$pI_{apparent}$ values from chromatofocusing profiles					
Peak no.	4.3.01	4.3.02	4.3.03	4.3.04	4.3.06[1]	[2]
1	8·18	8·20	8·39	8·30	8·40	8·40
2	8·10	8·08	8·28	8·15	8·25	8·30
3	8·00		8·22	8·10	8·10	8·10
4	7·88	8·08	8·00	8·07	7·95	7·85
5	7·72	8·00		8·05	7·75	7·70
6		7·82	7·82	8·00		
7				7·90		

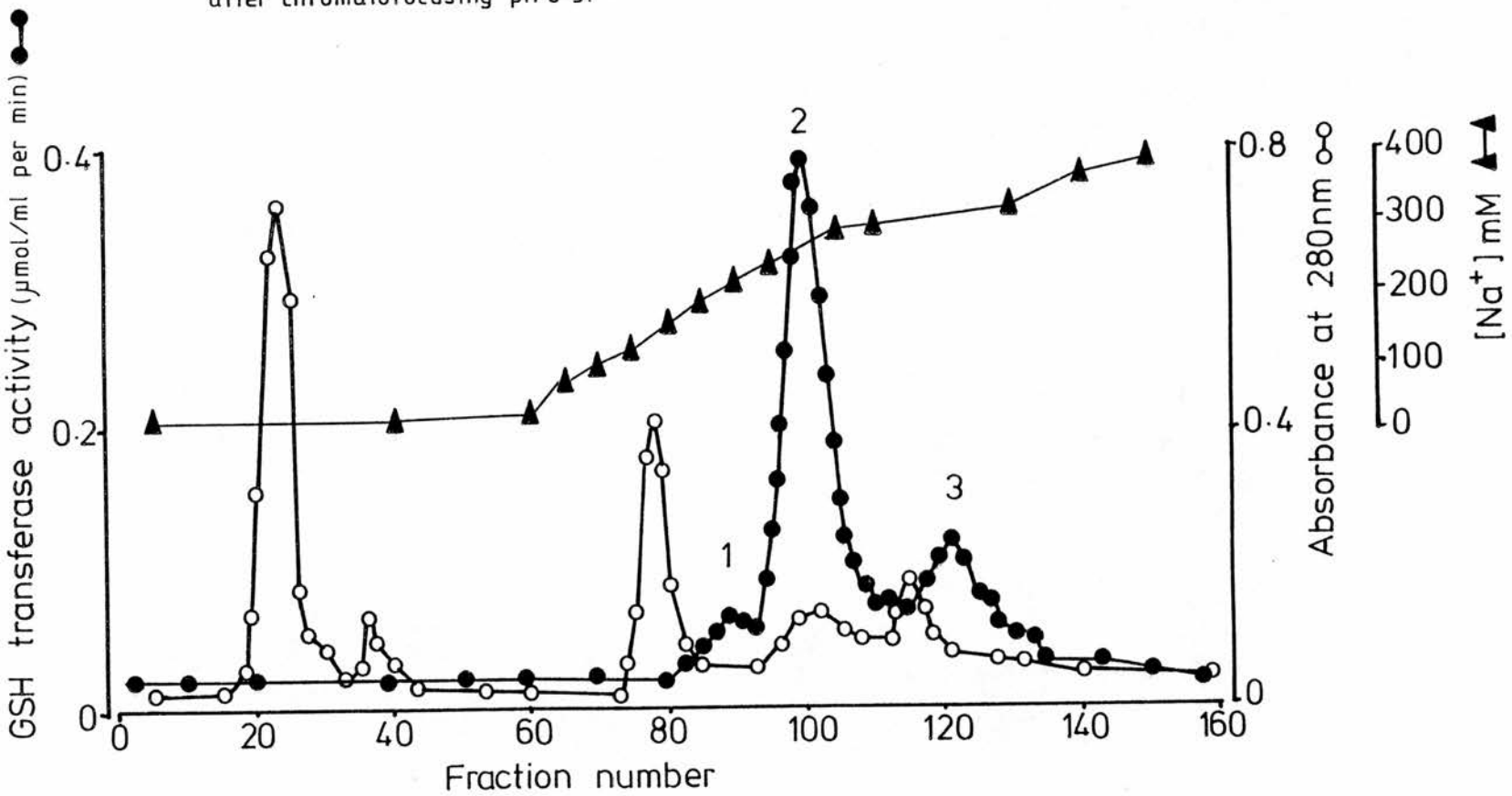
repeated using a different gradient (pH 9-7) because the basic peaks all eluted in the pH range 8-7. The profile obtained in Fig. 4.3.01 was unacceptable because the pH gradient fell too much at the start of the run. Although not shown in the figure, the pH of fraction 1 was only 8.5. A drop in pH is experienced at the start of a run (probably caused by a pulse of ions as the effect is almost instantaneous) but for a column properly equilibrated at pH 9.4 a drop to about pH 8.9 is the smallest drop obtained and is deemed acceptable.

Several chromatofocusing runs (pH 9-7) were carried out and the profiles obtained showed a certain amount of variation (Figs. 4.3.02; 4.3.03; 4.3.04). In preparations using a greater number of livers a number of extra cationic peaks (6- 8) were found, but in such small amounts that they were impossible to look at in any detail. The rapid drop in pH during the start of elution profile 4.3.04 led to the assumption that the extra cationic peak (1a) was part of peak 1. To compare the results the apparent pI's (pI_{app}'s) of the peaks were measured (the pH's at which they were eluted) for all four profiles and are shown in Table 4.3.01.

A number of differences are seen in the pI_{app} values for the Penicuik fish, particularly in the peaks eluted first from the columns. It can also be seen that in all the Penicuik profiles except 4.3.03, proteins were beginning to elute before the pH gradient had properly stabilized. This is due to inadequate equilibration of either the column or of the sample itself. As a result as far as pI_{app} values are concerned Fig 4.3.03 is the most reliable.

Because about 5% of the enzyme activity passed straight through the affinity column (S-hexylGSH) in the purification step prior to chromatofocusing, this non-bound material was itself chromatofocused (pH 9-6) with a view to finding out what type of enzymes it was composed of. Increasing the size of the affinity column failed to prevent

Fig. 4.3.05 Elution profile from hydroxylapatite of the acidic fraction from trout liver cytosol after chromatofocusing pH 8-5.



this material from passing straight through, indicating that the capacity of the column was not being exceeded. The results (not given) showed clearly that the bulk of the activity occurred in an acidic GSH transferase peak.

This meant that there were at least 2 forms of acidic transferase with different affinities for S-hexylGSH-sepharose. To see if the two forms could be separated by chromatofocusing, cytosol was applied to a column (pH 8-5 gradient) and omitting the affinity chromatography step. The basic forms were removed after elution and purified by affinity chromatography and re-chromatofocused (pH 9-7; Fig. 4.3.04) so as not to waste material.

The lower pH gradient failed to resolve the acidic forms, nor were they resolved by a linear 0-1M-NaCl gradient that was applied (elution profile not shown).

In a final attempt at resolution, the acidic peak was dialysed (100 vol.; 24h; 3 changes) against 10mM-sodium phosphate buffer pH 6.7 and applied to a hydroxylapatite column (methods section, 3.03.07). The resultant elution profile (Fig. 4.3.05) showed that three forms of acidic GSH transferase had been resolved. This meant that as many as eight cationic and three anionic forms had been found; whether these represented distinct transferases or merely charge isomers remained to be seen.

Because of the proposed role of the transferases in detoxication, a preparation of microsomal material was also made to look for the presence of microsomal forms of the enzyme (methods section, 3.14). However, no activity towards 1-chloro-2,4-dinitrobenzene was detected in the fraction and as the material was unsuitable for assaying with other substrates because of turbidity problems due to the impure nature of the material, the work was carried no further. Multiple forms of cytosolic transferase had been found, but no such activity in the microsomal fraction.

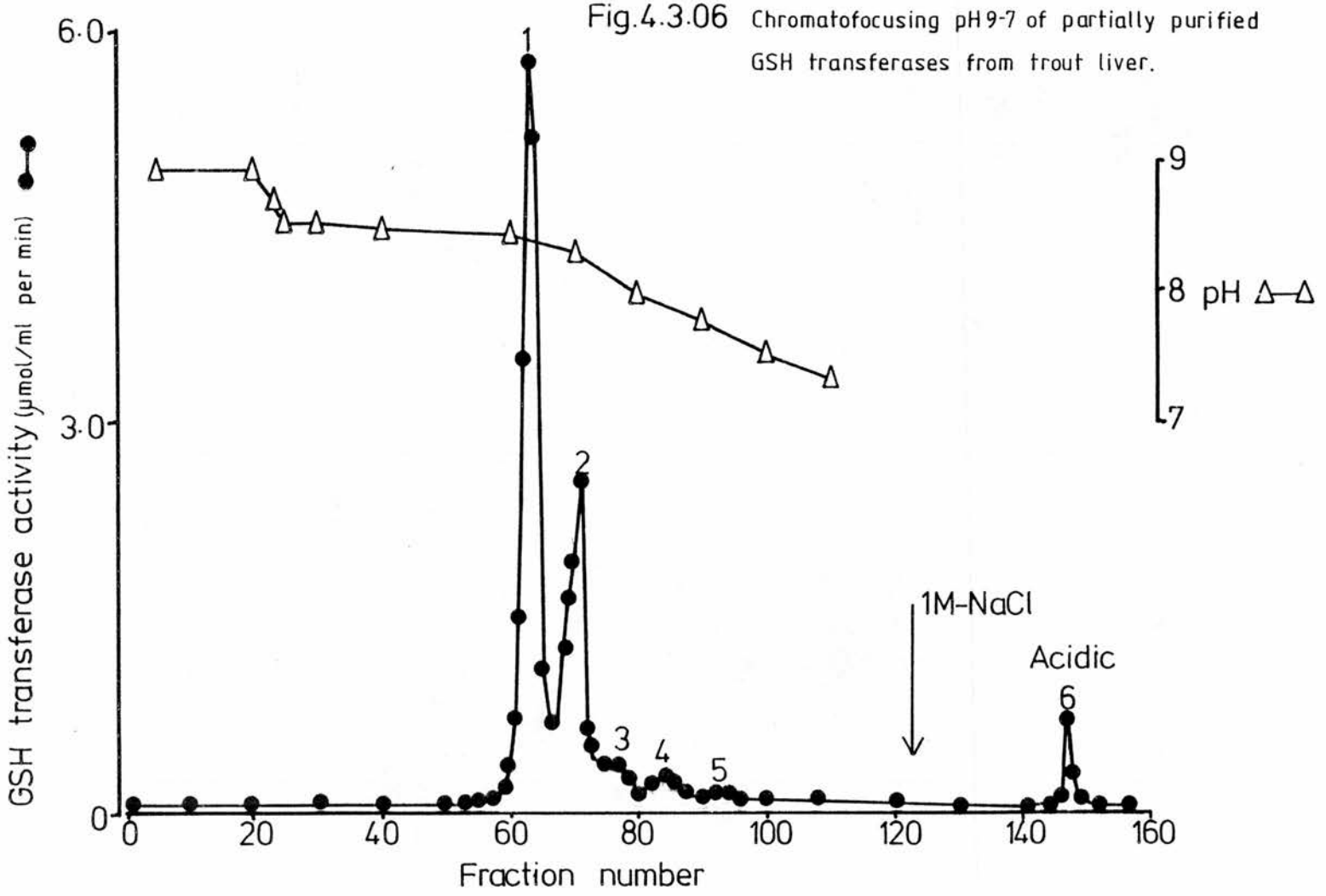


Fig.4.3.06 Chromatofocusing pH 9-7 of partially purified GSH transferases from trout liver.

The pI_{app}'s of the basic forms were in the range 8.4 - 7.0, whilst those of the acidic forms were less than 5.0 and the ratio of the amounts of individual peaks showed considerable variation. As samples were taken in a five week period during the summer of 1983, seasonal differences can be ruled out. The fish were also feeding well in the warm summer months. Preparations containing up to ten livers were used so that the variations due to individuals were minimized, which suggests either different batches of fish were in the capture tanks on days of sampling or feed variations were causing differential induction of individual enzymes. As no facilities were available for keeping fish and feeding them specific diets no real conclusion can be made other than that the samples used were subject to constant variation. A considerable amount of care and attention had been applied to the chromatofocusing technique and apart from some pH fluctuations the method was very reproducible. pH meters were carefully calibrated to ensure that the pH gradients obtained were correctly recorded. Any elution profiles that were obtained to which any element of doubt could be attached were discarded. As a result, the above differences were regarded as being real and not artefactual.

Later, when the Penicuik trout farm closed, fish were taken from another source (Beecraigs trout farm). When chromatofocusing (pH 9-7) was carried out on the transferases from these fish the resultant profiles obtained were quite distinct (Fig. 4.3.06) and very reproducible.

In Fig. 4.3.06 peaks 1 and 2 comprised over 90% of the transferase activity eluted from the column, three other visible cationic peaks were also seen, although the scales used in the figure tend to mask their presence. These samples were taken in November 1983 and the profile differences could be due to a seasonal change, a feeding difference or different stock. The only way to have investigated this would have been through constant routine

analysis throughout the seasons which could not be accommodated in a project of such a short duration. A closer look at the Beecraigs trout results revealed that the pI_{app}'s of the proteins were very similar to those obtained in Fig. 4.03.03 which was the best of the previous chromatofocusing runs as far as the pH gradient was concerned. A comparison of the pI_{app}'s can be seen in Table 4.3.01. The "poorer" results are included because they are genuinely different and because later substrate specificity and K_{emp} value determinations were carried out using this material (so as not to waste any purified enzymes).

To investigate further the differences and similarities between the different proteins, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out. The aim was to relate differences in chromatographic properties to the subunit composition of the proteins and to test if some of the different forms were the products of *in-vivo* hybridization.

4.4: SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE GSH S-TRANSFERASES FROM RAINBOW TROUT LIVER CYTOSOL

SDS-PAGE was carried out according to the methods described previously (section 3.07) and showed up several relationships between the different elution profiles.

Analysis of the major enzyme peaks from the elution profiles shown in Figs. 4.3.02 and 4.3.04 revealed strong similarities in subunit compositions of the enzymes from both profiles. The results (Figs. 4.4.01a and 4.4.01b) showed that peaks 1,2 and 5 all contained one major band migrating as if it had a M_r of about 22,400.

The acidic peak contained two subunits in approximately equal amounts and was therefore assumed to be a heterodimeric protein. The M_r's of the subunits were approximately 22,400 and 25,000. Two distinct

Fig. 4.4.01a

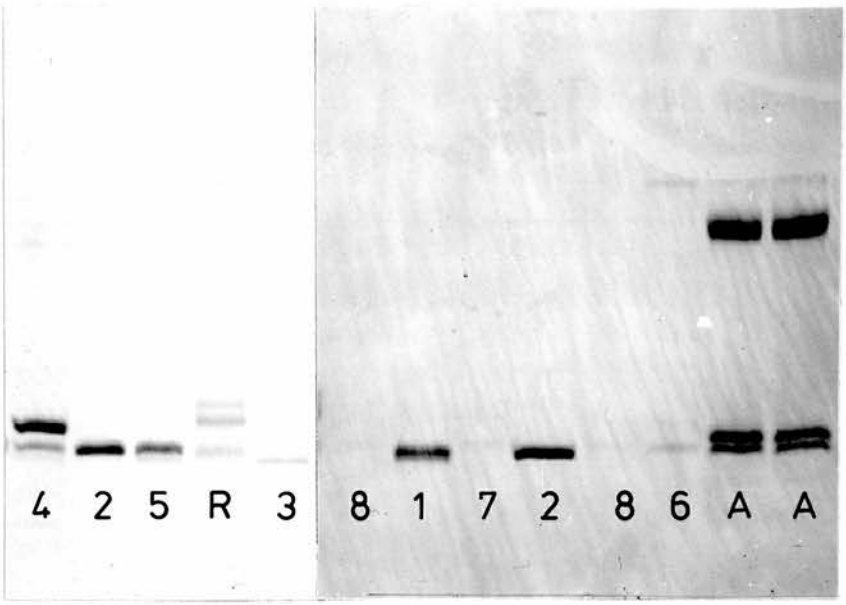


Fig. 4.4.01b

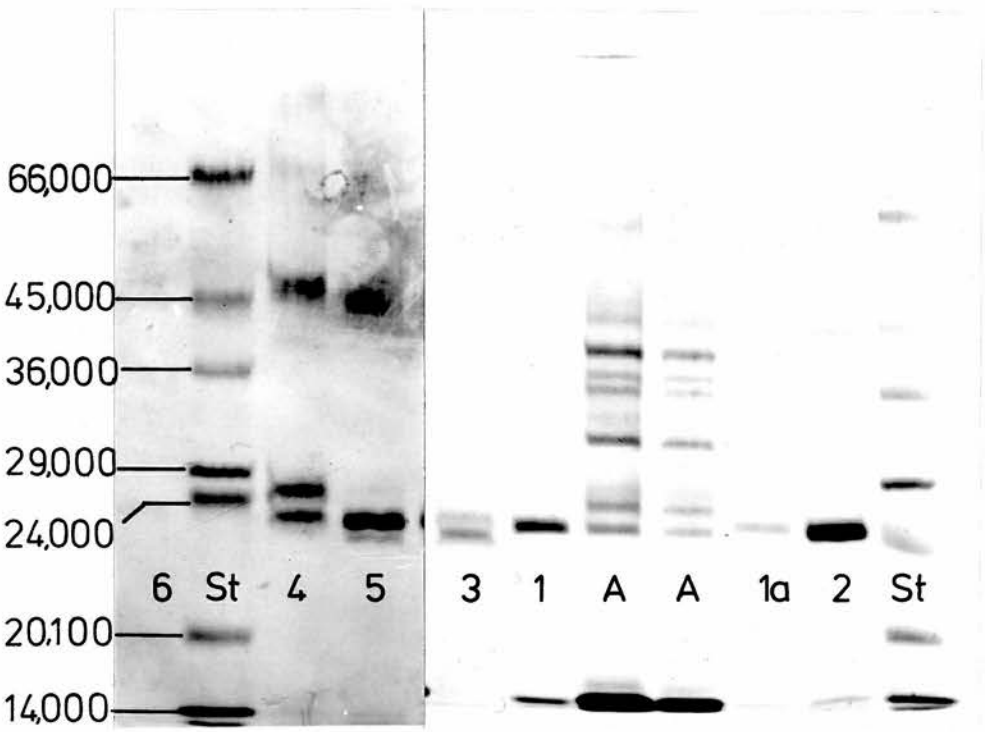


FIG. 4.4.01A.

SDS-PAGE of rainbow trout GSH transferases from the elution profile shown in Fig. 4.3.02. All basic forms are numbered and the acidic form is labelled A. This nomenclature is adhered to in all the subsequent figure legends. R represents rat GSH transferase Ya, Yb and Yc subunits.

FIG. 4.4.01B.

SDS-PAGE of rainbow trout GSH transferases from the elution profile shown in Fig. 4.3.04. St represents Sigma standard molecular weight markers (MW-SDS-70L) and this nomenclature is adhered to in all subsequent figures.

subunits mean that three forms of enzyme are possible by hybridization which may relate to the elution profile from hydroxylapatite chromatography. Peaks 3 and 4 both contained a band of approximate M_r 22,400 but in addition contained a lower and a higher M_r band respectively, indicating that both proteins were distinct heterodimers. Peak 1a from Fig. 4.3.04 had an identical subunit composition to peak 1 and was therefore assumed to be identical to this peak and produced by a fluctuation of the pH gradient on the chromatofocusing column.

The proteins eluted in Fig. 4.3.03 appeared to be different (Fig. 4.4.02) in that although peak 1 was apparently a 22,400 M_r homodimer, peak 2 appeared to comprise a higher M_r band plus two protein bands of equal intensities but smaller M_r. Subsequent analysis showed that these were a contaminant from a fraction between the two peaks, which itself might have been a distinct heterodimeric transferase. Peaks 3,4 and 5 appeared to comprise a subunit of M_r 22,400 and contained contaminants of the same M_r as the peak 2 major band. The acidic protein contained two protein bands; a dominant M_r 22,400 band and a band migrating slightly slower. The acidic transferase which was chromatofocused (pH 8-5) prior to affinity chromatography (Acidic X) is also shown and is observed to contain bands which have distinct mobilities from the subunits of the affinity purified form indicating that the two acidic proteins are distinct.

The material from Beecraigs trout (Fig. 4.3.06) was shown by electrophoresis to be different to the other material (Figs. 4.4.03). Although all the fractions contained a band of M_r approximately 22,400, the basic proteins all contained a band migrating slightly slower whereas the acidic protein contained in addition a more rapidly migrating component. Peak 1 contained far more of the M_r 22,400 form and was probably a mixture of transferases. Peaks 2-5 all contained more of the lower M_r component whilst the

Fig.4.4.02

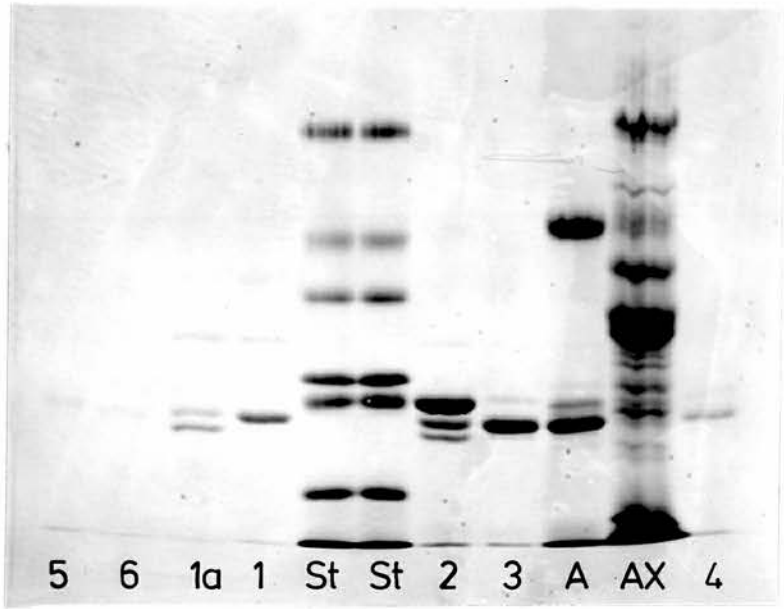


Fig.4.4.03

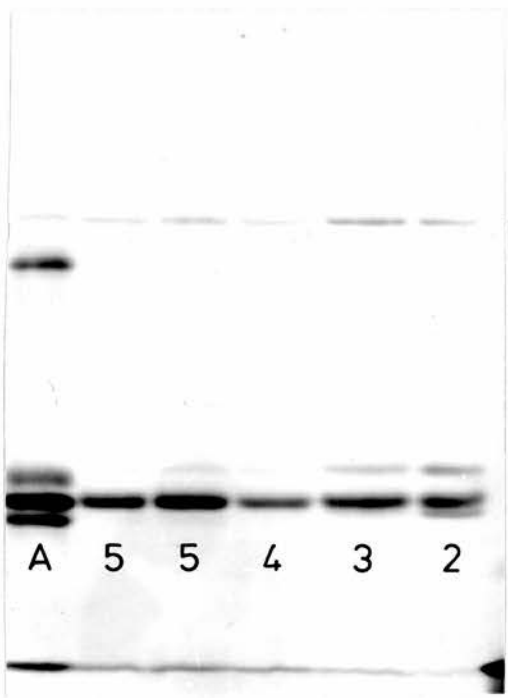


Fig.4.4.04

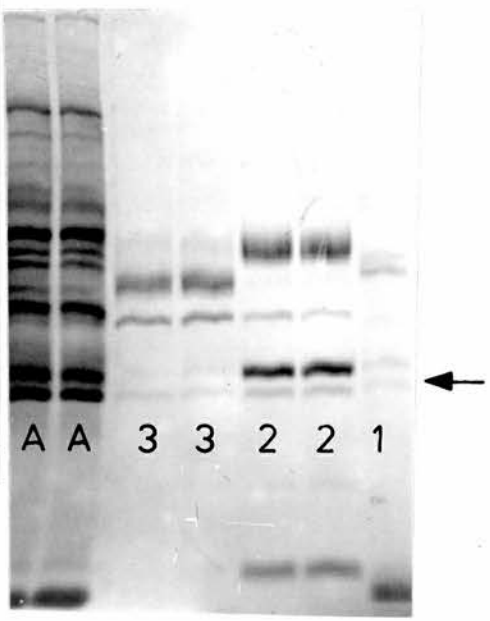


FIG. 4.4.02.

SDS-PAGE of rainbow trout GSH transferases from the elution profile shown in Fig. 4.3.03. AX represents the acidic material not bound by S-hexylGSH-sepharose.

FIG. 4.4.03.

SDS-PAGE of rainbow trout GSH transferases from the elution profile shown in Fig. 4.3.06.

FIG. 4.4.04.

SDS-PAGE of the acidic GSH transferase fraction from rainbow trout (A) after hydroxylapatite chromatography. The position of the GSH transferase subunits is indicated with an arrow.

staining intensities of the bands in the acidic fraction suggested a M_r 22,400 homodimer plus a heterodimer comprised of proteins migrating more slowly and more rapidly than the dominant subunit.

The strong similarity between the subunit compositions of the proteins from Figs. 4.3.02 and 4.3.04 is also seen in the elution profiles. Fig. 4.3.04 differs in that a shallower pH gradient between peaks 1 and 2 allows greater separation. The similarities in pI_{app} seen between Figs. 4.3.03 and 4.3.06 would be mirrored in the subunit composition of the peaks with the exception of the higher M_r band found in the transferases in Fig. 4.3.06.

No correlation can be made between pI_{app}'s and M_r's of the different enzymes in the profiles such as the one made by Mannervik & Jensson (1982). However, SDS-PAGE has shown that elution profiles containing peaks with related pI_{app}'s show similarities in subunit composition. Likewise distinct profiles contain peaks with distinct subunit compositions which indicates that the differing elution profiles obtained using chromatofocusing represented real differences in GSH transferase content and not poor laboratory technique.

Hydroxlapatite chromatography separated an acidic transferase peak from chromatofocusing into three forms (Fig. 4.3.05). Analysis using SDS-PAGE (Fig. 4.4.04) shows that in the major peak (2), the higher M_r acidic subunit predominates. Peak 3 contains slightly more of a lower M_r subunit and peak 1 contains the two peaks in approximately equal amounts. This distribution does not confirm the prediction of hybridization but might indicate the presence of two lower M_r subunit types as peaks containing a lower M_r subunit straddle a peak containing the higher M_r form in the elution profile.

Overall, the results from SDS-PAGE and chromatofocusing show that the chromatographic similarities

Table 4.5.01

Substrate specificities of the glutathione S-transferases from trout liver

Peak	Substrate						
	ENPP	NPA	BSP	NBC	TBO	Δ^5-A	ETHA
1	0	0·2	0·3	1·3	0·2	0	4·7
2	0	0	1·0	0·5	0·3	0	6·7
4	40	0	0	0	0·3	0	9·4
5	34	0·9	0	0·2	0·3	0	0
Acidic	0	10·7	0	318	0	7·1	0

The results are expressed as percentages of those with 1mM-GSH and 1mM-1,chloro-2,4-dinitrobenzene. Abbreviations: ENPP, 1,2-epoxy-3-(p-nitrophenoxy)propane; NPA, p-nitrophenyl acetate; BSP, bromosulphophthalein; NBC, p-nitrobenzyl chloride; TBO, trans-4-phenyl-3-buten-2-one; Δ^5-A, Δ^5-androstene-3,17-dione; ETHA, ethacrynic acid.

and differences between proteins are matched by similar trends in their subunit compositions. To see if this extended to their catalytic properties the substrate specificities of a number of GSH transferases were investigated.

4.5: THE SUBSTRATE SPECIFICITY OF THE GSH S-TRANSFERASES FROM RAINBOW TROUT LIVER

4.5.01: Conjugation of GSH with substrates

The substrate specificities of the GSH transferases separated by chromatofocusing and shown in Fig. 4.3.02 were studied following the methods laid down in methods section 3.05. The purity of the synthesized substrate Δ^5-androstene-3,17-dione was checked (see methods, 3.5.01), mainly to determine whether or not the desired product was present.

The rates of reaction obtained using different substrates were expressed as a percentage of the rate achieved using 1-chloro-2,4-dinitrobenzene as substrate and are shown in Table 4.5.01. Activity was not detected when p-nitrophenethyl bromide was used as a substrate. Activity with 1,2-dichloro-4-nitrobenzene as substrate was tested but some of the observed absorbance increase was due to the substrate coming out of solution. As a result neither of these substrates are included in Table 4.5.01. On the whole, these assays were rather unsatisfactory as the non-enzymic rates of reaction were sometimes very high and also unsteady, making it difficult to obtain a value for the enzymic rate. Because the enzymic rates themselves were so low the percentage error in calculating rates was probably very high. In addition, the high background absorbances experienced mean that Beer's law was probably not obeyed, leading to a masking of small absorbance changes.

The enzymes were dealt with in two groups; acidic and

basic; the results showed some interesting trends. The acidic peak showed very high activity towards p-nitrobenzyl chloride and also activity towards Δ^5-androstene-3,17 -dione and p-nitrophenyl acetate. In this respect it differed considerably from the basic forms, which had activity with trans-4-phenyl-3-buten-2-one and ethacrynic acid; two compounds which appeared not to act as substrates for the acidic form.

Peaks 4 and 5 showed the ability to catalyse the conjugation of 1,2-epoxy-p-(nitrophenoxy)propane. These peaks have a slightly different subunit composition (assuming that the bands discussed previously were all GSH transferase subunits). Peak 4 contained a M_r 22,400 band and a higher M_r band whilst peak 5 contained only the lower M_r component. This suggests that the M_r 22,400 band in peaks 4 and 5 is catalytically distinct from the one seen in peaks 1 and 2 (which would seem to catalyse the conjugation of bromosulphophthalein). The other activities cannot be easily related to subunit composition of the proteins.

Nevertheless, these assays were able to distinguish between the acidic and basic transferases and also suggested that the basic forms existed in two groups.

These results showed that the GSH transferases have the ability to catalyse the conjugation of a number of electrophilic compounds with GSH. The rates of reaction were very low, but different proteins appeared to work with different substrates. This would appear to confirm the theory that multiple forms of the enzymes function to deal with a wide range of xenobiotics.

One of the proposed functions in detoxication of the transferases is the covalent binding of electrophilic compounds (Jakoby & Keen, 1977). Because the conjugation

Table 4.5.02

The inhibition of the conjugation of 1mM-GSH with 1mM-CDNB by other second substrates expressed as a percentage.

Peak	Second substrate		
	BSP (0·03mM)	NBC (0·5mM)	ETHA (0·01mM)
1	66	3	90
2	71	31	83
4	52	19	81
5	52	14	86
Acidic	85	69	59

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; BSP, bromosulphophthalein; NBC, p-nitrobenzene; ETHA, ethacrynic acid.

activity with most of the substrates was so low it was decided to see what effect preincubation with a number of these substrates had on the ability of the proteins to catalyse the conjugation of 1-chloro-2,4-dinitrobenzene with GSH.

4.5.02: The inhibition of activity towards 1-chloro-2,4-dinitrobenzene by preincubation with other second substrates

Assays were carried out as in methods section 3.06.03. Plots of the reciprocal of initial velocity against inhibitor (other second substrate) concentration were curved and difficult to interpret. Because of this, the results were expressed in their simplest form; as the percentage inhibition of enzyme activity.

The results (Table 4.5.02) help to further differentiate between the different groups of transferases and apply to peaks eluted from chromatofocusing and shown in Fig. 4.3.04 (a very similar profile to 4.5.02 which was used in the previous subsection). The acidic form again differs from the basics as it is inhibited to a greater degree by bromosulphopthalein (BSP) and p-nitrobenzyl chloride (NBC) and is also inhibited significantly by ethacrynic acid (ETHA). It is interesting to note that neither BSP or ETHA are substrates for the acidic transferase. ETHA was a very powerful inhibitor of all the cationic forms tested. The use of BSP suggested that the basics might comprise two distinct groups as judged by the greater inhibition of peaks 1 and 2 than peaks 4 and 5. The effect with NBC is less clear but shows that the catalytic activity shown by the acidic form towards NBC is related to its ability to bind the substrate.

Overall the results showed the differences observed in catalytic activity are not reflected in low affinities for

Table 4.5.03 Fluorescence quenching by a number of second substrates.

Substrate	% Quenching of fluorescence	
	Basic	Acidic
1-chloro-2,4-dinitrobenzene	43·7	21·0
bromosulphophthalein	33·2	9·5
p-nitrobenzyl chloride	29·7	13·1
1,2-epoxy-3-p-(nitrophenoxy)propane	22·6	6·1
ethacrynic acid	8·9	<1·0
Δ^5-androstene-3,17-dione	<1·0	<1·0
rose bengal	<1·0	<1·0
cholic acid	<1·0	<1·0
lithocholic acid	<1·0	<1·0

the substrates. Compounds which do not act as substrates serve as inhibitors, indicating that they do indeed bind to the proteins. To take the investigation further, the ligand 1-anilino-8-napthalenesulphonic acid (ANS) was used. ANS fluoresces when bound to proteins (Sugiyama et al., 1978); binding by other ligands causes a quenching of the fluorescence.

The fluorimetric assay of binding to the GSH S-transferases

Following the methods laid down in section 3.16.02 the quenching of ANS fluorescence by electrophilic compounds was investigated and the results shown in Table 4.5.03. The protein material used in these experiments were some pooled basic and acidic fractions from chromatofocusing.

Overall the quenching of the acidic fluorescence was lower than the basic quenching, indicating that the basic proteins bound electrophilic ligands more strongly or that the acidic material bound the ligands at a site which did not neccessarily cause fluorescence quenching. Alternatively the ligand might be bound less strongly and therefore be more readily displaced. This also suggests that the M_r 22,400 subunits of the acidic and basic proteins are different. The two bile acids at the bottom of the table had no effect and are therefore assumed not to be bound by the transferases. Ethacrynic acid does not cause as much quenching as might be expected from the data in the previous subsection; again this indicates that binding does not neccessarily affect ANS fluorescence. Similarly BSP which inhibits the acidic enzyme more strongly than the basic enzymes causes a far greater quenching of fluorescence in the basics than the acidic protein. This probably means that ligands and substrates bind at different sites on the GSH transferases and that the effects depend on the proximity of the different sites.

Fig. 4.5.06

A typical distribution of points during K_m determinations using double reciprocal plots.

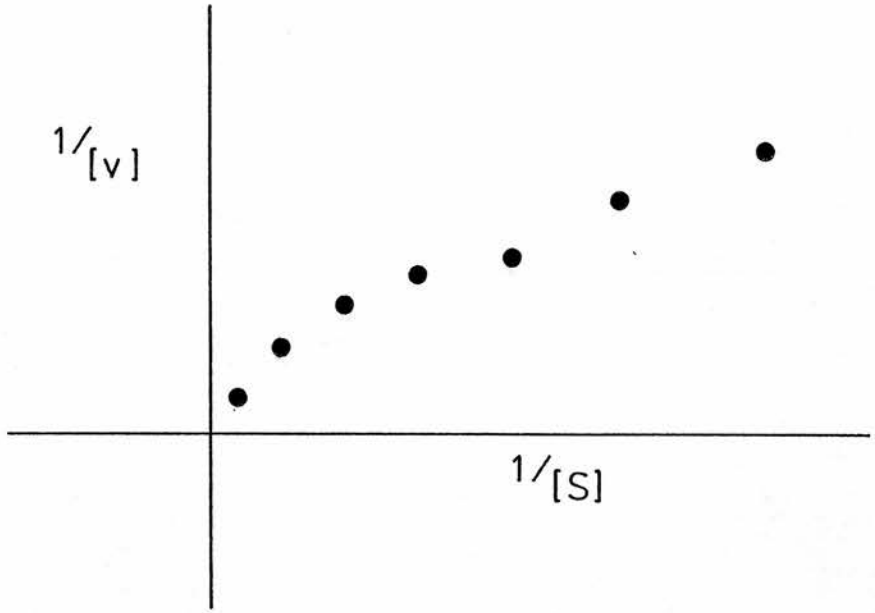


Table 4.5.06

Empirical half-saturation concentrations for GSH and 1-chloro-2,4-dinitrobenzene.

Peak	K_{emp} GSH (mM)	K_{emp} 1-chloro-2,4-dinitrobenzene (mM)
1	0·2	0·5
2	0·2	0·4
4	0·3	0·4
5	0·2	0·4
Acidic	0·7	1·9

The standard errors of the above values, calculated by the jack-knife technique (Miller, 1974) were less than 15% of the values themselves.

Such differences may be reflected in affinities for substrates and to test differences in the binding capacities of the different enzymes, half-saturation concentrations for GSH and 1-chloro-2,4-dinitrobenzene were investigated.

4.5.06: Half-saturation concentrations for GSH and 1-chloro-2,4-dinitrobenzene

These determinations were carried out as in methods section 3.06. When double reciprocal plots were drawn the resultant line was curved in the fashion indicated by Fig. 4.5.06. This meant that half-saturation concentrations could not be determined; instead an empirical method of determining the values was used (methods section 3.06.02). These values are shown in Table 4.5.06.

The results again show clearly that the acidic protein differs from the basic forms, the half-saturation concentrations being much higher for both substrates. The results for the basic enzymes suggest that their capacities for the two substrates are closely related. One of the consequences of the higher values for the acidic transferase, is that at higher substrate concentrations the role played by this fraction will be greatly increased. When assayed with both substrates at 1mM this fraction only represented about 3% of the total GSH transferase activity.

To test that the higher values obtained for the acidic fraction were not caused by the high salt concentration (chloride ions can produce product inhibition; Clark et al., 1984), empirical half-saturation concentrations (K_{emp}'s) were carried out with some basic enzymes as they were or made 1M with respect to NaCl. The results (not shown), demonstrated that if anything, NaCl decreased the K_{emp} values.

The most likely cause of the curved double reciprocal plots was that the fractions investigated contained more

than one enzyme (Mannervik, 1984). Because the GSH transferase subunits are thought to be catalytically independant from one another (Mannervik, 1984) it follows that more than one enzyme can also be taken to mean more than one subunit type. Alternative explanations are either that the enzyme subunits exhibit negative cooperativity or that the reaction mechanism itself which causes the effect (Mannervik & Askelof, 1975).

Because protein fractions after affinity chromatography contained 5mM-S-hexylGSH, the effect of this ligand on kinetic behaviour was also tested.

4.5.07: The effect of S-hexylGSH on enzyme activity

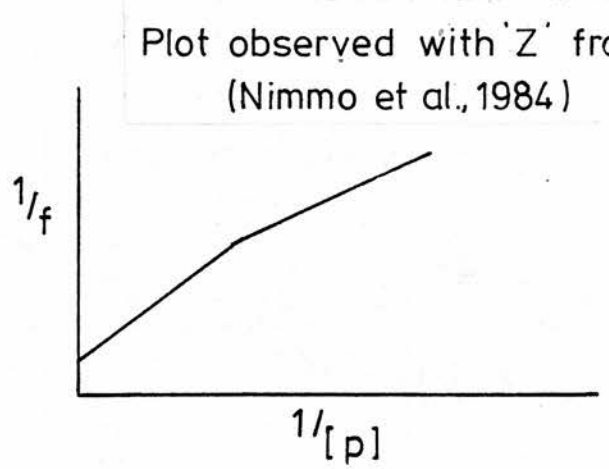
S-HexylGSH was preincubated with a number of purified GSH transferases following the methods laid down in section 3.06.04. The resultant plots of the reciprocal initial velocity against inhibitor concentration were curvilinear ; curving more steeply upwards as the inhibitor concentration increased (not shown). The curvature of the plots prevented accurate calculations of Ki values.

The results could be interpreted on the basis of the degree of inhibition observed. Peaks 1 and 2 were both inhibited to a small degree, peak 4 was the most strongly inhibited and peak 5 and the acidic fraction were inhibited to an intermediate degree.

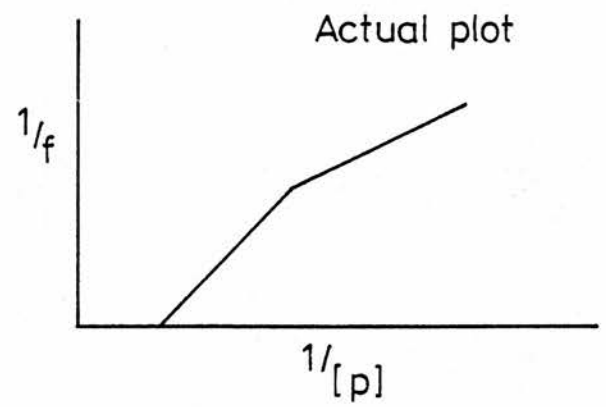
The results show that S-hexylGSH inhibits the transferases to differing degrees. The difference between peaks 1,2 and 4 and 5 are further highlighted. The basic proteins seem split into two groups. The inhibition of the acidic fraction is suprising because previous investigations had shown that this fraction had a lower affinity for S-hexylGSH-sepharose than the basic forms. S-Hexyl GSH would

Fig. 4.5.08. Double reciprocal plots of fluorescence (f) v protein concentration ([p]).

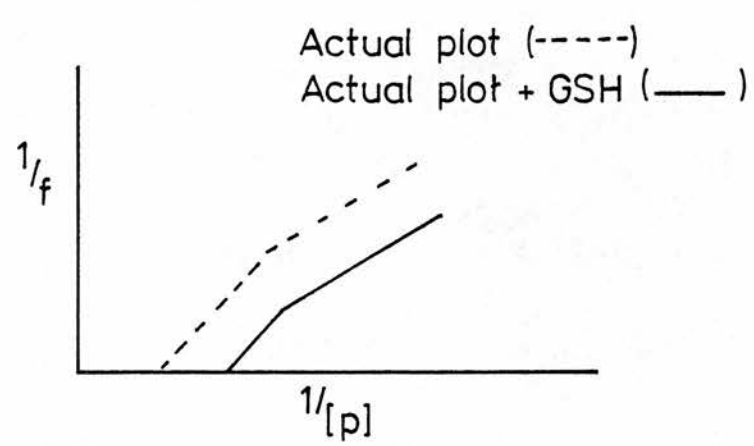
a)



b)



c)



not therefore be expected to inhibit the fraction as much (as binding is required) as the basic fractions.

Finally the binding to the ligand ANS was investigated in a little more detail.

4.5.08: ANS binding by the GSH transferases

The methods shown in section 3.16.01 were followed in this investigation. The material used was from trout from Beecraigs trout farm (elution profile Fig.4.3.06); all the previous substrate specificity and related work had been carried out on fish from Penicuik trout farm.

The results of the double dilution titrations were plotted as double reciprocals of fluorescence against protein concentration. The form the plots should have taken is illustrated in Fig. 4.5.08a, instead the plots appeared as in Fig. 4.5.08b. As a result quantum yields could not be estimated.

Why the results should have taken such a form is a mystery. All the results were corrected for intrinsic protein and ligand fluorescence so no errors could have crept in there. As a control the titrations were carried out with material purifed by S-hexylGSH affinity chromatography and GSH affinity chromatography; no differences were observed, so the effect was not due to ligands such as S-hexylGSH binding to the proteins. The effect of adding GSH to the ANS buffer enhanced the effect by lowering the line (Fig. 4.5.08c) as it increased the fluorescence. The overall effect is that the fluorescence is higher than predicted for a set protein concentration.

As the substrates for the GSH transferases are theoretically activated by microsomal enzymes and then

partition out of the membranes into the cytosol, it was thought important to see how 1-chloro-2,4-dinitrobenzene behaved.

4.6: PARTITIONING OF 1-CHLORO-2.4-DINITROBENZENE INTO MEMBRANES

Membranes were produced from the livers of brown trout *Salmo trutta* as described in methods section 3.15 and the partitioning of 1-chloro-2,4-dinitrobenzene (CDNB) recorded. The results that were obtained were treated in a qualitative manner so precise figures for partitioning were not obtained.

The results (not shown) showed partitioning of CDNB into the membrane fraction. The membranes themselves were then fractionated to see which partitioned the greater proportion of CDNB per mg of protein. Five different fractions were produced and it was fractions of comparable density to Golgi and endoplasmic reticulum fractions in rat brain that partitioned most CDNB. It would be expected that the endoplasmic reticulum might play the most important role in the partitioning of electrophiles. This is likely to be the case as the observed partitioning by the Golgi is likely to be artificially high because of its highly encapsulated structure which may trap extra CDNB.

In summary the GSH transferases can be divided into two groups; acidics and basics. Chromatofocusing shows at least five major basic peaks and several minor ones. Several acidic forms have been shown but these are more difficult to purify and are less stable. The acidic fraction from chromatofocusing has higher K_{emp} values for both GSH and CDNB. This is similar to the case with elasmobranchs (Sugiyama et al., 1981), where two forms of enzyme were separated by gel-filtration and observed to have Km values

for GSH differing by almost an order of magnitude. The enzymic properties of the two groups differ widely which effectively covers a broader spectrum of substrates.

The use of GSH affinity chromatography causes the pI_{app}'s of the GSH transferases to change, making them more acidic. This is believed to be due to covalent linkage of GSSG to the proteins to form mixed-disulphides.

The relative amounts of different components differ widely in a non-seasonal variation. These differences are seen not only as changed elution profiles, but as different subunit compositions as well.

The proteins seem to be able to conjugate a wide range of substrates with low efficiency. They also bind covalently to a large number of compounds which are not conjugated, but do not appear to bind bile-acids. This indicates that the binding of bile-salts and bile-acids in other species is non- physiological. However, they do bind to the organic anion ANS. It therefore seems likely that their high concentration reflects their enzymic inefficiency and the covalent suicide- binding role in detoxication.

4.7: THE PURIFICATION AND CHARACTERIZATION OF THE GSH S-TRANSFERASES FROM THE ATLANTIC SALMON (Salmo salar)

This section deals with salmon raised in a fish farm on a sea-loch and these fish are referred to throughout as sea salmon. They differ from the fish in section 4.8 which are migrating salmon, line-caught in fresh water and referred to as river salmon. The Atlantic salmon was investigated because of its economic importance and for comparison with the other salmonid investigated *Salmo gairdneri.* Because material was obtained in batches often six months apart

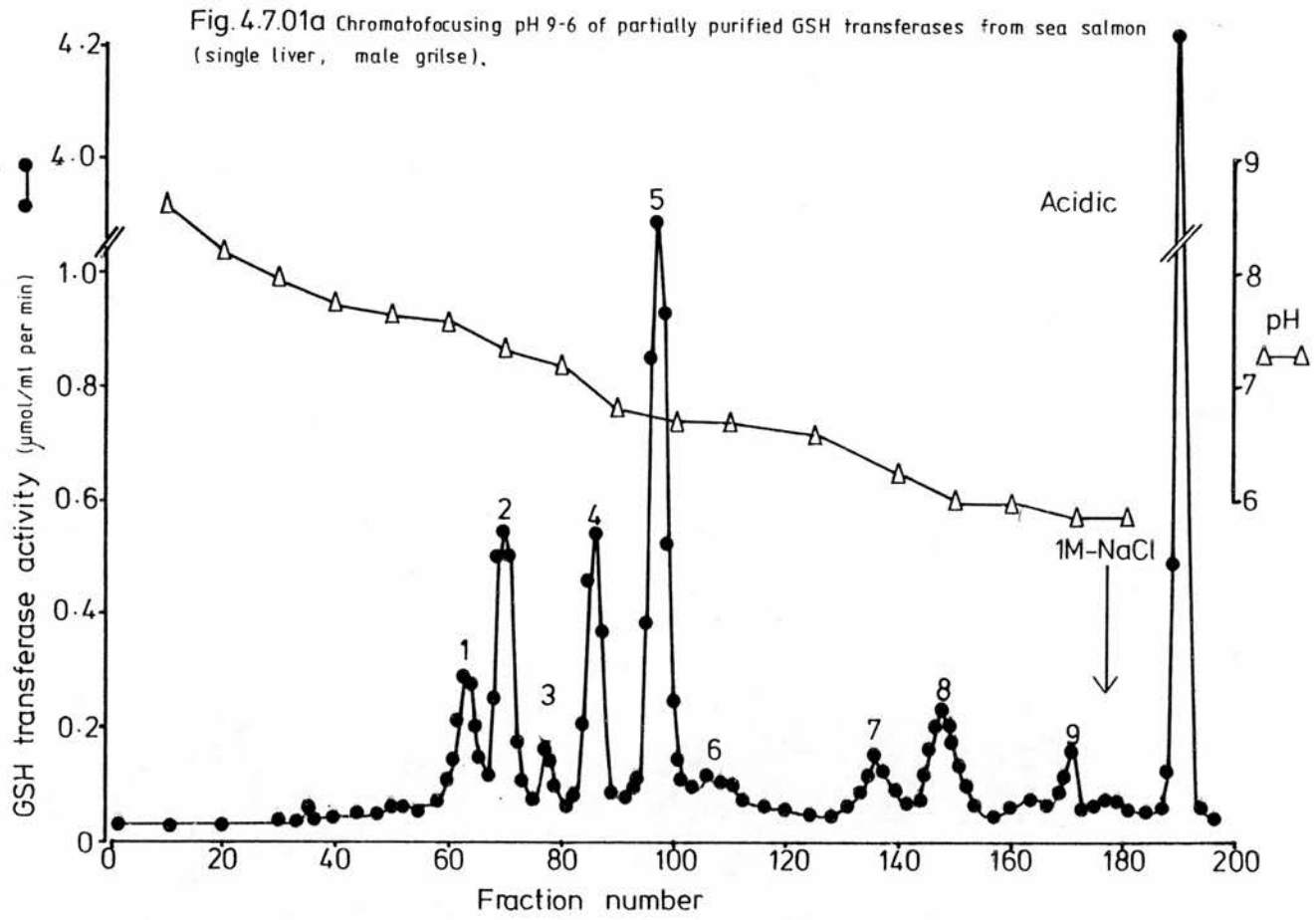


Fig. 4.7.01a Chromatofocusing pH 9-6 of partially purified GSH transferases from sea salmon (single liver, male grilse).
GSH transferase activity (µmol/ml per min)
4·2
4·0
1·0
0·8
0·6
0·4
0·2
0
0
20
40
60
80
100
120
140
160
180
200
Fraction number
Acidic
1M-NaCl
pH
9
8
7
6
1
2
3
4
5
6
7
8
9

Table 4.7.01 Apparent pI values for the GSH transferases from grilse and trout. excluding those eluted by salt.

Peak	Apparent pI values		
	Trout 4.3.06	Grilse ♂ 4.7.01	Grilse ♀ ——
1	8·40	7·50	7·45
2	8·25	7·38	7·35
3	8·10	7·25	7·20
4	7·95	7·00	7·00
5	7·75	6·75	6·80
6	——	6·70	6·70
7	——	6·40	6·30
8	——	6·05	6·00
9	——	5·80	——

this section is subdivided according to the work carried out on individual batches of material.

4.7.01: The purification of GSH S-transferases from sea salmon: August 1983

GSH transferases were purified from salmon-liver cytosol by S-hexylGSH affinity chromatography and chromatofocusing (Methods sections 3.03.05 and 3.03.06). The livers were obtained from male and female grilse and the separations obtained using chromatofocusing were reproducible (the male elution profile is shown in Fig. 4.7.01a). The only difference between the two sexes was that female grilse lacked peak 9 and as the female livers were larger and had a greater total transferase activity. This would appear to be a real difference as opposed to one caused by failure to detect a peak of very low activity.

The profiles were similar to those of the rainbow trout except for the pI_{app}'s of the proteins which were on the whole lower (see Tables 4.7.01 and 4.8.02) and the relative proportion of the acidic form which contributed over 25% of the total activity when assayed using 1mM-CDNB and 1mM-GSH. This indicates that the protein is either more stable than the trout acidic form(s) or is present in greater amounts. If the Km of the acidic form is high, the percentage activity contributed by it could be increased by raising the GSH concentration above 1mM.

In addition to the material which bound to the S-hexylGSH affinity column over 30% of the total activity was not bound by the matrix. When the preparation was repeated using an affinity column with double the quantity of matrix the same effect was observed which indicated that the capacity of the column was not exceeded but that these enzymes differed in their affinity for the matrix. To investigate this excluded material was dialysed and applied

Fig.4.7.01b Elution profile from DEAE sepharose 6B of GSH transferase activity from female grilse liver which did not bind to S-hexylGSH-sepharose 6B.

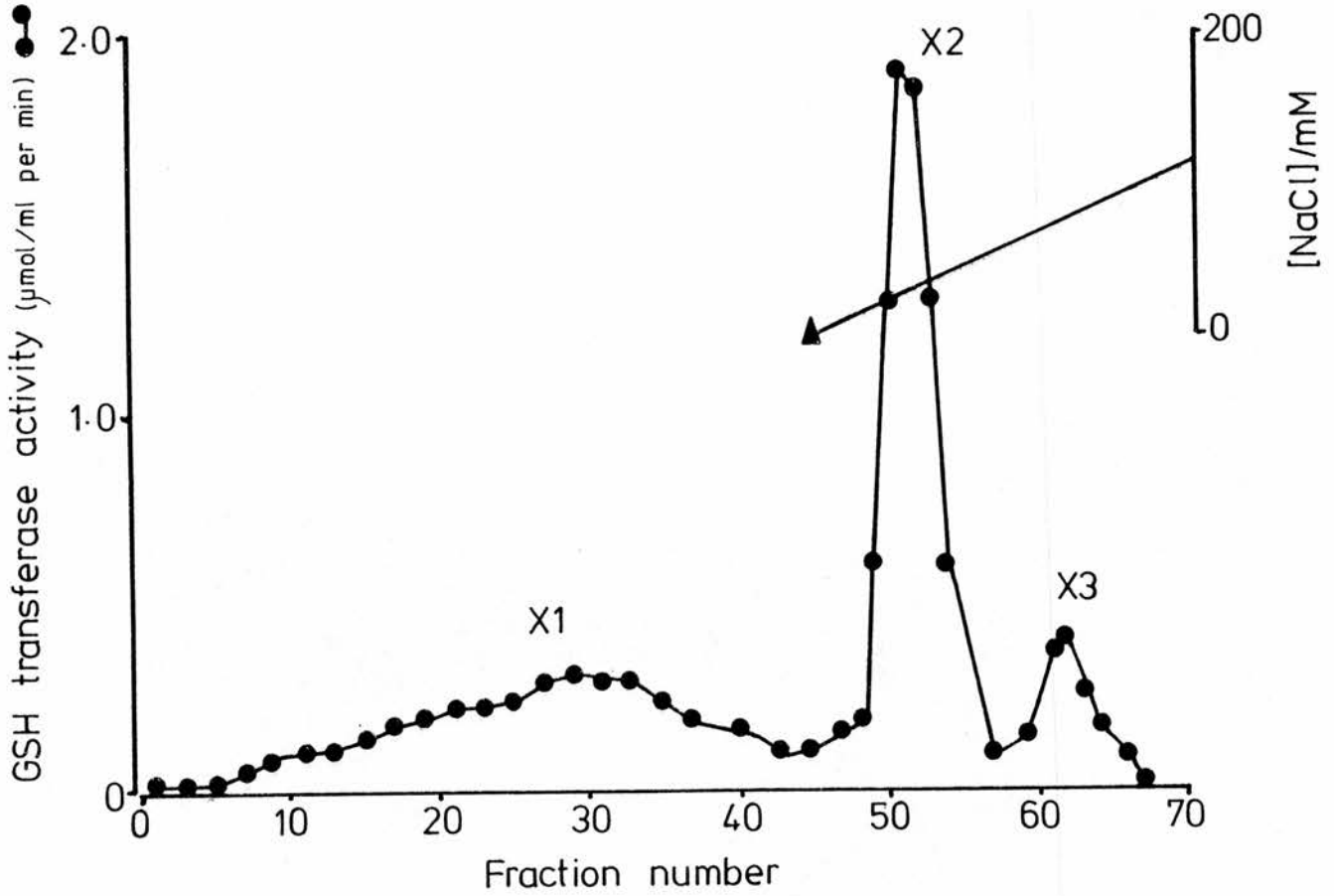


Table 4.7.02a Substrate specificities of some GSH transferases from grilse liver.

Substrate	GSH transferases which were bound by S-hexylGSH-sepharose 6B				GSH transferases excluded from S-hexylGSH-sepharose 6B		
	2	4	5	Acidic	X1	X2	X3
NBC	3·55	3·30	2·34	0·31	3·30	0·84	52·50
ENPP	Activity not detected above background rate				0·13	5·97	37·08
NPA	0	0	0	0	0	0·14	0·92
BSP	0	0	0	0·02	0·14	0·15	0·17
DCNB	7·63	6·11	1·35	0·21	18·42	0·40	1·50
TBO	6·18	5·62	7·01	0·35	0	0·21	0
ETHA	0	0	0	0·05	0·13	0·08	0·12
Δ^5-A	0	0	0	0	0·53	0·13	0·03

The activities are expressed as percentages of the rates obtained using 1mM-CDNB and 1mM-GSH.
Abbreviations: NBC, p-nitrobenzyl chloride; ENPP, 1,2-epoxy-3-p-(nitrophenoxy)propane; NPA, p-nitrophenyl acetate; BSP, bromosulphophthalein; DCNB, 1,2-dichloro-4-nitrobenzene; TBO, trans-4-phenyl-3-buten-2-one; ETHA, ethacrynic acid; Δ^5-A, Δ-androstene-3,17-dione; CDNB, 1-chloro-2,4-dinitrobenzene.

to DEAE sepharose 6B (see Methods 3.03.04).

The elution profile obtained (Fig. 4.7.01b) shows that more than one form of GSH transferase from grilse liver has a low affinity for S-hexylGSH sepharose. There appeared to be an acidic form X3, a near neutral form X2 and several basic forms X1. To see if the differential affinities for S-hexylGSH sepharose of the GSH transferases from sea grilse were reflected in differential catalytic properties and kinetics, the substrate specificities and K_{emp}'s for several enzymes were determined.

4.07.02: Substrate specificities and K_{emp} values for some grilse hepatic GSH S-transferases (August 1983)

The substrate specificities for the transferases from grilse are shown in Table 4.7.02a. As the activities are expressed as a percentage of those with CDNB as substrate, it should be noted that the activities of peaks 2,4,5 and X1 towards CDNB were low and therefore any errors incurred in obtaining the percentage rates of reaction towards other substrates would be higher. The activities shown in the first half of the table for peaks 2,4 and 5 are probably artificially high as with most of the GSH transferase assays with other substrates it was often very difficult to distinguish from small reaction rates and fluctuations in background rates. However, peak X2 had a high activity towards CDNB indicating that the activity recorded towards ENPP was genuine.

The results showed clear differences in substrate specificities between the transferases which bound and did not bind to S-hexylGSH sepharose. Those that were excluded from the affinity matrix had higher activities towards other substrates such as X1 towards DCNB, X2 towards ENPP and X3 towards NBC and ENPP. The chromatofocused forms showed limited substrate specificites and worked poorly

Table 4.7.02b

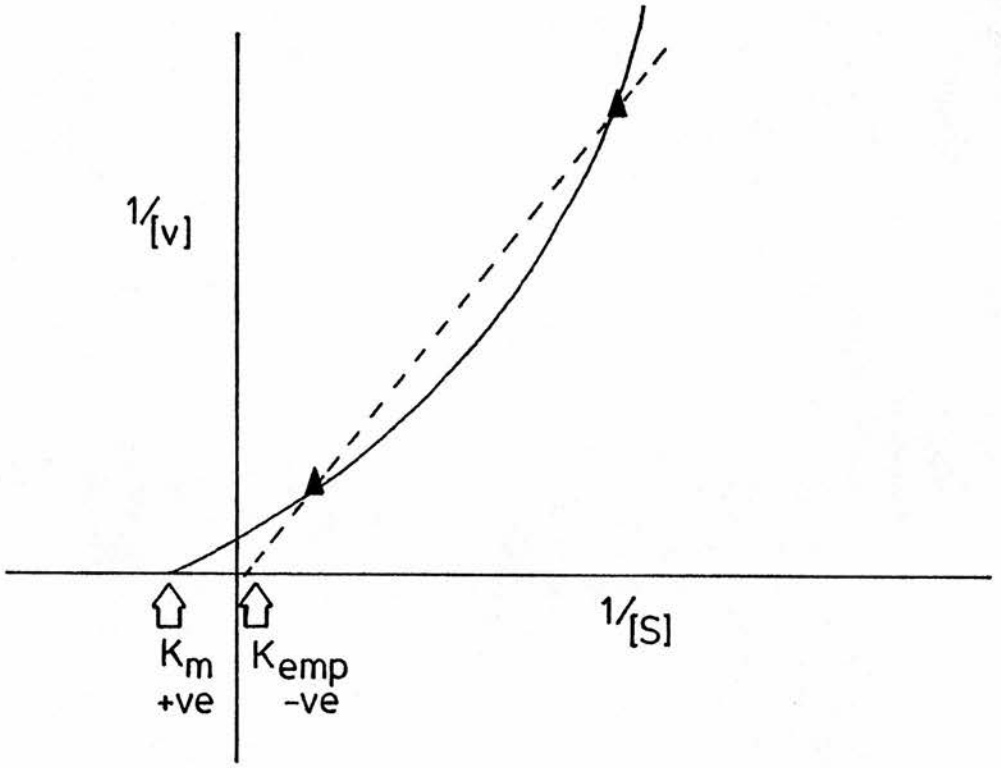
Empirical half-saturation concentrations for GSH and CDNB for some GSH transferases from grilse liver.

Peak	K_{emp} values	
	GSH (mM)	CDNB (mM)
1	0·89	1·71
2	0·37	1·36
3	0·26	3·57
4	0·27	0·99
5	0·43	1·57
6	0·36	1·36
7	0·30	0·62
Acidic	-45·20	-3·38
X1	1·17	1·48
X2	0·74	3·84
X3	0·84	0·24

The standard errors for the above values (calculated using the method of Miller, 1974) were always less than 15% of the values themselves.

Fig. 4.7.02c

Theoretical double reciprocal plot to explain the negative K_{emp} values obtained for the acidic GSH transferase eluted from the chromatofocusing column.



with all of the substrates except CDNB, although some activity towards TBO and DCNB were recorded. The activities towards DCNB were probably under-estimated due to inhibition of the enzymes by the substrate. High initial velocities were recorded; these very quickly tailed off and were assumed to be caused by alkylation of the proteins by the substrate. The initial high rates of activity were of insufficient duration to be recorded accurately.

The K_{emp} determinations (carried out as shown in Section 3.06.02) suggested some significant differences between the proteins and are shown in Table 4.7.02b. For the cationic forms which were bound by S-hexylGSH sepharose, the values for GSH for peaks 2,3,4,6 and 7 were all in the same range. Peak 5 was probably different and peak 1 was very definately distinct. Using CDNB, the observed values showed a wider deviation: peaks 3,4 and 7 were clearly distinct and other peaks showed slight differences. The use of these two substrates was therefore able to show distinctions between all the cationic forms except peaks 2 and 6 which were apparently identical.

The acidic form bound by the affinity matrix showed a massive deviation in that K_{emp} values were negative for both substrates. Fig. 4.7.02c illustrates an explanation of this strange phenomenon. If a double reciprocal plot had been drawn it might have curved in a convex manner in such a way that a straight line drawn between the points indicated would give a negative K_{emp} value. As mentioned previously (Section 4.5.06) such behaviour can be explained by the presence of a mixture of enzymes, or in the case of the transferases by the presence of two subunits with distinct catalytic properties or by the mechanism of action of the enzymes themselves.

K_{emp} values for the excluded fractions showed that these differed from those of the bound forms. X1 had higher GSH K_{emp} than any of the bound cationic forms. X2

and X3 showed a similar trend differing in this respect to all but peak 1. The X3 K_{emp} value for CDNB was far lower than any of the other forms whilst X1 and X2 values fell in the range of values obtained for the bound cationic forms.

These results show minor differences within the bound and excluded forms and also show significant differences between the forms. To carry the comparisons further, the subunit compositions of the various fractions were investigated electrophoretically.

4.7.03: SDS-polyacrylamide electrophoresis of grilse hepatic GSH transferases; August 1983

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to methods section 3.07.

The gels (Fig. 4.7.03) showed very clearly that peaks 1-6 contained subunit species migrating together on SDS-PAGE. Peak 7 contained in addition a subunit migrating more slowly and because the staining intensity of this band was lower it is assumed peak 7 was a mixture of at least two proteins. The acidic peak contained two bands of equal intensities; one band migrated at the same rate as the bands in peaks 1-6 and the slower migrating band at about the same rate as its equivalent in peak 7. It appears therefore that the acidic peak is either a heterodimeric protein or comprises two transferases in equal proportions. This agrees with the suggested composition of this peak from the K_{emp} data.

The excluded fractions contained some bands with similar rates of migration to those in the bound peaks. X1 and X3 consisted predominantly of bands with similar mobilities to the slower migrating component of the acidic peak. As the properties of these three transferases differ, then it follows that these subunits are distinct. X2 has a component which migrates at the same rate as the band in

Fig.4.7.03.

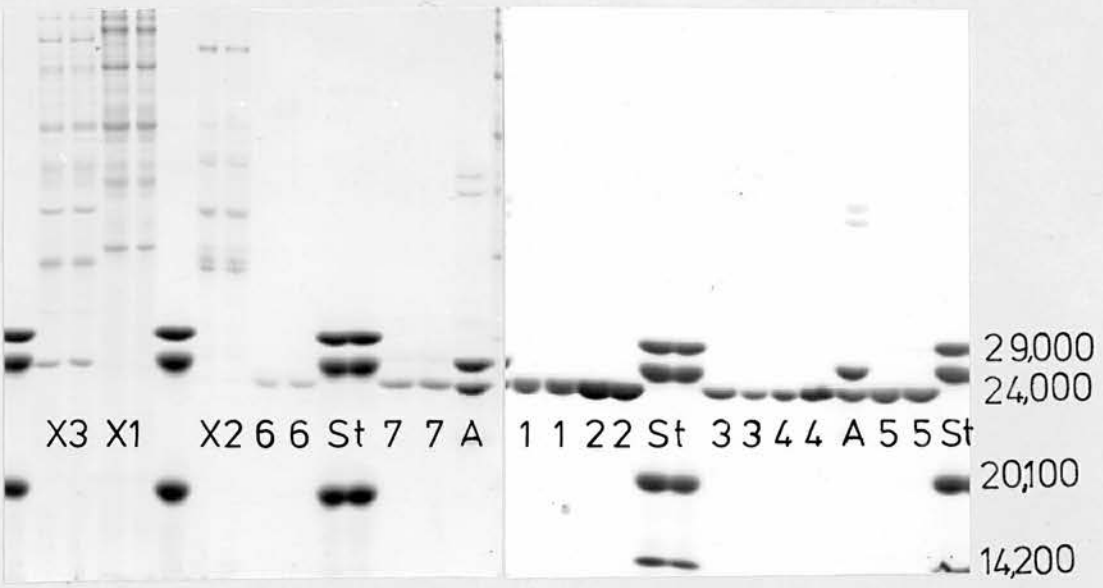


Fig.4.8.01b.

Fig.4.8.01c.

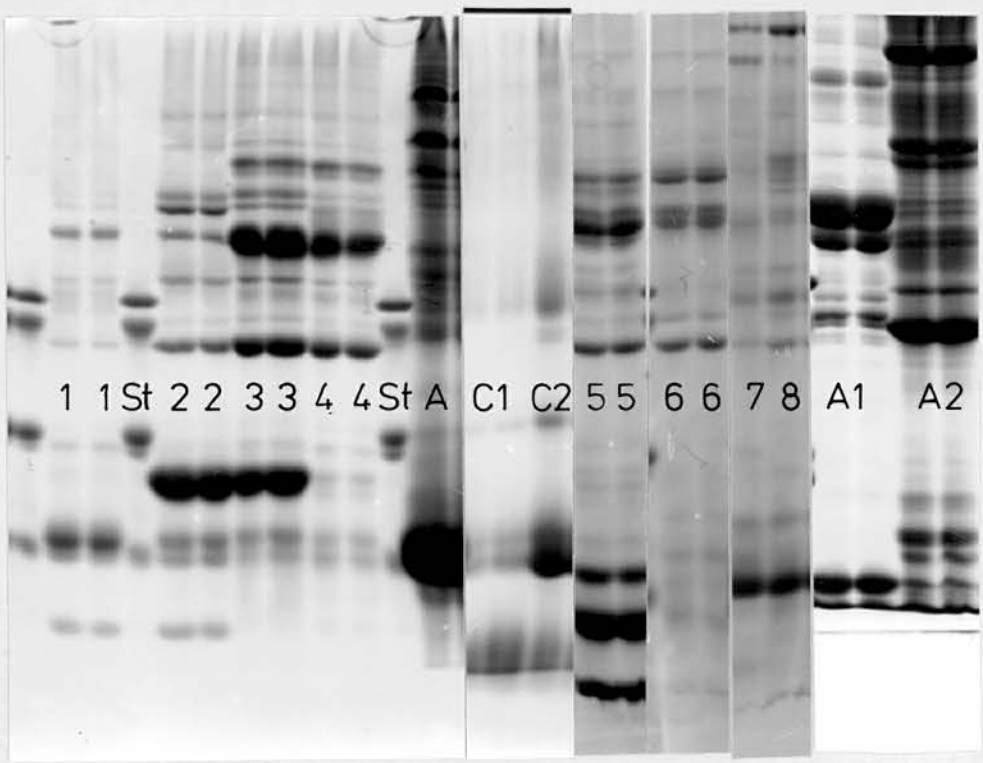


FIG. 4.7.03.

SDS-PAGE of sea salmon GSH transferases from the August 1983 batch. X1, X2 and X3 represent the transferases excluded from S-hexylGSH-sepharose and subsequently separated on DEAE-sepharose.

FIGS. 4.8.01B. AND C

SDS-PAGE of river salmon GSH transferases separated by chromatofocusing (pH 9-6) after lyophilization. A1 and A2 represent the acidic fraction from a later preparation which were resolved by the application of a salt-gradient. C1 and C2 represent the most basic fractions separated using chromatofocusing (pH 11-8).

peaks 1-6 and also contains two slower migrating components (in approximately equal proportions) with distinct mobilities to any of the other forms. It was difficult to say exactly what the subunit compositions of the excluded fractions were due to the impurity of the material. However, the bands that were observed migrated at similar rates to those of the transferases which bound to the affinity matrix. It must be said that the bands mentioned cannot be proven to be GSH transferase subunits, but were predominant and had M_r's in the range predicted.

Calibration using molecular weight standards indicated that the M_r's of the bands in the acidic peak were around 22,400 and 24,000. Similarly those for the cationic peaks 1-6 were around 22,400.

In summary, a number of different forms of GSH transferase have been purified from sea salmon (grilse). Apart from peaks 2 and 6 (which appeared to be identical in all respects except pI_{app}) the various forms all showed some differences. The total number of fractions with transferase activity was thirteen and some of these may have contained more than one transferase. The substrate specificity and K_{emp} data suggest that several forms of subunit with M_r's 22,400 and 24,000 exist. The forms which had a low affinity for the S-hexylGSH matrix had distinct characteristics from the higher affinity forms. The purification scheme used was very successful and allowed purification to near homogeneity with two chromatographic steps. The high degree of reproducibility of the elution profiles is probably due to increased familiarity with the techniques.

The elution profiles had the same appearance as those from rainbow trout and the substrate specificities of the peaks after chromatofocusing were also similar. In the case of the trout the acidic form(s) differed more strongly from the basic forms. The relatively high K_{emp} values for the proteins indicates that they have a high capacity for CDNB

and GSH. Why certain forms should have much broader specificities than others is unclear and does not fit in with the theory that the high concentration of these enzymes is due to a non- specific detoxication role. It is conceivable that the narrower substrate specificities of the chromatofocused forms is related to the S-hexylGSH affinity chromatography step. If these forms had a higher affinity for the ligand, its binding to the enzymes might lower their catalytic reactivity, although this does not seem to effect activity towards CDNB.

To follow on from this, the GSH transferases from river salmon were investigated to see if they differed in any way from the forms in sea salmon, bearing in mind the enormous physiological changes that have taken place in these fish.

4.8: THE PURIFICATION AND CHARACTERIZATION OF THE GSH S-TRANSFERASES FROM RIVER SALMON

4.8.01: Purification using lyophilization and chromatofocusing

When hepatic cytosol from river salmon was applied to a S-hexylGSH affinity column over 90% of the total activity was excluded from the matrix. As the column had been used extensively and was slightly discoloured it was discarded and a new affinity matrix synthesized. The experiment was repeated and the same result was observed. To test if the affinity matrix had been correctly synthesized, trout cytosol was applied and was observed to bind. Clearly, the GSH transferases from river salmon did not bind well to S-hexylGSH sepharose, unlike most of the GSH transferases from sea salmon.

Before the cause of this difference could be investigated, the transferases had to be purified. As

Fig. 4.8.01a Chromatofocusing pH 9-6 of lyophilized river salmon cytosol.

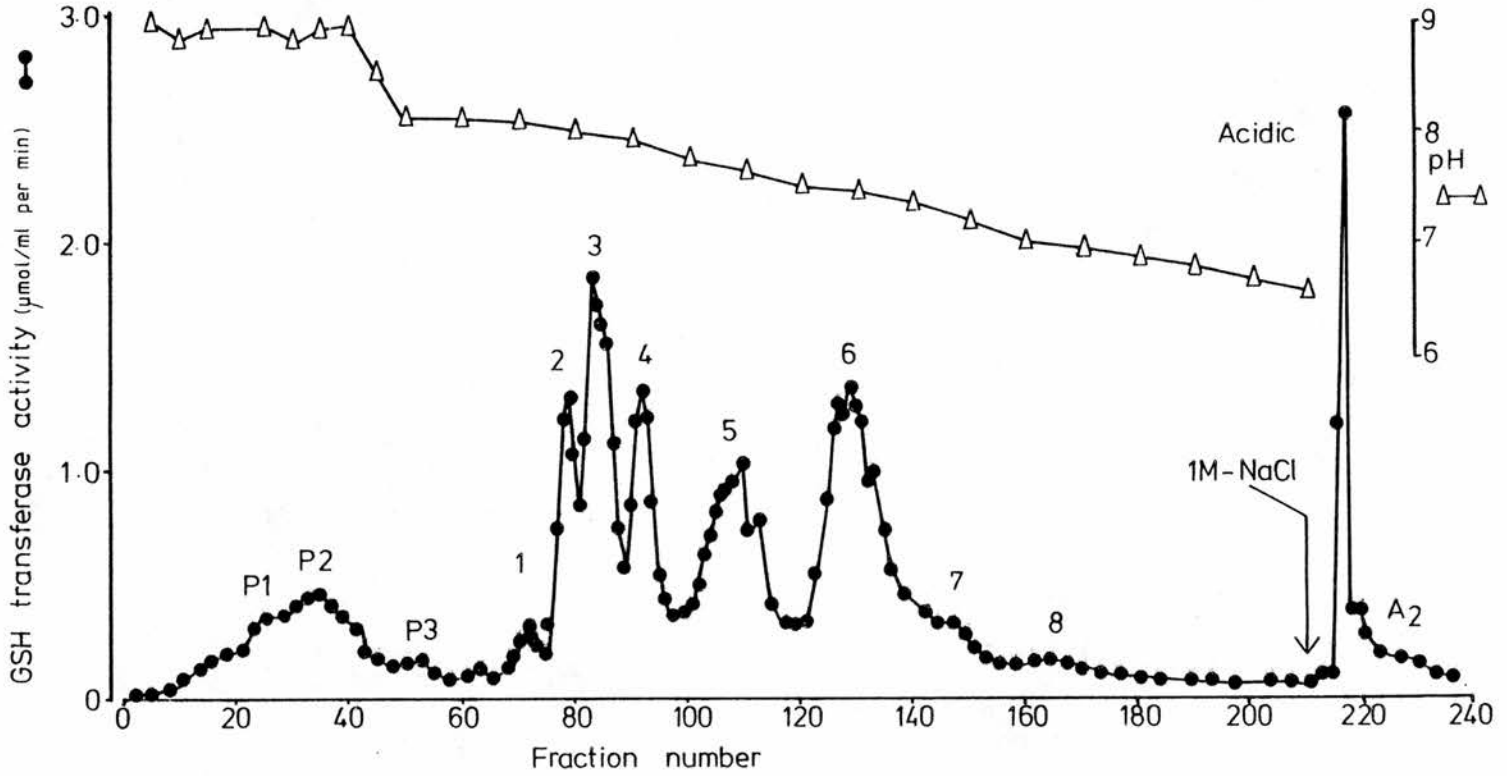


Table 4.8.02 Comparative apparent pI values for salmon GSH transferases excluding acidic forms.

Peak	$pI_{apparent}$ River salmon Lyophilized Fig. 4.8.01a	River salmon GSH affinity Fig. 4.8.02a	Sea salmon (grilse) S-hexyl GSH affinity Fig. 4.7.01a
1	8·20	8·30	7·50
2	8·15	8·20	7·38
3	8·00	8·00	7·25
4	7·80	7·80	7·00
5	7·50	7·60	6·75
6	7·20	7·15	6·70
7	7·10	6·85	6·40

chromatofocusing was the most powerful technique available, its use was continued. Ideally for chromatofocusing, small volumes of well equilibrated material should be applied. Concentration of the cytosol using ultrafiltration was too slow, so lyophilization was used instead. Cytosol was dialysed, lyophilized, redissolved in dialysis buffer and dialysis continued before chromatofocusing (see sections 3.03.06 and 3.04). Because the material to be applied was very impure resolution was expected to be impaired.

The resultant elution profile (Fig 4.8.01a) is very similar in appearance to the profiles obtained for sea salmon. The main difference is the region P1 - P3, which is very basic material eluted from the column before stabilization of the pH gradient. However, it is probably similar to the X1 fraction from sea salmon. As expected the band widths of the other peaks are broader due to the high protein concentration of the applied material.

The pI_{app}'s of the river salmon transferases were observed to differ from those from sea salmon (grilse). The transferases from river salmon began to be eluted from the chromatofocusing column at a higher pH than did the sea salmon transferases. The pI_{app}'s of the sea salmon were nearly one pH unit lower (see Table 4.8.02) if the pI_{app}'s were compared directly from river salmon peak 1 to sea salmon peak 1 and so on.

The substrate specificities were not investigated because of time needed to carry out a whole batch of assays. The enzymic activities of the individual peaks were beginning to decrease and substrate specificity tests with such material (which was also relatively impure; see Fig: 4.8.01b) were likely to yield unreliable results. It was felt that more useful comparisons could be obtained between the two kinds of salmon using K_{emp} determinations. In addition, the peaks were assayed using a fluorescent substrate, monobromobimane (see section 3.05.05). The sensitivity and

Table 4.8.01 Empirical half-saturation concentrations for GSH and CDNB and substrate specificity comparisons between CDNB and mBrB for certain GSH transferases from river salmon.

Peak	K_{emp} values		CDNB activity / Δ fluorescence from mBrB-GSH
	GSH (mM)	CDNB (mM)	
1	0·35	0·92	1·18
2	0·23	1·61	0·38
3	0·20	1·90	0·31
4	0·16	1·34	0·33
5	0·15	0·97	0·37
6	0·13	0·93	0·54
7	0·07	0·42	0·11
Acidic	4·64	0·87	2·06

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; mBrB, monobromobimane. Standard errors calculated for the K_{emp} values were all less than 15% of the values themselves.

reliability of this assay meant that the rate of reaction (expressed as the change in fluorescence with time) could be expressed as a ratio of the activity towards CDNB and be used as a simple test for differential substrate specificity.

The results for both K_{emp} and substrate specificity comparisons are shown in Table 4.8.01. The K_{emp} values for GSH and CDNB were slightly lower than those obtained for grilse (see Table 4.7.02b for a comparison). The K_{emp} for the acidic form for GSH was very high (4.64mM), similar to the situation with trout. The K_{emp} values for GSH indicated that peaks 4,5 and 6 were similar, but the values for CDNB showed that peak 4 was distinct. Overall only peaks 5 and 6 appeared to be closely related.

The ratio of CDNB activity to activity with the fluorescent substrate (Table 4.8.01) showed that the values for peaks 1,6,7 and the acidic peak were distinct and that those for peaks 2 - 5 were in the same range. Peaks 5 and 6 appeared distinct therefore all the forms differed in one respect or another.

To investigate further the region P1 - P3, all the activity which was eluted before fraction 60 was pooled, ultrafiltered, dialysed and applied to a pH 11-8 chromatofocusing column (see 3.03.06). The resultant elution profile (not shown) indicated the presence of two peaks C1 and C2, which eluted at pH 8.9 and 8.8 respectively.

The impurity of the different fractions was clearly seen when the samples were subjected to SDS-PAGE (Figs. 4.8.01b and 4.8.01c). In a later experiment (elution profile not shown) the acidic peak was split into two fractions by the use of a salt-gradient. These peaks contained different subunits (shown in Fig. 4.8.01c ; labelled A1 and A2).

To investigate fully the differences between the sea and river forms a purer preparation of river salmon

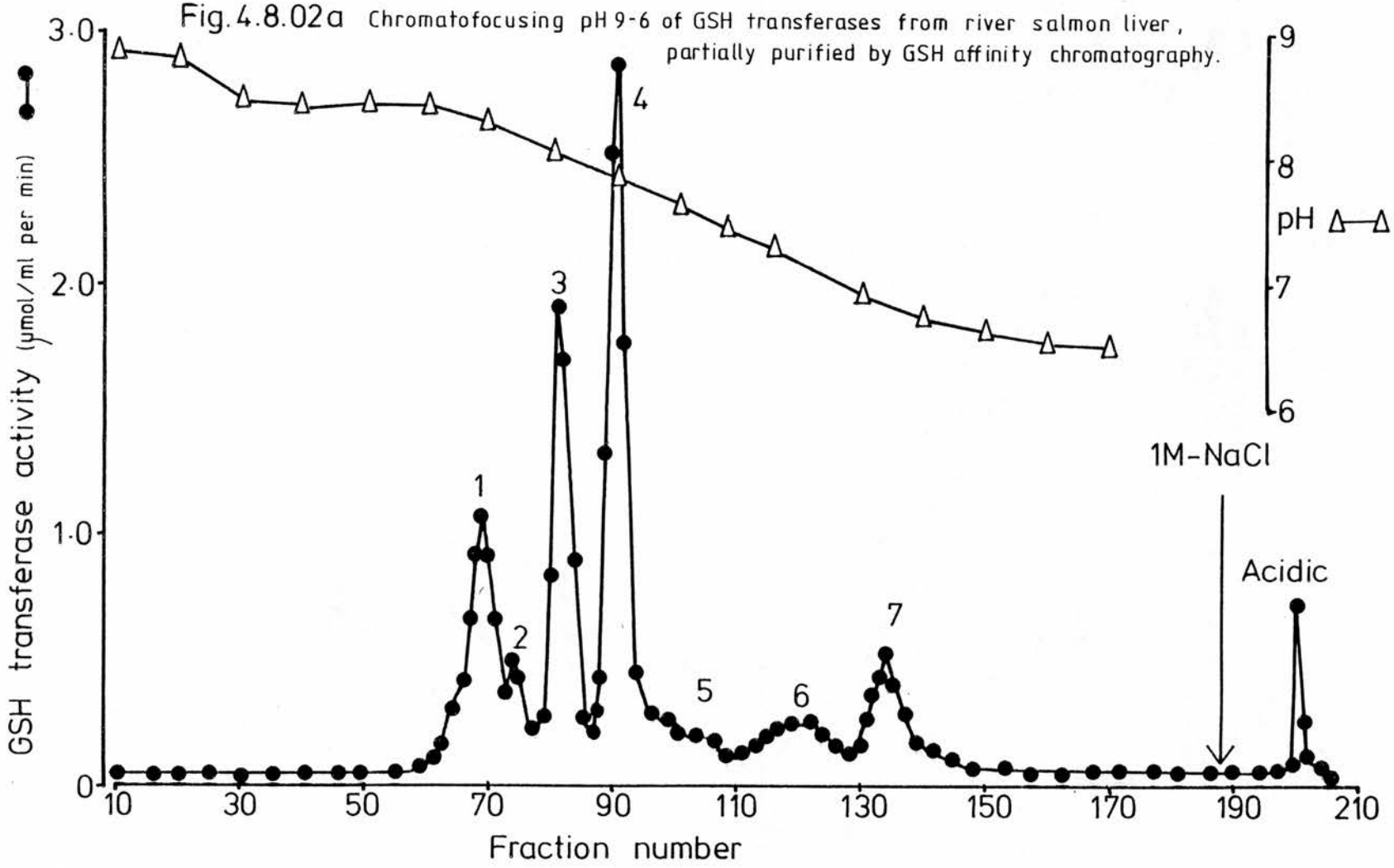


Fig. 4.8.02a Chromatofocusing pH 9-6 of GSH transferases from river salmon liver, partially purified by GSH affinity chromatography.
GSH transferase activity (μmol/ml per min)
3·0
2·0
1·0
0
pH
9
8
7
6
1M-NaCl
Acidic
1
2
3
4
5
6
7
10
30
50
70
90
110
130
150
170
190
210
Fraction number

material was required. For this reason it was decided to use GSH affinity chromatography despite its effect on the pI's of the transferases from rainbow trout.

4.8.02: The purification of river salmon GSH S-transferases using GSH affinity chromatography and chromatofocusing

Hepatic cytosolic GSH transferases from river salmon bound strongly to the GSH-sepharose matrix. This is interesting as it is the first reported case of a differential affinity between the two matrices for a GSH transferase (Mannervik, 1984; personal communication). The bound material was chromatofocused as before and the elution profile obtained is shown in Fig. 4.8.02a.

This profile is very similar to that obtained for sea salmon (Fig. 4.7.01a). The criteria used for this similarity being the relative peak heights, band widths and resolution. Peaks 1 - 7 from 4.8.02a appear to correspond to peaks 2 - 8 for the grilse. Although the grilse appears to have an extra peak, this might be hidden in the shoulder of peak 1 from the river salmon. The only significant difference in the profile is the smaller size of the acidic peak in Fig. 4.8.02a. Because the acidic peak for river salmon cytosol chromatofocused after lyophilization is larger (Fig. 4.8.01a) it is very likely that a proportion of the acidic material is not bound to GSH-sepharose, which in turn suggests at least two forms of the acidic protein exist with differing affinities for the matrix.

The pI_{app} values obtained after chromatofocusing of lyophilized and affinity purified river salmon cytosol (Table 4.8.02) are very similar; clearly no major alteration of pI occurred. The elution profiles themselves differ, probably due to differential affinities for the GSH- sepharose matrix; a certain amount of material was not bound (including a

high proportion of the acidic fraction). This highlights a drawback of affinity chromatography; a certain proportion of activity may not be bound, giving a false distribution of activity.

A number of other factors might contribute to the differences observed between the profiles. With the lyophilized material, the peaks are poorly separated and the band widths are large, making it difficult to compare with the affinity purified material. In addition, differences in stability between enzymes at low and high protein concentrations may contribute and these salmon preparations all used one liver; clearly any difference in peak heights could also be explained as a difference between individuals.

To compare the river and sea salmon transferases, the peaks from Fig. 4.8.02a were then subjected to SDS-PAGE.

4.8.03: SDS-PAGE of the GSH S-transferases from river salmon hepatic cytosol after purification by affinity chromatography and chromatofocusing

The resulting gel is shown in Fig. 4.8.03. Peaks 1 - 6 (peak 7 not shown as it had insufficient protein) all comprised one protein band migrating as if had a M_r of 22,400. The acidic peak contained in addition to the M_r 22,400 band a band of equal staining intensity with a M_r of approximately 24,000.

No apparent differences in the mobility of the staining components was seen when sea and river salmon transferases were run on adjacent tracks of SDS-PAGE gels (not shown). From this the sea and river salmon GSH transferases can be seen to be very closely related. Their elution profiles after affinity chromatography and chromatofocusing are similar, their K_{emp} values for GSH and CDNB are in the same range, their subunit compositions are similar and their subunit M_r's are identical. The only clear differences remain in the pI_{app}'s of the enzymes and in the

Fig.4.8.03.

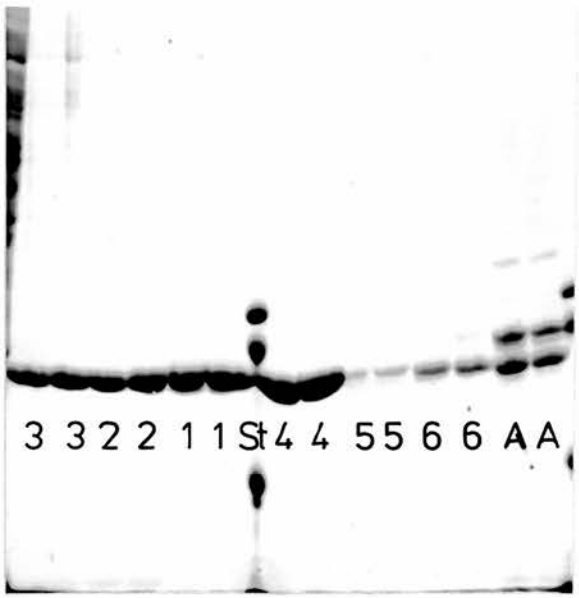


Fig.4.9.01a.

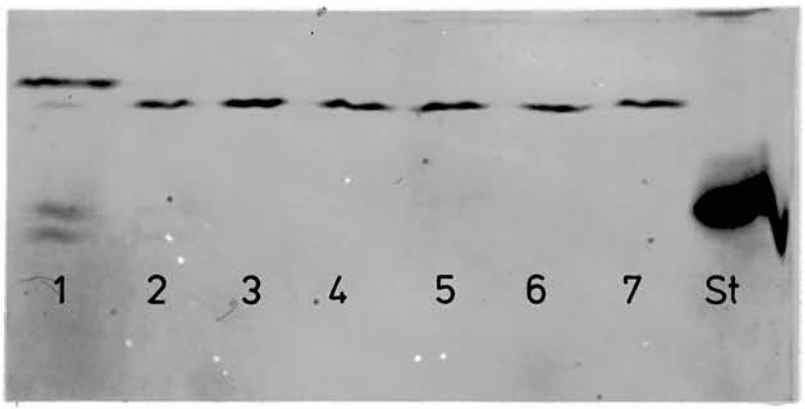


Fig.4.9.01b.

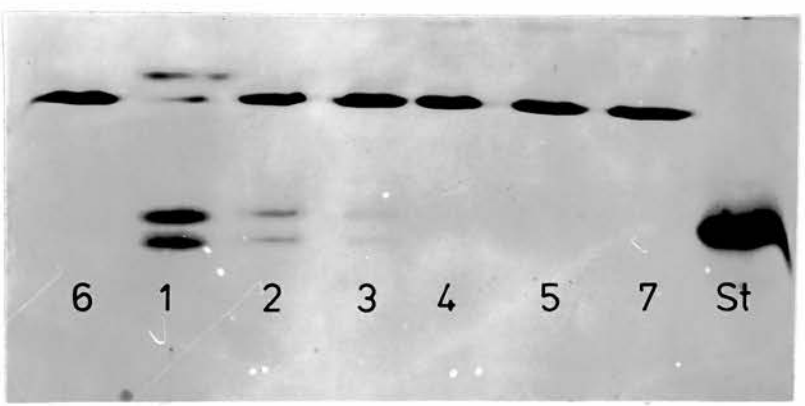


FIG. 4.8.03.

SDS-PAGE of river salmon GSH transferases separated by chromatofocusing (pH 9-6) after partial purification by GSH-affinity chromatography.

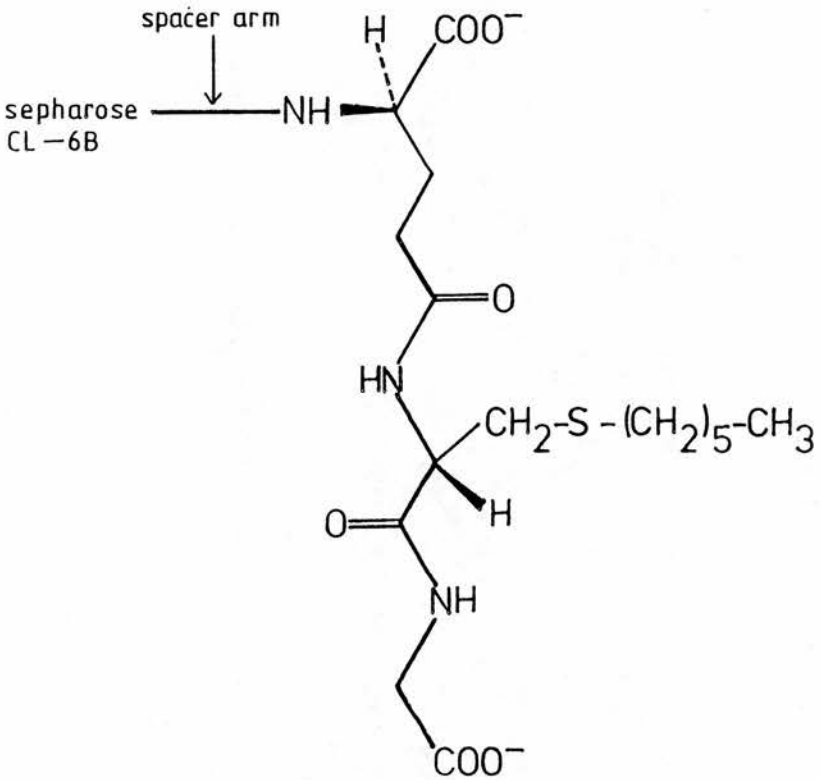
FIG. 4.9.01A.

Limited proteolysis of purified sea salmon GSH transferases by *Staphylococcus aureus* V8 protease. 7 represents pure protein with no added protease whilst 1-6 represent different concentrations of protease added; these were 0.5, 0.05, 0.005, 0.0005, 0.00005 and 0.000005mg/ml respectively. In this case St represents only the α-lactalbumin (M_r 14,200) standard.

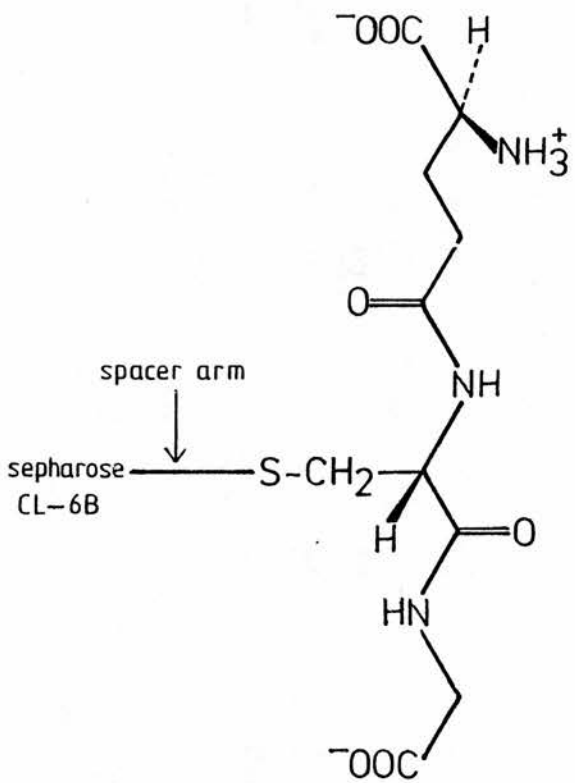
FIG. 4.9.01B.

Limited proteolysis of purified river salmon GSH transferases by *Staphylococcus aureus* V8 protease. Otherwise this figure is identical to 4.9.01a.

Fig.4.9.01 The S-hexyl GSH and GSH affinity matrices used.



Coupled through the glutamic acid amino group
Hydrophobic, net charge 1-ve.



Coupled through the sulphydryl group of the cysteine residue. Net charge 1-ve.

affinities for both kinds of affinity matrices.

Both sea and river salmon have a number of basic GSH transferases with similar properties and have acidic forms with distinct properties. This bears a strong similarity to the case of the human GSH transferases (Kamisaka et al., 1975b). Certain distinctions can be observed between the basic forms of salmon GSH transferase to indicate that the different basic forms are distinct and are not merely charge isomers formed by deamidation.

To investigate the cause of the differences between sea and river salmon a number of experiments were carried out.

4.9: AN INVESTIGATION OF THE DIFFERENCES BETWEEN SEA SALMON AND RIVER SALMON

In section 4.8, two clear differences were observed between the hepatic GSH transferases of sea salmon and river salmon. River salmon transferases were not bound by S- hexylGSH sepharose whereas the sea salmon forms were. In addition, the sea salmon enzymes had lower pI_{app}'s than the river salmon enzymes.

The two affinity matrices used in section 4.8 are illustrated in Fig. 4.9.01. The differences between them are the couplings and the ligands used. S-hexylGSH is coupled by the glutamic acid amino group to the spacer arm whereas GSH is coupled via the cysteinyl sulphydryl group (if it is coupled via the amino group the resultant matrix has a low affinity for the transferases: Simons & Vander Jagt, 1977). In addition S-hexylGSH is hydrophobic because of the hexyl group which is attached to the cysteinyl sulphydryl group.

At this stage it was thought that the pI_{app} difference and affinity difference were related. It seemed likely that the differences could be explained by a small difference in amino acid compositions or by the binding of charged hydrophobic ligands. To investigate these possibilities a number of experiments including limited proteolysis were carried out.

4.9.01: Limited proteolysis of salmon GSH transferases

Peak 4 from Fig. 4.7.01a and peak 4 from Fig. 4.8.02a (sea salmon 4 and river salmon 4) were prepared and loaded onto Cleveland gels (Methods; 3.07.04) and digested with *Staphylococcus aureus* V8 protease (at concentrations ranging from 0.5 - 0.00005 mg/ml). The results are shown in Figs. 4.9.01a and 4.9.01b. No differences in cleavage between the two salmon peak 4 proteins can be seen. As only very limited proteolysis occurred it must be assumed that the aspartic acid and glutamic acid residues in the proteins are either few or well hidden in the centre of the protein structures. This experiment indicates that the distribution of these two negatively charged amino acids between the two proteins is even and that they do not appear responsible for the greater negativity of the sea salmon transferases.

Further limited proteolysis was carried out using electroeluted materials and SDS-PAGE urea gels were used (Methods; 3.10 and 3.08). The proteins (peak 4) were digested with *Staphylococcus aureus* V8 protease and chymotrypsin. The results (not shown) indicated no differences between the peak 4 proteins although the gels were silver-stained and visually less clear than SDS-PAGE gels. These results indicated no differences in the content of the negatively charged amino acids or in the content of aromatic amino acids. Proteolysis using cyanogen bromide (cleaving at residues such as methionine) carried out later revealed no differences although the gels (not shown) were very poor.

Limited proteolysis proved unsuccessful in resolving the differences in behaviour between the river and sea salmon. It must be said that this work was only carried out with one transferase peak from each fish type, but to have done the work with all the proteins would have been very time consuming. It was better to look for a difference with one protein and then if one were found, to apply the technique successively to the other proteins. If the difference were caused by a pocket of hydrophobic amino acids then limited proteolysis would show cleavage at apparently only one point due to the low resolving power of the gels. A protein with one hydrophobic amino acid in the pocket would show the same cleavage pattern as a protein with say three such amino acids in the pocket.

It was decided therefore to use the more powerful technique of amino acid analysis.

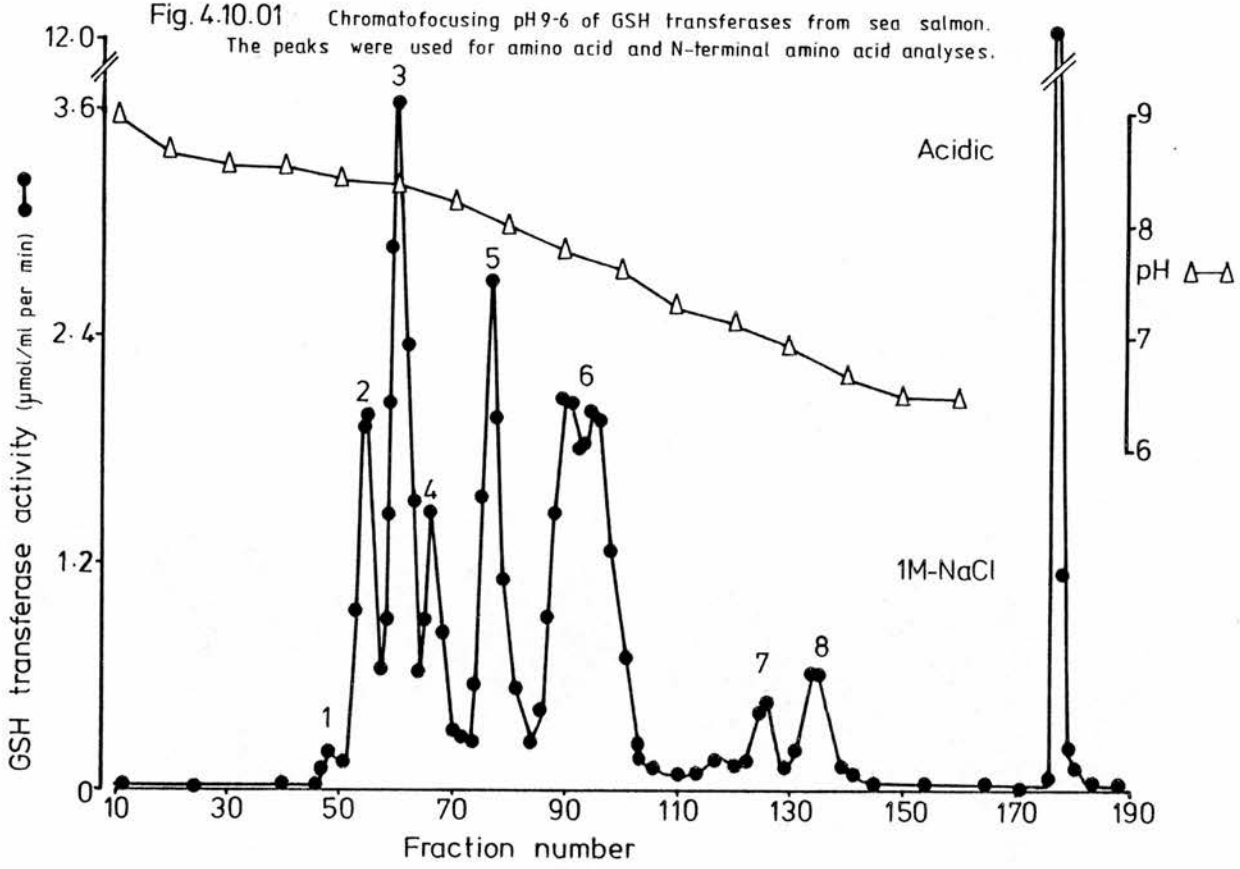


Fig. 4.10.01 Chromatofocusing pH 9-6 of GSH transferases from sea salmon. The peaks were used for amino acid and N-terminal amino acid analyses.

Table 4.10.01 Amino acid compositions of sea salmon GSH transferase peaks.

AMINO ACID	1	2	3	4	5	6	7	8	Acidic
ASP	10·96	13·65	14·98	12·10	13·27	16·42	6·27	9·45	10·15
THR	5·68	2·81	2·93	2·66	3·12	3·75	1·55	2·11	2·43
SER	7·92	5·43	6·26	4·55	5·66	6·15	4·01	3·96	6·21
GLU	15·72	8·10	8·17	6·61	8·81	9·99	25·44	17·89	13·31
PRO	4·86	2·88	3·48	2·76	2·79	4·01	0·81	1·41	3·32
GLY	8·48	9·56	10·32	8·52	9·10	10·37	26·46	20·39	6·77
ALA	7·43	6·51	7·10	6·62	6·83	8·45	3·56	4·86	7·50
CYS/2	0·31	1·69	1·98	1·30	0·23	2·05	0·41	2·66	0·41
VAL	9·78	4·23	4·67	4·30	4·99	6·39	1·80	2·66	3·15
MET	5·23	3·80	4·07	3·77	3·59	5·38	0·96	2·00	2·83
ILE	5·76	4·28	4·91	4·06	4·38	5·21	1·80	2·34	3·47
LEU	14·14	9·58	10·63	8·61	9·56	12·50	3·30	5·35	8·85
STANDARD	10·00	10·00	10·00	10·00	10·00	10·00	10·00	10·00	10·00
TYR	4·32	4·14	4·70	3·75	3·39	5·57	1·68	2·26	4·53
PHE	5·28	4·66	5·11	4·33	4·42	6·08	1·94	2·64	3·57
HIS	2·88	2·15	2·44	3·62	7·75	7·62	20·85	20·82	2·64
LYS	10·59	8·33	9·17	7·02	8·00	11·06	2·65	4·24	5·25
ARG	3·95	2·82	3·01	2·28	2·72	3·71	0·86	1·55	3·36

4.10: AMINO ACID ANALYSIS

By this time all the river salmon livers obtained had been used as had the sea salmon (grilse) livers from August 1983. Because of difficulties in obtaining river salmon livers, amino acid analysis was carried out on a new batch of sea salmon livers (February 1984) from the same source.

The preparation was scaled up, three livers being used. The profile produced after affinity chromatography and chromatofocusing is shown in Fig. 4.10.01. The profile obtained was very similar to previous profiles except that the pI_{app}'s of the proteins were very similar to those of river salmon; this meant that it was the August 1983 batch of sea salmon which had transferases with lower pI_{app}'s and that the only difference between sea and river salmon was the behaviour on affinity chromatography. In all cases the pH measurements were only carried out after calibration of the pH meter and it is therefore reasonable to assume that these differences were real and not artefactual.

When the purity of the preparation was tested using SDS- PAGE (Fig. 4.10.02) a new protein (peak 1) was observed and the composition of the acidic peak was observed to have changed. These differences are discussed in more detail in section 4.12. The peaks were then prepared and subjected to amino acid analysis using the methods laid down in section 3.17. The results were then analysed by the method of Cornish-Bowden (1983). The overall analysis is shown in Table 4.10.01.

Cornish-Bowden analysis of the results showed that peaks 2 - 6 were closely related and that peaks 2 and 3 were very similar. Peaks 1,7,8 and the acidic peak were all distinct. The SDS-PAGE analysis had already indicated that peak 1 and the acidic protein were distinct from the other

Fig.4.10.02.

FIG. 4.10.02.

SDS-PAGE of sea salmon GSH transferases (material obtained in February 1984). The staining at the lower end of the gel is caused by polybuffer 96 in the samples.

forms from the subunit composition. This data showed therefore, that the multiple forms of cationic transferase were not simply charge isomers but distinct enzymes. This agrees with the findings that the different subunits had different kinetic and catalytic characteristics.

The problem with such analyses is that the assumption is made that one migrating component on a gel represents one subunit type and not one or more of similar M_r. Earlier kinetic and catalytic data suggests that several types of M_r 22,400 subunit exist. This means that the results do not neccessarily represent one subunit type but might be an average of several. These differences apply to all species investigated as it is becoming apparent that as many as five different Yb type subunits are found in the rat (Hayes, 1984; personal communication).

To test for differences between sea and river salmon, more river salmon material was required. Unfortunatley such material has proved impossible to obtain.

To study the relationships further, studies using antibodies were carried out. No new material was needed for these because using the immune replica technique, samples prepared for SDS-PAGE (unsuitable for amino acid analysis) could be used.

4.11: THE USE OF ANTIBODIES TO TEST THE RELATIONSHIPS BETWEEN SEA SALMON, RIVER SALMON AND RAINBOW TROUT HEPATIC GSH S-TRANSFERASES

Antibodies were raised to the low M_r band of the basic sea salmon transferase peak 2 and to the higher M_r band of the acidic sea salmon GSH transferase (Methods; 3.12) from livers obtained in February 1984. Their reactivity towards different GSH transferase subunits was tested using the immune replica technique (Methods; 3.12.01).

4.11.01: Sea salmon GSH S-transferase immune replicas

The photographs (Figs. 4.11.01a and 4.11.01b) show the developed autoradiographs of immune replicas carried out on sea salmon GSH transferases reacted with antibodies to the low and high M_r subunit types respectively.

In 4.11.01a, it is shown that the anti-low M_r serum reacts strongly with the basic GSH transferase low M_r subunits, but does not react with the intermediate M_r subunit from protein 1. This agrees with the amino acid analysis data which shows that peak 1 is distinct. The antigenic dissimilarity between the two migrating components of peak 1 might suggest that this peak contains two homodimeric transferases. A reaction is also seen between the acidic protein low M_r band and the antiserum, but not between the high M_r band and the antiserum.

In 4.11.01b, using antibodies raised against the higher M_r subunit little or no reaction can be seen with transferases containing either the low or intermediate M_r subunits. The antiserum does react with the acidic high M_r protein and also reacts with X2, one of the sea salmon transferases excluded from S-hexylGSH sepharose. It did not react with X3 (although this fraction had a component

Fig.4.11.01a

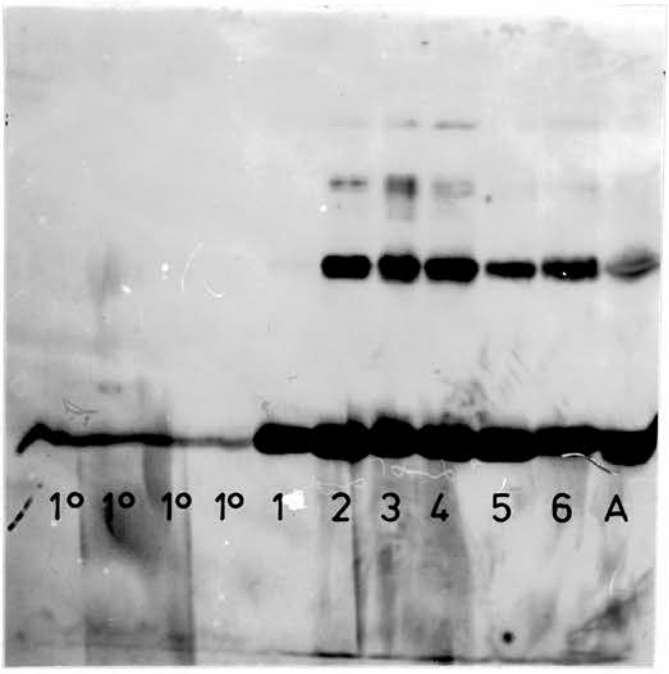


Fig.4.11.01b.

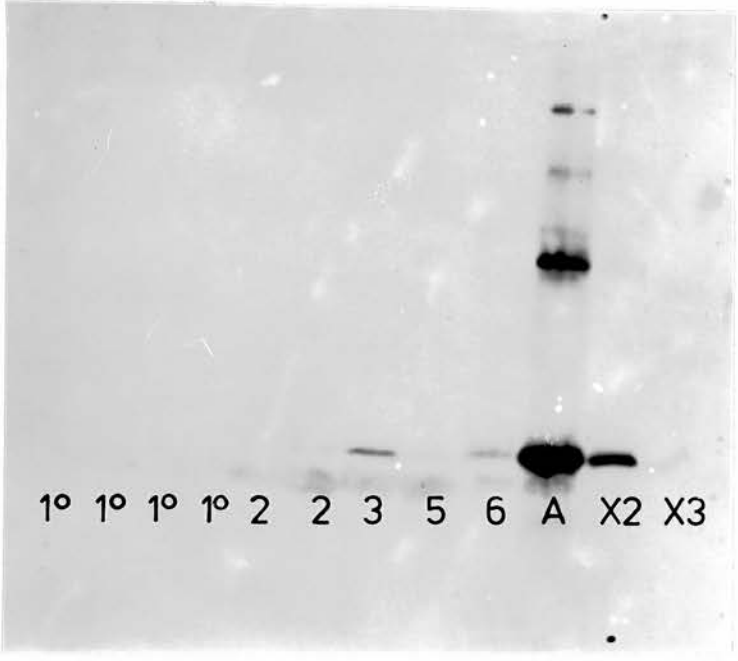


FIG. 4.11.01A.

Immune replica using sea salmon GSH transferases (August 1983). 1° represents peak 1 (February 1984) and contains in addition, an intermediate M_r component. The blotted proteins were probed with antiserum raised against the lower M_r subunit of the sea salmon transferases.

FIG. 4.11.01B.

As for Fig. 4.11.01a, but using antiserum raised against the higher M_r subunit of the acidic transferase to probe the blotted proteins.

migrating at a similar rate to the acidic higher M_r component).

These results indicate that the low and higher M_r components of the sea salmon GSH transferases are immunologically distinct. This also indicates that the two bands found together in the "heterodimeric" acidic protein of grilse (August 1983) were two homodimers as by analogy with the rat (Mannervik & Jensson, 1982), immunologically distinct (therefore differently shaped) subunits are less likely to form a dimer.

The power of this technique is demonstrated by the number of other bands seen on the autoradiographs and not seen in Coomassie stained gels. Either the "pure" proteins injected into the rabbits contained traces of highly antigenic proteins or pre-immune rabbit serum cross-reacts with these bands.

4.11.02: River salmon immune replicas

The antisera were tested against river salmon material which had been lyophilized before chromatofocusing. The aim was to use this far less pure material because it would be a better test of the specificity of the antibodies.

The autoradiographs of the replicas are shown in Figs. 4.11.02a and 4.11.02b. Fig. 4.11.02a using antibodies raised against the lower M_r sea salmon transferase subunits shows reaction with all of the cationic forms and the acidic forms (A1 and A2; separated by the application of a salt-gradient).

In Fig. 4.11.02b the high specificty of the high M_r antiserum can be seen as this reacts only with the two acidic proteins and with peak 5. Why peak 5 should react is not known; contamination from the next well can be ruled out due to the absence of any higher M_r bands on the autoradiograph. There is a possibility that lyophilized peak 5 contains more than one transferase; one of which has a low affinity for GSH-sepharose and contains a subunit immunologically similar to the acidic higher M_r subunit. This is confirmed by SDS-PAGE of the lyophilized river salmon transferases (Fig. 4.11.02c) which shows that peak 5 contains a higher M_r component.

Rat transferases C and AA, loaded onto the other tracks of the gel did not react with either of the antisera, suggesting that these rat transferases are immunologically distinct from the salmon forms, although anti-rat GSH transferase antisera would have to be used against salmon replicas to confirm this.

These results show that the transferases from river

Fig. 4.11.02a.

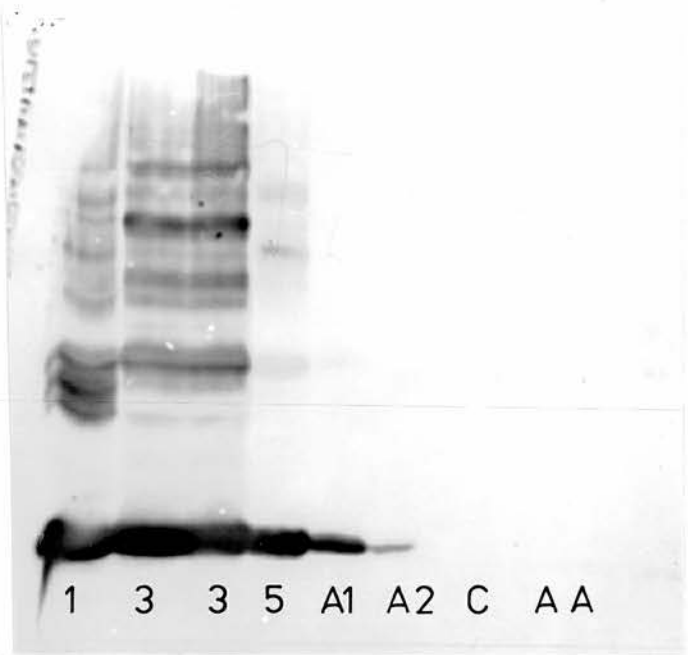
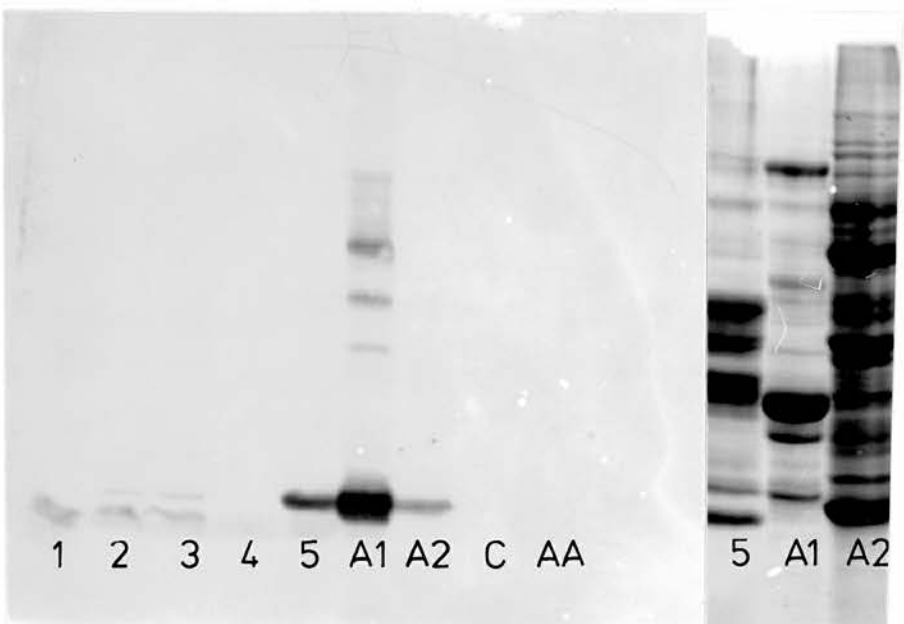


Fig. 4.11.02b.



4.11.02A.

Immune replica using river salmon GSH transferases. The blotted proteins were probed with antiserum raised against the lower M_r subunit of the sea salmon transferases. C and AA represent rat GSH transferases C and AA.

4.11.02B.

As for Fig. 4.11.02a., but using antiserum raised against the higher M_r subunit of the sea salmon acidic transferase. The final three tracks are normal SDS-PAGE of peaks 5, A1 and A2.

and sea salmon cannot be distinguished on the basis of their immunological properties. To test the relationships between the different salmonids still further, replicas were carried out using rainbow trout transferases.

4.11.03: Rainbow trout GSH S-transferase immune replicas

To test the immunological similarities between the salmon and trout transferases, Beecraigs trout transferase fractions were tested against antisera raised to the salmon high and low M_r subunits using the immune replica technique.

Figs. 4.11.03a, 4.11.03b and 4.11.03c show the replicas using the anti-low M_r serum, the anti-high M_r serum and a control SDS-PAGE gel respectively. The results are very interesting; the anti-low M_r serum reacts with the major staining component of the basic and acidic forms (M_r 22,400) and also with a lower M_r component in the acidic fraction, but the anti-high M_r serum reacts highly specifically with a slightly higher M_r component (only a minor band on SDS-PAGE). This indicates that the Beecraigs trout GSH transferase peaks contain two immunologically distinct subunit types, indicating that each peak is a mixture of enzymes. The higher M_r band is not stained as intensely as the lower M_r band on SDS-PAGE, indicating the presence of more than one enzyme and not just one heterodimer.

Antiserum was also raised to the Beecraigs trout basic GSH S-transferases. Replicas were carried out using salmon and trout transferases but the antiserum was very non-specific as impure pure material was injected into the rabbits. Cross-reactivity was observed with both sea and river salmon transferases (not shown) and also a reaction with the rat Yc (but neither Yb type) subunits. This indicates that the trout enzymes are more closely related

Fig.4.11.03a.

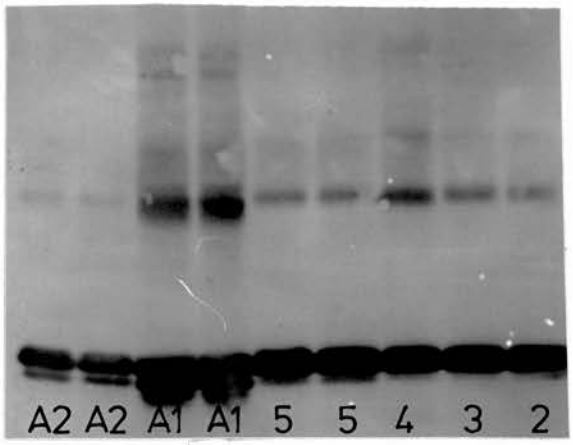


Fig.4.11.03b.

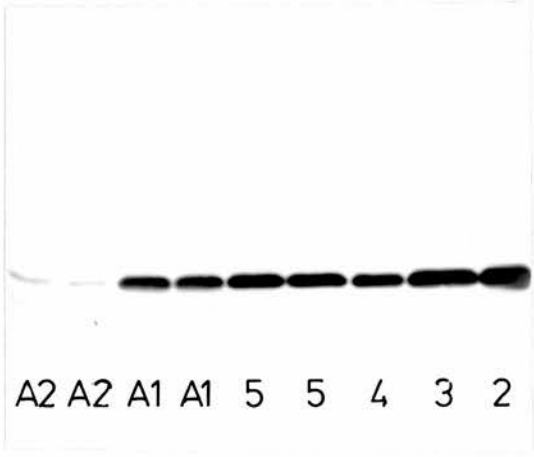
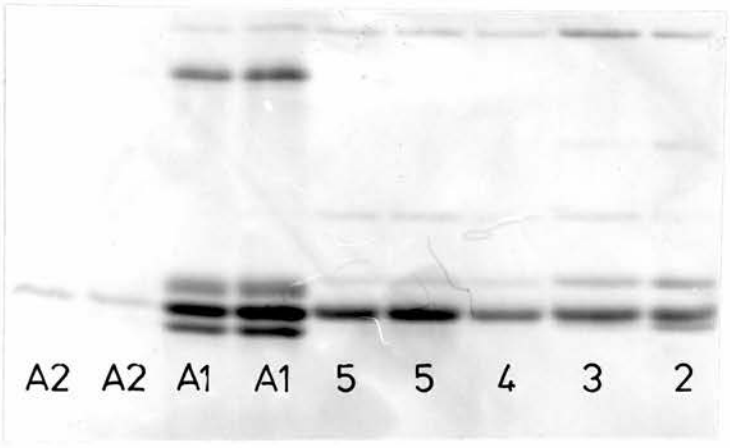


Fig.4.11.03c.



4.11.03A.

Immune replica using GSH transferases from rainbow trout obtained from the Beecraigs trout farm. The blotted proteins were probed with antiserum raised against the lower M_r subunit of the sea salmon transferases.

4.11.03B.

As for Fig. 4.11.03a., but using antiserum raised against the high M_r subunit of the sea salmon acidic transferase.

4.11.03C.

SDS-PAGE of rainbow trout GSH transferases (as above).

to the rat enzymes than are the salmon enzymes.

The use of antibodies failed to show any difference between the sea and river salmon transferases indicating that the two groups of proteins have a similar shape. Neither limited proteolysis nor gel electrophoresis were able to distinguish between them.

A number of different theories can be put forward to account for the difference in binding to the affinity columns. Firstly the enzymes could be completely different; the data above does not support this theory. The difference could be due to a change in the amino acid composition; this would require a complex mechanism and is unlikely to occur in a species moving from salt-water to fresh-water (here the assumption has to be made that the river and sea salmon are the same species). This theory remains untested because no further river salmon material has been obtained to carry out amino acid analysis.

Another explanation might be that the configuration of the GSH binding site was changed in the river salmon, making it smaller and not allowing the hydrophobic tail of S-hexylGSH to enter. Again this would require a complex mechanism and is also very difficult to either prove or disprove with the techniques available . The possibility that the change might have been caused by the different treatment of livers after removal has been disproved by leaving some sea salmon livers unperfused on ice for a day (to no effect); ideally some of the farmed salmon should have been line-caught or some of the river salmon netted to get the two treatments as close as possible. Another possibility (and probably the most realistic) is that the river salmon transferases have bound to them close to the GSH binding site some small hydrophobic ligand which repels the S-hexyl tail. It is not close enough to the hydrophobic binding site(s) or large enough to prevent hydrophobic substrates from binding (CDNB activity is not apparantly inhibited as the enzyme activities for the river and sea

salmon transferases were in the same order of magnitude). If river salmon material were readily available this could be tested by comparing the inhibitory powers of hydrophobic inhibitors (at saturating concentrations) on river and sea salmon transferases. In addition the bound group might well show up if difference spectra were carried out and a hydrophilically bound ligand would also be removed by the introduction of an ammonium sulphate precipitation step.

It is difficult to predict what such a ligand might be. Whether biliverdin or bilirubin accumulating in starving salmon with occluded guts bind to the transferases is unknown, and for any binding studies river salmon transferases are needed. Another possibility is the binding of furanoid fatty acids. Such fatty acids have been found in the livers of a number of fresh-water fish (Glass et al., 1977). These lipids appear to be transported from the liver to the gonads prior to spawning and afterwards accumulate in the liver. Their physiological function is unknown, but if transport does occur then GSH transferases may be involved. However, with all of these ligands a slight pI shift should also be seen due to the charge on the ligands.

The final possibility for the differences between the river and sea salmon is that the two might be distinct species, however, the similarities between the transferases from the two suggests that this is unlikely.

A factor which kept occurring throughout this work was the variability of the sea salmon material; this is discussed in the following section.

4.12: VARIABILITY OF THE SEA SALMON GSH TRANSFERASES

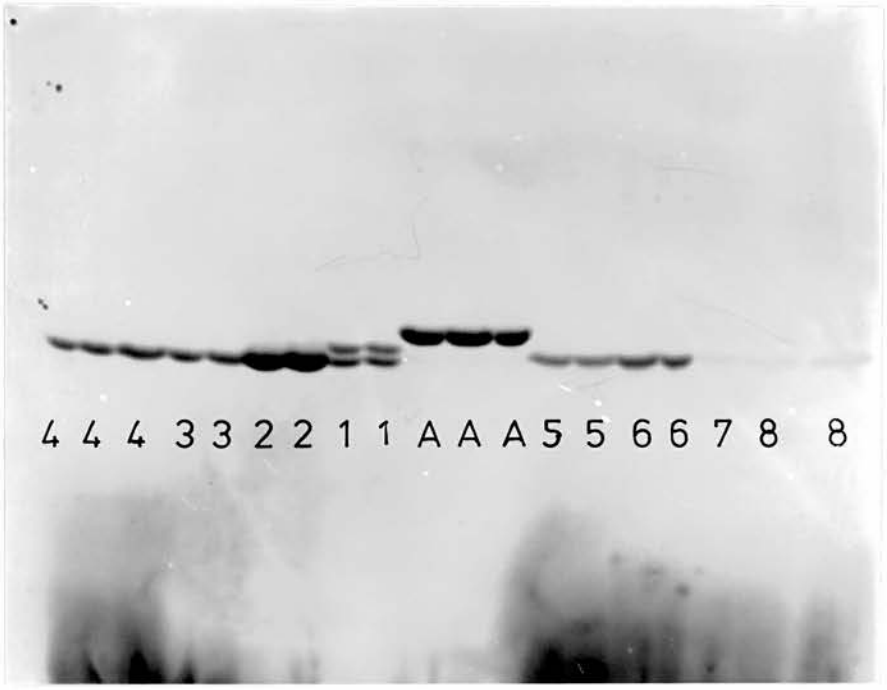
When material collected from Loch Sunart (August 1983; elution profile Fig. 4.7.01a) was compared with material collected in February 1984 (elution profile Fig. 4.10.01) a number of differences were noticed. A comparison of the elution profiles revealed that the pI_{app}'s of the August 1983 fish were lower than those for the February 1984 fish. The later preparation also had an extra peak (peak 1); probably not noticed previously because of the smaller preparative scale. The subunit compositions of the two (Figs. 4.12.01 and 4.12.02; August and February respectively) was also seen to change. The acidic fraction in the later preparation contained only the higher M_r component and the extra peak contained an additional intermediate M_r band (shown earlier to be immunologically distinct from the lower M_r component.

The pI_{app} difference might have either been seasonal summer or winter) or developmental (grilse and post-grilse) respectively. Because of the earlier experience with covalent binding of mixed disulphides, to see if the pI_{app} change was caused by GSH addition, N-terminal amino acid analyses were carried out. These were to test for an increase of N-terminal glutamic acid residues in the lower pI_{app} material over the higher pI_{app} material. This assumed that the γ-carboxyl glutamic acid residue was susceptible to cleavage using the DABITC method.

By this time all the August 1983 material had been consumed so N-terminal microsequencing was carried out (Methods; 3.18) on the February 1984 material to give control values. The results (not shown) indicated that each of the N- terminals were blocked; TLC analysis had shown spots that did not correspond to the standards clearly and HPLC confirmed that these were merely by-products. This

Fig.4.12.01.

Fig.4.12.02.



4.12.01.

SDS-PAGE of sea salmon GSH transferases (grilse, obtained in August 1983).

4.12.02.

SDS-PAGE of sea salmon GSH transferases (post-grilse, obtained in February 1984).

was not too surprising because in most if not all of the GSH transferases tested in other species the N-termini are blocked by proline residues (Beale et al., 1983; Dao et al., 1984).

Material was then collected from Loch Sunart (July 1984) comprising smolts and post-grilse; this was to cover the seasonal and developmental possibilities. However, chromatofocusing of both samples produced profiles with transferases having normal (February 1984) pI_{app}'s. Consequently no further N-terminal microsequencing was carried out; had the pI_{app} values been lower, microsequencing might have shown glutamic acid residues from bound GSH molecules (as the protein N-termini were blocked).

SDS-PAGE of the post-grilse material (Fig. 4.12.03) revealed that the acidic fraction (elution profile not shown but resembling 4.10.01 very closely) contained two migrating components again; similar to August 1983 and river salmon. In addition, the presence of an extra, more basic peak (P) was noticed. This contained only the component of intermediate M_r and suggests that peak 1 might be a hybrid of peak P and peak 2. When the smolt material was chromatofocused (there was little working material because of reduced liver size), the acidic fraction was the only one with a significant amount of activity and this contained predominantly the higher M_r component (Fig. 4.12.04).

In summary, every batch of salmon from Loch Sunart has been distinct in at least one way. The differences cannot be attributed to either seasonal or developmental variation without more work, but merely serve to highlight the problems faced when carrying out a study of this kind. Because batches of material were obtained at long time intervals some of the differences were not noticed until previous material had been consumed preventing definitive experiments from being performed. However, this work has provided a reference point so that anybody continuing the

Fig.4.12.03.

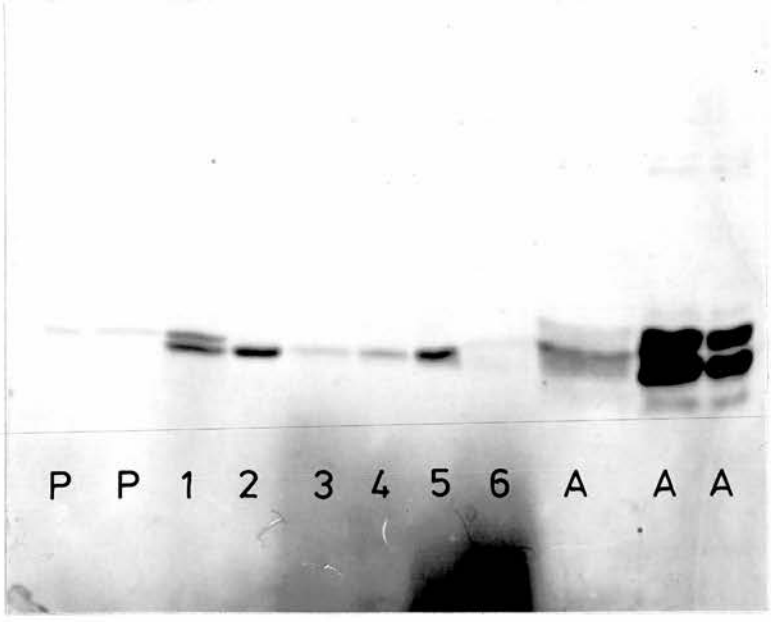


Fig.4.12.04.

4.12.03.

SDS-PAGE of sea salmon GSH transferases (post-grilse, obtained in July 1984). P represents the most basic transferase, eluted prior to peak 1.

4.12.04.

SDS-PAGE of sea salmon GSH transferases (smolt, obtained in July 1984).

study can be prepared for such variations and investigate the causes.

Salmon and rainbow-trout possess closely related GSH S- transferases. In all cases a number of similar basic forms and distinct acidic forms were found (as determined immunologically, kinetically and by their catalytic properties). These proteins possess the ability to detoxify certain xenobiotics although the specificities of the different forms are not broad enough to confirm the theory that they may act by providing a blanket approach to detoxication. No evidence was found for the trout liver enzymes binding non-covalently to bile-acids; covalent binding was however observed with a number of electrophiles. This suggests that the covalent binding function of the transferases may explain their large concentrations.

SECTION 5

SUMMARY

SECTION 5: SUMMARY

This work has shown that the rainbow trout and the Atlantic salmon possess multiple forms of GSH transferase. The forms from the different species appeared to be immunologically similar and eluted in a similar manner from chromatofocusing matrices.

Some of the enzymes from both species were able to use alkyl epoxides as substrates. This gave an indication that the enzymes were capable of detoxifying electrophilic compounds activated by microsomal enzyme systems. A number of other compounds served as substrates, but with the exception of 1-chloro-2,4-dinitrobenzene (CDNB) all were poor substrates for the transferases.

With both species multiple basic enzymes with similar properties were observed. The acidic form(s) were quite distinct. Most of the basic forms in the salmon comprised a single M_r component on SDS-PAGE; similar to the case with the human and murine forms. Comparisons with other fish data are difficult because very little detailed work has been carried out. Epoxide conjugation activity was found in a number of marine species (James et al., 1976) although the substrates used were mainly polyaromatic epoxides. The most detailed work so far has been carried out on the elasmobranch liver transferases (Sugiyama et al., 1981). This work was concerned with the identification of ligandin- like activity. Direct comparisons are difficult to make because the elasmobranch forms were separated by gel- filtration and not ion-exchange; however, some similarities can be seen. Activity towards p-nitrobenzyl chloride was 300% of that for CDNB. This bears a striking similarity to the results obtained for the trout acidic form and for the sea-salmon X3 protein. In addition a form with a high Km for GSH was shown which is similar to the trout and salmon

acidic proteins. The substrate specificity data for the Northern pike (Balk et al., 1980) is not directly comparable because assays were only carried out with crude extracts and not purified enzymes.

No binding of bile-acids by the trout transferases was detected although the organic anions ANS and BSP were bound. Inhibition data shows that many compounds which were not conjugated bound to the transferases.

Much detail has been paid to the binding function of the rat enzymes (Hayes et al., 1979, 1980; Strange 1981) but the physiological significance of the function is still not known. No attempt has been made to explain why transferases from tissues such as the spleen and lung bind bile-salts (Mannervik, 1984). It is therefore uncertain whether or not bile-salt binding is physiological or merely coincidental.

The number of different transferase forms remains a mystery. If for example 5 different Yb type subunits exist (Hayes, 1984; personal communication), then a possible 15 YbYb containing proteins exist. One of the theories behind GSH transferase function is the blanket approach. In this a large number of enzymes with overlapping substrate specificities are produced to deal with potentially harmful compounds. However, the substrate specificities of the salmonid enzymes (as assayed) appear too limited to offer any kind of cover by this method. This may be due to the wrong substrates being chosen; the different forms may provide good cover for a range of endogenous compounds.

The proposed role in the detoxication of xenobiotics is questionable. If highly reactive electrophiles are activated by the microsomal enzyme systems then the GSH transferases would be expected to be more closely associated with the particulate region. No microsomal GSH transferase activity was detected in the trout, although microsomal GSH

transferases have been found in the rat (Morgenstern et al., 1979; Bach et al., 1984).

The microsomal forms are not apparently induced by the same compounds that induce the cytosolic forms (Morgenstern et al., 1980). In fish, other microsomal enzyme systems are induced (Chambers & Yarborough, 1976) whilst the cytosolic GSH transferases are not induced (Sherwood & Mearns, 1977). These deviations make it very difficult to believe that the GSH transferases are closely involved with the microsomal enzyme systems.

The fact that the GSH transferases are so widely distributed in tissues and species indicates that their original role was not detoxication (Grover, 1982). The results presented in this work and by others (Lee, 1984; Stenersen & Øien, 1981; Balabaskaran & Muniandy, 1984) show that although a great number of species have GSH transferases, significant differences occur between the enzymes from different species.

Although variations in the protein content of salmonids are documented (Love, 1970; Sedgewick, 1982) the causes of the variations seen with the sea salmon material were not determined. These differences and those observed with the binding behaviour of the river forms could best be determined using pure-bred stocks of fish. Samples could be taken at regular intervals from smolt stage right through to the spawning adult stage. This work has shown that other methods are prone to too much variation.

This study is the most detailed so far carried out on the GSH transferases in fish. The salmonids are vunerable to xenobiotics yet have the potential to detoxify them using the GSH S-transferases. As yet there is no conclusive evidence that the transferases have evolved as a mechanism for the detoxication of xenobiotics, indeed the literature would appear to suggest that these enzymes evolved to deal

with endogenous compounds and that they are of sufficiently broad spectrum to catalyse the conjugation of certain xenobiotics.

SECTION 6

REFERENCES

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APPENDIX

Appendix 3.05.04

Parameters used for the centrifugal analysis of GSH transferase activity using CDNB as substrate.

CARDIOVASC RES UNIT EDINBURGH

TEST NR 28 + GST

PARAMETER LISTING

1	UNITS	UMOL/L	
2	CALCULATION FACTOR	5208	
3	STANDARD 1 CONC	0	
4	STANDARD 2 CONC	0	
5	STANDARD 3 CONC	0	
6	LIMIT	12000	
7	TEMPERATURE [DEG.C]	25.0	
8	TYPE OF ANALYSIS	3	
9	WAVELENGTH [NM]	340	
10	SAMPLE VOLUME [UL]	05	
11	DILUENT VOLUME [UL]	25	
12	REAGENT VOLUME [UL]	300	(GSH in assay buffer)
13	INCUBATION TIME [SEC]	60	
14	START REAGENT VOLUME [UL]	10	(CDNB)
15	TIME OF FIRST READING [SEC]	30.0	
16	TIME INTERVAL [SEC]	10	
17	NUMBER OF READINGS	09	
18	BLANKING MODE	1	
19	PRINTOUT MODE	1/2	

24 Samples are assayed per run.

Appendix 3.06.02

Derivation of the equation for K_{emp} Atkins and Nimmo (1981).

$$V_{max} = \frac{v_1(K_m + S_1)}{S_1} \qquad \ldots\ldots (1)$$

$$v_1 = \frac{V \cdot S_1}{K_m + S_1} \qquad \ldots\ldots (2)$$

$$v_2 = \frac{V \cdot S_2}{K_m + S_2} \qquad \ldots\ldots (3)$$

substituting (1) into (3) we get

$$v_2 = \frac{v_1(K_m + S_1)S_2}{S_1(K_m + S_2)} \equiv v_2 = \frac{v_1}{S_1}(K_m + S_1)\frac{S_2}{K_m + S_2} \qquad \ldots\ldots (5)$$

rearranging ..... (5)

$$\frac{v_2(K_m + S_2)}{S_2} = \frac{v_1}{S_1}(K_m + S_1)$$

$$v_2 S_1 (K_m + S_2) = v_1 S_2 (K_m + S_1)$$

$$K_m v_2 S_1 + v_2 S_1 S_2 = v_1 S_2 K_m + v_1 S_2 S_1$$

rearranging

$$v_2 S_1 S_2 - v_1 S_2 S_1 = v_1 S_2 K_m - v_2 S_1 K_m \qquad \ldots\ldots (9)$$

rearranging ·····⑨

$$S_1 S_2 (v_2 - v_1) = K_m (v_1 S_2 - v_2 S_1)$$

$$K_m = \frac{S_1 S_2 (v_2 - v_1)}{v_1 S_2 - v_2 S_1}$$

dividing through by $S_1 S_2$

$$K_m = \frac{v_2 - v_1}{\frac{v_1}{S_1} - \frac{v_2}{S_2}}$$

because of the empirical nature of the half-saturation concentration K_m, it is called K_{emp}.

PUBLICATIONS

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DISTRIBUTION AND SOME PROPERTIES OF THE GLUTATHIONE *S*-TRANSFERASE AND γ-GLUTAMYL TRANSPEPTIDASE ACTIVITIES OF RAINBOW TROUT

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(*Received* 31 *March* 1982)

Abstract—1. Gills, kidney, intestinal caeca and liver of trout have glutathione *S*-transferase activity with 1-chloro-2,4-dinitrobenzene (200–500 nmol/min/mg protein), and reduced glutathione (0.5–2.0 mmol/kg tissue).

2. Only kidney and intestinal caeca have substantial γ-glutamyl transpeptidase activity with γ-glutamyl-*p*-nitroanilide (2–9 nmol/min/mg protein).

3. Renal γ-glutamyl transpeptidase is membrane-bound and has similar kinetic properties to its mammalian counterparts.

4. The data are consistent with the presence of a mercapturic acid pathway in trout.

INTRODUCTION

In mammals, one way of detoxifying foreign compounds is to convert them to *N*-acetylcysteine *S*-conjugates (mercapturic acids) which are then excreted in the urine or faeces (Boyland & Chasseaud, 1969; Meister, 1981). The first enzymic step in mercapturic acid formation is the conjugation of the foreign compound with reduced glutathione (GSH)‡, and is catalysed by a glutathione *S*-transferase (Jakoby, 1978). The γ-glutamyl group is subsequently removed by γ-glutamyl transpeptidase (GGT) and the glycyl group by a peptidase. Finally the cysteine conjugate is acetylated by an *N*-acetyl transferase (Boyland & Chasseaud, 1969). The initial reaction is thought to occur predominantly in the liver, and the remaining ones in the kidney (Hughey *et al.*, 1978; Meister, 1981).

The presence of a similar pathway in fish has been inferred, though not established. For example, it is known that isolated hepatocytes, together with renal tissue from trout, can convert acetaminophen to a cysteine conjugate (Parker *et al.*, 1980, 1981), and that in this species the liver and some other tissues have glutathione *S*-transferase activity (Nimmo *et al.*, 1979, 1981). To define further the potential of the trout to metabolize xenobiotics by a mercapturic acid pathway, we have extended our previous data on the tissue distribution of glutathione *S*-transferase activity (Nimmo *et al.*, 1979), and have studied the distribution and some of the properties of the second enzyme in the putative pathway, namely GGT.

MATERIALS AND METHODS

Chemicals

Bovine albumin, L-γ-glutamyl-*p*-nitroanilide, glycylglycine and GSH were purchased from Sigma London Chemical Co., Poole, UK, and all other reagents from BDH Chemicals, Poole, U.K.

Fish

Rainbow trout (*Salmo gairdnerii*; 150–200 g) were collected from a local trout farm, transported to the laboratory, and used within 2 hr.

Preparation of tissue extracts

Fish were killed by cervical dislocation, and blood was taken into tubes containing lithium heparin. The other tissues (gills, kidney, intestinal caeca and liver) were dissected out, washed in 0.154 M NaCl, chopped with scissors in 5 vol. phosphate medium (20 mM KH_2PO_4 adjusted to pH 7.4 with 20 mM K_2HPO_4; 0.25 M sucrose), and homogenized with a glass–teflon homogenizer (all at about 4°C). Cytosol for the determination of glutathione *S*-transferase was prepared by centrifuging the homogenate (100,000 *g*; 60 min; 4°C).

When kidney was subjected to differential ultracentrifugation the phosphate medium was replaced by Tris buffer (50 mM Tris adjusted to pH 7.4 at room temperature with HCl; 25 mM KCl; 5 mM $MgCl_2$; 0.25 M sucrose). Homogenate was fractionated into preparations called nuclei (sedimented at 600 *g* for 20 min), heavy mitochondria (sedimented at 8000 *g* for 10 min), light mitochondria (sedimented at 13,300 *g* for 10 min), microsomes (sedimented at 100,000 *g* for 60 min) and cytosol (Statham *et al.*, 1977). The subcellular fractions (except cytosol) were washed once with 10 ml of Tris buffer/sucrose and resuspended in 2–5 ml of this medium.

Analytical methods

Glutathione *S*-transferase activity was assayed spectro-

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‡ Abbreviations: GSH, reduced glutathione; GGT, γ-glutamyl transpeptidase or (γ-glutamyl)-peptide: amino acid γ-glutamyltransferase (EC 2.3.2.2); EDTA, ethylenediamine-tetra-acetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoate); K_m, Michaelis constant; V, maximum velocity.

photometrically at 25°C and 344 nm using 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene (Habig *et al.*, 1974; Nimmo *et al.*, 1979). The buffer was sodium phosphate (100 mM phosphate; pH 6.5) and the reaction volume 1.25 ml.

GGT activity was routinely assayed spectrophotometrically at 410 nm with L-γ-glutamyl-*p*-nitroanilide as substrate (Orlowski & Meister, 1963). A stopped assay was used with tissue homogenates and a continuous one with subcellular fractions, which were less turbid. In the former, 100 µl of homogenate was incubated at 38°C with 1 ml of reaction mixture (100 mM Tris adjusted to pH 8.4 at room temperature with HCl; 20 mM glycylglycine; 13 mM $MgCl_2$; 2 mM γ-glutamyl-*p*-nitroanilide), and the reaction terminated after 60 min by adding 1 ml of 10% (w/v) perchloric acid. The supernatant was neutralized with an equal volume of 0.5 M $NaHCO_3$ and its absorbance measured once CO_2 evolution had ceased. Under these conditions absorbance was directly proportional to length of incubation and volume of homogenate. In the continuous assay, 50–100 µl of enzyme was incubated at 25°C with 2.3 ml of reaction mixture (composition as above unless otherwise stated). The rate of reaction was directly proportional to the volume of enzyme. The molar absorption coefficient of the product (*p*-nitroaniline) was the same as that in the continuous assay, and was taken as 8800 per cm (Connell & Adamson, 1970).

GGT activity with GSH as substrate was measured by monitoring the disappearance of GSH using alloxan as colour reagent (Patterson & Lazarow, 1955; Young *et al.*, 1975). Enzyme preparation (1 ml) was incubated at 37°C with 10 ml of reaction mixture (100 mM Tris adjusted to pH 8.4 at room temperature with HCl; 20 mM glycylglycine; 13 mM $MgCl_2$; 0.2 mM GSH). The reaction was terminated by adding 0.5 ml of the incubation mixture to 1 ml of metaphosphoric acid containing ethylenediamine-tetraacetic acid (EDTA) (200 µl of 25% (w/v) metaphosphoric acid; 700 µl of 14.3 mM EDTA (disodium salt); 100 µl of water) and centrifuging. The supernatant (1 ml) was adjusted to pH 7.5 with 0.5 ml of approx. molar NaOH and reacted with 1 ml of 0.1 M alloxan. Its absorbance at 305 nm was recorded 10 min later.

GSH in tissue homogenates was determined using 5,5′-dithiobis(2-nitrobenzoate) (DTNB) as colour reagent (Ellman, 1959). Homogenate (0.5 ml) was added to perchloric acid (12% (w/v); 0.5 ml) and centrifuged. Supernatant (40 µl) was added to 2.5 ml of water and 0.5 ml of 1 mM DTNB in sodium phosphate buffer (0.5 M phosphate; pH 8.0), and its absorbance at 412 nm measured.

Protein was determined by the method of Bradford (1976) with minor modifications, using bovine albumin as standard.

RESULTS

Distribution of glutathione S-transferase and GGT activities

The activities were determined in gills, kidney, intestinal caeca, liver and blood, the first three because they interact with the external environment. Table 1 shows that the tissues had both glutathione *S*-transferase activity and its substrate GSH, whereas GGT activity predominated in kidney and intestinal caeca.

Since the kidney is thought to play an important part in mercapturic acid synthesis in mammals (Hughey *et al.*, 1978), subsequent experiments on GGT were carried out using renal preparations.

Subcellular distribution of GGT in kidney

The specific activities of GGT (nmol/min/mg at 25°C) in the subcellular fractions were: microsomes, 41; light mitochondria, 58; heavy mitochondria, 13; and nuclei (which contained cell debris as well), 11. No activity was detected in the cytosol.

To find out if there were qualitative differences between the GGT activities in these fractions, their thermal stabilities were determined, as this is a sensi-

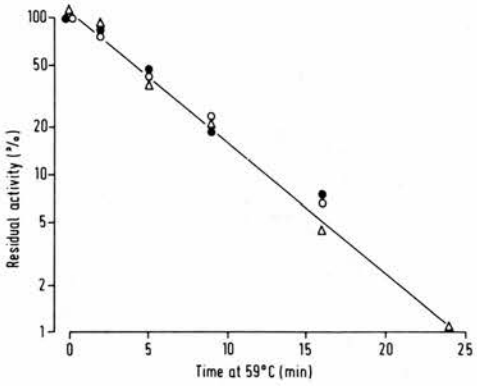

Fig. 1. Thermal stability of GGT in subcellular fractions of kidney. Subcellular fractions were prepared in Tris buffer, heated at 59°C for the times indicated, cooled and assayed at 25°C with γ-glutamyl-*p*-nitroanilide as substrate (see Materials and Methods). △ microsomes; ○ light mitochondria; ● heavy mitochondria.

Table 1. Distribution of glutathione *S*-transferase, GGT and GSH

Tissue	Specific activity		Concentration of GSH (mmol/kg)
	Glutathione *S*-transferase	GGT	
Gills	260 ± 60	0.1 ± 0.1	0.5
Kidney	340 ± 160	8.5 ± 2.0	2.0
Caeca	220 ± 80	2.4 ± 0.5	1.7
Liver	510 ± 400	<0.1	1.8
Blood	4 ± 2	<0.1	ND

Specific activities are in nmol/min/mg protein (mean ± SD of 4 fish). Cytosolic glutathione *S*-transferase was measured at 25°C with 1-chloro-2,4-dinitrobenzene as substrate, homogenate GGT at 38°C with γ-glutamyl-*p*-nitroanilide, and GSH using DTNB (see Materials and Methods).

ND, not determined.

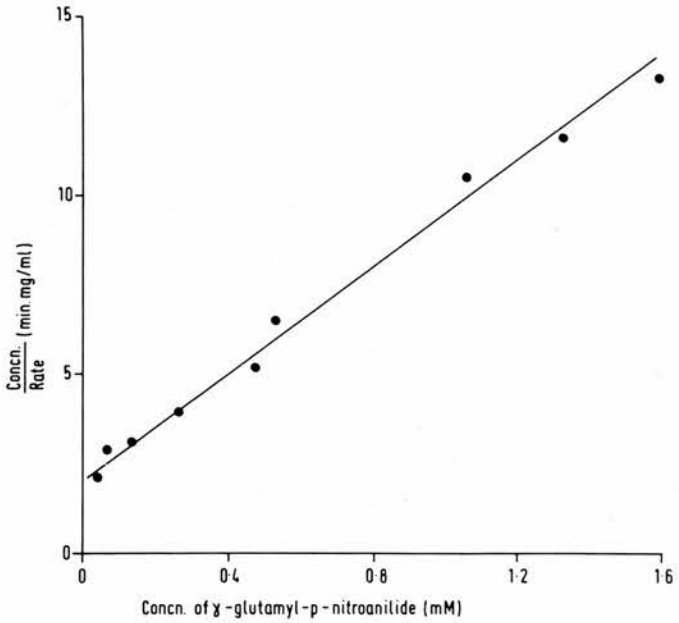

Fig. 2. Dependence of renal GGT activity on concentration of γ-glutamyl-*p*-nitroanilide. Particulate GGT prepared in phosphate buffer (see Results) was assayed at 25°C (see Materials and Methods). The Michaelis constant (K_m) and maximum velocity (V), calculated assuming constant relative error in initial rates (Cornish-Bowden, 1979), were: $K_m = 0.28$ mM; $V = 130$ nmol/min/mg.

tive index of differences between proteins (Clarke, 1979). At 59°C the activity of GGT in each of the fractions tested (microsomes, light mitochondria and heavy mitochondria) declined exponentially with a half-life of about 4 min (Fig. 1). There is no evidence here for qualitative differences between the activities of these fractions, or for the presence of more than one activity in any of them. In subsequent experiments, therefore, the enzyme was studied in a combined particulate fraction prepared by homogenizing kidney in phosphate medium (see Materials and Methods), centrifuging it at 10,000 *g* for 20 min, re-centrifuging the supernatant at 100,000 *g* for 60 min, and retaining the pellet.

Kinetic properties of kidney GGT

GGT activity increased by 30% as the pH was raised in steps of 0.2 from 8.1 to 8.9, showing that its

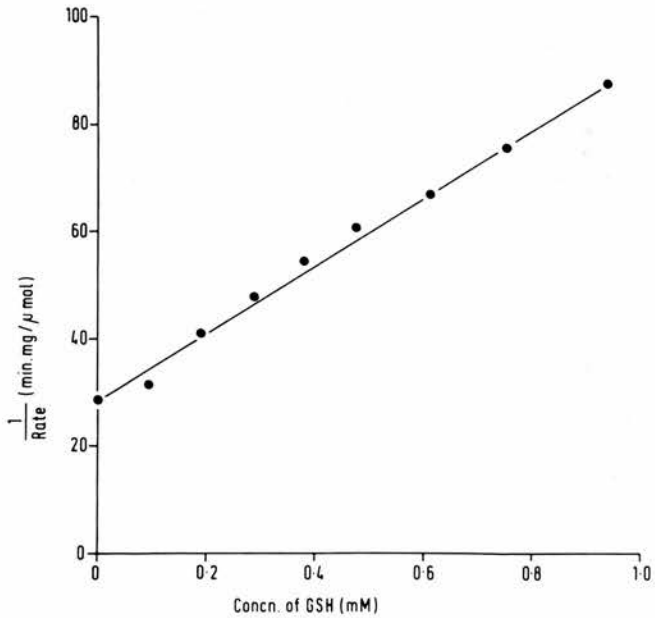

Fig. 3. Effect of GSH on renal GGT activity with γ-glutamyl-*p*-nitroanilide. Particulate GGT was prepared in phosphate buffer (see Results), and its activity at 25°C with 0.8 mM γ-glutamyl-*p*-nitroanilide determined at different concentrations of GSH (see Materials and Methods). The straight line was fitted by unweighted least-squares.

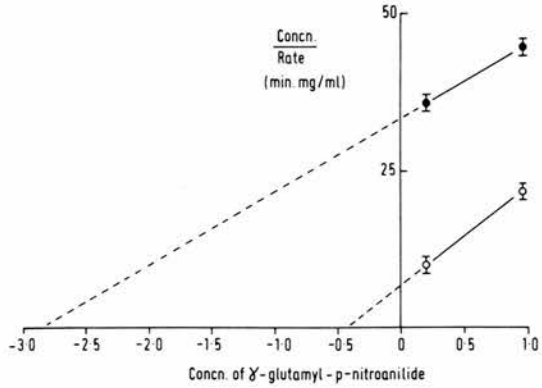

Fig. 4. Inhibition by GSH of renal GGT activity with γ-glutamyl-*p*-nitroanilide. Particulate GGT prepared in phosphate buffer (see Results) was assayed at 25°C in the presence (●) or absence (○) of 0.56 mM GSH (see Materials and Methods). Each point is the mean ± SD of 3 determinations. GSH absent: K_m = 0.37 mM; V = 57 nmol/min/mg. GSH present: K_m = 2.7 mM; V = 82 nmol/min/mg.

pH optimum must be at least 8.9. For convenience the enzyme was routinely assayed at pH 8.4.

The activity also increased when glycylglycine was included in the assay medium to act as γ-glutamyl acceptor (Orlowski & Meister, 1963). The optimal concentration of glycylglycine was about 20 mM, and caused an increase in activity of 120%. The presence of 13 mM $MgCl_2$ raised the activity by a further 10%.

The dependence of activity on the concentration of the substrate γ-glutamyl-*p*-nitroanilide is shown as a Hanes plot in Fig. 2. The plot is close to linear with a half-saturation concentration of about 0.3 mM. GSH inhibited the breakdown of γ-glutamyl-*p*-nitroanilide (0.8 mM), 0.45 mM GSH producing 50% inhibition (Fig. 3).

Since GSH is presumably an alternative substrate for GGT, it should inhibit γ-glutamyl-*p*-nitroanilide breakdown competitively. Figure 4 shows that 0.56 mM GSH raised the half-saturation concentration for γ-glutamyl-*p*-nitroanilide from 0.37 to 2.7 mM, and that it also increased the apparent maximum velocity (i.e. the reciprocal of the slope of the Hanes plot) by about 44%. (Note that to increase the precision of this experiment, replicate measurements were made at only two concentrations of substrate, one low and the other high (Atkins & Nimmo, 1981).)

These experiments suggest that the particulate GGT activity of kidney can degrade GSH as well as γ-glutamyl-*p*-nitroanilide, but do not prove it. The capacity of the particulate fraction to degrade GSH was therefore measured directly, by using alloxan to assay GSH. At pH 8.4 and 25°C the initial rate of disappearance of GSH (0.2 mM) was 60 nmol/min/mg protein, which was some 20 times faster than the rate in the absence of enzyme.

DISCUSSION

Our data (Table 1) confirm that substantial glutathione *S*-transferase activity is present in the gills, kidney, intestinal caeca and in particular the liver of rainbow trout, and that all four tissues have GSH in millimolar concentration as well (cf. Nimmo *et al.*, 1979). Since the half-saturation concentration of GSH for the hepatic glutathione *S*-transferases is about 0.4 mM (Nimmo *et al.*, 1979), these tissues should all be able to catalyse the first step in the synthesis of mercapturic acids. In contrast the distribution of the second enzyme in the putative pathway, namely GGT, is much more restricted, only kidney and intestinal caeca having appreciable activity (Table 1). For comparison, the specific activities of GGT in porcine kidney and intestine from carp are 96 and 2.1 nmol/min/mg protein respectively (Orlowski & Meister, 1965; Sobiech, 1981). Since in mammals the kidney rather than the intestine is thought to play an important part in the synthesis of mercapturic acids (Hughey *et al.*, 1978), further experiments were carried out on the GGT activity of kidney but not intestinal caeca.

The renal GGT activity was found to be particulate, appearing in both heavy and light mitochondria and in microsomes. Its subcellular distribution in the rat is similar, and is thought to reflect the presence of the enzyme on the outside of the cells of the proximal straight tubules (e.g. Hughey *et al.*, 1978).

Thermal stability studies (Fig. 1) suggested that each of the subcellular fractions tested has only one sort of enzyme and that there are no differences between fractions. The half-life of the enzyme at 59°C was 4 min, so it is rather more thermolabile than the GGT from porcine kidney, whose half-life at 58°C is 25–30 min (Orlowski & Meister, 1965). The enzymes from the two species are both stimulated by the presence of Mg^{2+} and glycylglycine, and have similar pH-dependencies and half-saturation concentrations for γ-glutamyl-*p*-nitroanilide (see Orlowski & Meister, 1965). Thus they seem to have comparable kinetic properties.

This conclusion is important, because it follows that the enzymes may have similar substrate specificities. It is known that mammalian GGT has a broad specificity (e.g. Orlowski & Meister, 1965; Tate & Meister, 1974), and could therefore degrade a wide range of glutathione *S*-conjugates. We have not demonstrated that trout GGT also has such a wide specificity, but we have indirect evidence that it can act on GSH as well as γ-glutamyl-*p*-nitroanilide. First, the particulate preparation from kidney catalysed the disappearance of both compounds. Secondly, GSH inhibited the breakdown of γ-glutamyl-*p*-nitroanilide (Figs 3 and 4). Although inhibition was not competitive, which would have implied the two peptides were competing as γ-glutamyl donors for the reaction, the kinetics that were observed (increased apparent half-saturation concentration and maximum velocity) could be caused by the interaction of GSH with both the γ-glutamyl donor and acceptor sites (see Tate & Meister, 1981). We therefore presume that the renal GGT has a specificity consistent with a role in the synthesis of mercapturic acids.

The current scheme for the synthesis of mercapturic acids in mammals envisages conjugation of the foreign compounds with GSH in the liver and the conversion of the conjugates to the corresponding mercapturic acids in the kidney (Hughey *et al.*, 1978; Meister, 1981). Our results, together with those of Parker *et al.* (1980, 1981), indicate that a similar pathway is present in trout: the liver, as well as other tissues which interact with the external environment

(gills, intestinal caeca and kidney) can form glutathione *S*-conjugates, and these can be metabolized further in the kidney.

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Biochem. J. (1983) **211**, 523–526
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The purification of the hepatic glutathione *S*-transferases of rainbow trout by glutathione affinity chromatography alters their isoelectric behaviour

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1. The basic glutathione *S*-transferases from rainbow-trout liver were more stable than the acidic ones. 2. The apparent pI values of these enzymes were lowered when they were eluted from a glutathione affinity column by reduced glutathione at pH 8.85. 3. The pI effect was not a function of the high pH alone, was diminished under conditions less favourable to glutathione oxidation, and did not occur when *S*-hexylglutathione affinity chromatography was used instead.

The glutathione *S*-transferases (EC 2.5.1.18) are a group of multifunctional detoxification enzymes with a wide distribution in the animal kingdom (Jakoby, 1978; Baars & Breimer, 1980). The enzymes in rat liver cytosol have been studied in much greater detail than those from other sources, and considerable structural information is available about them. They are dimeric proteins, each comprising two of at least four subunits, termed Ya (M_r 22000), Yb (M_r 23500), Yb′ (M_r 23500) and Yc (M_r 25000) (Bass *et al.*, 1977; Mannervik & Jensson, 1982). These dimers can be resolved from one another by ion-exchange chromatography (Hayes *et al.*, 1979) and chromatofocusing (Jensson *et al.*, 1982); at least seven separate enzymes have been identified by the use of these techniques.

A powerful and popular method for the partial purification of the glutathione *S*-transferases is affinity chromatography, with the use of columns developed with either reduced glutathione (GSH) (Simons & Vander Jagt, 1977) or *S*-hexylglutathione (Mannervik & Guthenberg, 1981). Transferases from guinea-pig liver (Irwin *et al.*, 1980), human liver (Awasthi *et al.*, 1980) and bovine ocular lens (Saneto *et al.*, 1980) have been purified with the former, and from rat liver with the latter.

We have used both columns in a study of the hepatic glutathione *S*-transferases from rainbow trout (*Salmo gairdnerii*), and now demonstrate that the elution of the enzymes from the glutathione column alters their behaviour on subsequent cation-exchange chromatography. By contrast, the *S*-hexylglutathione column does not change the properties of the enzymes so far as cation-exchange chromatography is concerned.

Abbreviation used: GSH, reduced glutathione.

Experimental

Materials

1-Chloro-2,4-dinitrobenzene and 2-mercaptoethanol were purchased from BDH Chemicals (Poole, Dorset, U.K.), and *N*-acetyl-L-cysteine was from Boehringer Corp. (Lewes, East Sussex, U.K.). CM-Sephadex C-50 was from Pharmacia (Hounslow, Middx., U.K.). DL-Dithiothreitol, GSH and epoxy-activated Sepharose 6B were all supplied by Sigma Chemical Co. (Poole, Dorset, U.K.).

Fish

Rainbow trout (100–300g) were collected from a local trout farm (Penicuik Trout Farm, Penicuik, Midlothian, Scotland, U.K.).

Preparation of cytosol

Fish were killed by cervical dislocation, and their livers were perfused *in situ* with ice-cold 154mM-NaCl. These were then removed, chopped and homogenized in 4vol. of ice-cold 22mM-potassium phosphate buffer, pH 7.4, in a motor-driven glass/Teflon homogenizer. Cytosol was prepared by centrifuging the homogenate [9000 ***g*** ($r_{av.}$ 7.0cm) for 25min at 4°C followed by 100000 ***g*** ($r_{av.}$ 5.95cm) for 80min at 4°C].

Analytical methods

Glutathione *S*-transferases activity was assayed spectrophotometrically at 25°C at 340nm, with 1mM-GSH and 1mM-1-chloro-2,4-dinitrobenzene as substrates (Habig *et al.*, 1974; Nimmo *et al.*, 1979). The buffer was 100mM-potassium phosphate, pH 6.5, and the reaction volume was 3ml.

CM-Sephadex chromatography

Cytosol or partially purified enzyme preparations were dialysed (for 20h at 4°C) against two changes of 20vol. of CM buffer (10mM-sodium phosphate buffer, pH7.5, containing 1mM-*N*-acetylcysteine). When partially purified enzyme was being applied after affinity chromatography, the CM buffer also contained 10mM-2-mercaptoethanol. The non-diffusible material (15ml) was applied to a column (2.2cm × 15cm) of CM-Sephadex C-50 equilibrated and eluted with CM buffer (38.4ml/h). After 35 fractions (2.5ml) had been collected, a linear gradient of NaCl was applied as previously described (Strange *et al.*, 1977).

Glutathione affinity chromatography

Cytosol (about 70ml) was applied to a glutathione–Sepharose 6B affinity column (1cm × 10cm) prepared by the method of Simons & Vander Jagt (1977), equilibrated with 22mM-phosphate (16mM-KH_2PO_4 + 28mM-Na_2HPO_4) buffer, pH7.0. The column was developed with 50ml of 100mM-Tris, pH9.6 (24ml/h), followed by 200ml of Tris/GSH (100mM-Tris containing 10mM-GSH, final pH8.85). Fractions (5ml) were collected and pooled. The pH values of all solutions were measured at 25°C.

Glutathione affinity chromatography with the use of deoxygenated buffers

The procedure differed from the above as follows. The phosphate buffer and the water used to prepare the Tris solutions were boiled, and then DL-dithiothreitol was added to 10mM. The Tris solution was titrated with HCl to pH 8.6, and had GSH added to 10mM immediately before use, lowering the pH to 8.4. All the solutions were gassed with N_2 and stored in air-tight vessels.

S-Hexylglutathione affinity chromatography

Cytosol (about 70ml) was applied to an *S*-hexylglutathione–Sepharose 6B affinity column (1cm × 30cm) and eluted (38.4ml/h) with *S*-hexylglutathione at pH7.8 as described by Mannervik & Guthenberg (1981). Fractions (5ml) were collected, pooled and treated in the same way as those from the glutathione affinity column. No special precautions were taken to deoxygenate the buffers used.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This was performed as described previously (Laemmli, 1970; Nimmo *et al.*, 1981), with minor modifications.

Results

CM-Sephadex ion-exchange chromatography

The elution profile for cytosol that had been dialysed overnight is shown in Fig. 1(*a*). It demonstrates that the activity of the acidic transferases in fresh trout liver cytosol is greater than that of the basic transferases. The broad peak of acidic transferases (fractions 7–45) amounted to 77% of the total activity, the basic transferases being eluted in fractions 64–80. When identical cytosol was dialysed for 80h and then applied to the column

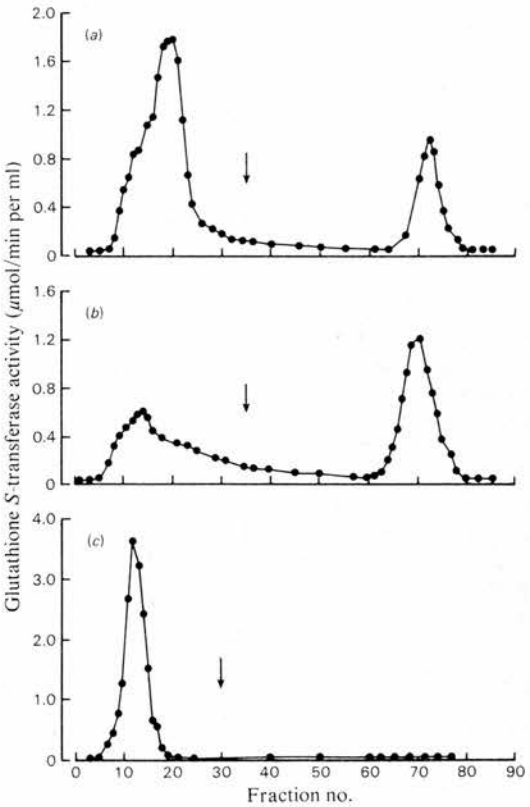

Fig. 1. *Elution profile from CM-Sephadex C-50 of* (*a*) *trout liver cytosol,* (*b*) *trout liver cytosol after extended dialysis against CM buffer, and* (*c*) *material partially purified by glutathione affinity chromatography and dialysed overnight against CM buffer*

Cytosol or preparation derived therefrom was dialysed against CM buffer (see the text). Non-diffusible material (15ml) was then applied and eluted with CM buffer (38.4ml/h). After 35 fractions (2.5ml) had been collected, a linear salt gradient (0–500mM-NaCl) was applied (arrow). Glutathione *S*-transferase activity (μmol of substrate conjugated/min per ml of fraction) was assayed with 1-chloro-2,4-dinitrobenzene as substrate.

(Fig. 1*b*), the acidic transferases accounted for 53% of the total activity. The change in the relative proportions of acidic and basic transferases is due mainly to a decrease in the activity of the acidic transferases and partly to a slight increase in that of the basic transferases. Although the reason for this increase has not been determined, there was no such increase in A_{280} for the basic transferase peak (not shown), implying that the change was not due to acidic transferases being eluted as basic ones. In contrast, when cytosol that had first been partially purified by glutathione affinity chromatography was eluted from the CM-Sephadex column (Fig. 1*c*), all the activity appeared in the acidic transferase fractions (6–21). Extended dialysis (48h) of this material produced a similar profile (not shown), but with a slight broadening of the acidic transferase peak and a very small basic transferase peak.

To determine whether the pattern of Fig. 1(*c*) was linked to the pH of elution (8.85) or to the presence of GSH at that pH, conditions were created to simulate elution. When cytosol (50ml; dialysed to remove endogenous GSH) was sequentially re-dialysed against Tris/GSH (100mM-Tris containing 10mM-GSH; 25vol.; 18h) followed by CM buffer (25vol.; two changes; 24h) and then eluted from the ion-exchange column, the profile (Fig. 2*a*) had both acidic and basic transferases, the latter comprising only 16% of the total activity. The profile is intermediate between those of Figs. 1(*b*) and 1(*c*), with the acidic transferases showing less retardation than those in Fig. 1(*b*). However, when the Tris/GSH solution was replaced with Tris (100mM; pH9.6), the basic transferases comprised 54% of the total activity (Fig. 2*b*). This profile closely resembles that for stored cytosol (Fig. 1*b*) and suggests that the pattern of Fig. 1(*c*) is due to the presence of GSH at a pH of 8.85 and not to pH alone.

To find out if the effect of GSH at an alkaline pH is connected with the oxidation of GSH to its disulphide under the conditions employed with the elution from the glutathione affinity column, the elution was repeated with deoxygenated buffers at a slightly lower pH and with the presence of a thiol reducing agent (DL-dithiothreitol). The profile (not shown) was very similar to that in Fig. 1(*a*). The acidic transferases appeared as a broad peak (fractions 7–50), and not as a sharp one as occurred when untreated buffers were used (Fig. 1*c*). The basic transferases were eluted as a sharp peak (fractions 58–75) similar in magnitude to that in Fig. 1(*b*). The acidic transferase peak constituted over 80% of the total activity. When cytosol partially purified by using *S*-hexylglutathione affinity chromatography was applied to the ion-exchange column, a sharp peak of basic transferase activity (accounting for 72% of the total activity) and a smaller peak of acidic transferase activity (showing some degree of retardation) were eluted (not shown). The profile showed a strong similarity to that of Fig. 1(*b*).

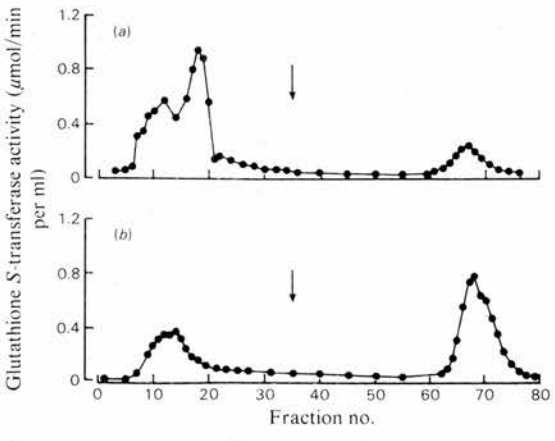

Fig. 2. *Elution profile from CM-Sephadex of trout liver cytosol after dialysis against* (*a*) *Tris/GSH or* (*b*) *Tris*
Cytosol (after initial dialysis against CM buffer) was dialysed first against Tris/GSH or Tris and then overnight against CM buffer before application. Otherwise treatment was identical with that indicated in Fig. 1.

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis

Electrophoresis on sodium dodecyl sulphate/polyacrylamide gels of samples before and after glutathione affinity chromatography showed that the most acidic transferases had components that migrated at essentially the same rates as the Ya and Yb subunits of rat liver glutathione *S*-transferases, whereas the remaining transferases had only the component migrating at the same rate as the Ya subunit. The subunit composition of the transferases was not changed by gluthatione affinity chromatography or *S*-hexylglutathione affinity chromatography.

Discussion

When the glutathione *S*-transferases from trout liver are stored before ion-exchange chromatography, the relative proportion of the acidic transferases decreases. If this is true for other species as well, it implies that the relative activities of their transferases will depend on the time taken to separate them. In contrast, when glutathione *S*-transferases are purified by glutathione affinity chromatography under standard conditions, their isoelectric points apparently become more acidic (see Fig. 1*c*). A comparison of Figs. 1(*b*) and 1(*c*)

reveals that the activity eluted entirely as acidic transferases (Fig. 1*c*) is very close to the total acidic and basic transferase activity eluted after dialysis (Fig. 1*b*). In contrast, transferases partially purified by *S*-hexylglutathione affinity chromatography did not behave anomalously on subsequent ion-exchange chromatography.

A possible explanation for the apparent decrease in isoelectric points of the transferases purified by glutathione affinity chromatography is that the enzymes were deamidated under the alkaline conditions. This was discounted because the fall in pI values was observed in proteins dialysed against Tris/GSH (pH8.85), but not in proteins dialysed against Tris alone (pH9.6). A second possible explanation is that the glutathione anion forms a mixed disulphide with the transferases. Either GSH could be oxidized to its disulphide at the alkaline pH employed and then react with thiol groups in the proteins, or GSH could give the mixed disulphides directly by reacting with cystine residues in the proteins. In either event the protein would gain one extra negative charge with each molecule of GSH incorporated. The experiment with deoxygenated buffers suggested that oxygen is a factor in the reaction, and that oxidized glutathione is the reactive intermediate. Co-purification of the enzyme glutathione thioltransferase (Mannervik & Eriksson, 1973) by glutathione affinity chromatography could further promote the reaction. On the other hand *S*-hexylglutathione would not react in this way, as its thiol group is blocked.

Although we have studied only the trout glutathione *S*-transferases, the observed change in the chromatographic properties of these enzymes may have a much wider significance. In a number of studies of the glutathione *S*-transferases, glutathione affinity chromatography has been used before pI determinations (Awasthi *et al.*, 1980; Irwin *et al.*, 1980; Nishiya *et al.*, 1981) and ion-exchange chromatography (Saneto *et al.*, 1980; Simons & Vander Jagt, 1980). If this effect occurs to the same extent with the glutathione *S*-transferases from other species, then a number of pI values and ion-exchange profiles may have to be re-evaluated. Likewise, the properties of commercially prepared glutathione *S*-transferases (from bovine, horse, pig, rabbit and rat livers; Sigma Chemical Co., 1983) may differ from those of the enzymes *in vivo*.

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THE SUBSTRATE SPECIFICITIES AND SUBUNIT COMPOSITIONS OF THE HEPATIC GLUTATHIONE *S*-TRANSFERASES OF RAINBOW TROUT (*SALMO GAIRDNERI*)

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(*Received* 13 *October* 1983)

Abstract—1. Chromatofocusing separated the glutathione *S*-transferases of trout liver cytosol into species termed cationic (eluted from pH 8 5) and anionic (eluted by 1.0 M NaCl at pH 5).

2. The cationic enzymes were separated from cytosol by *S*-hexylglutathione affinity chromatography, ultrafiltration and chromatofocusing (pH 9–7) into 4 major (C1, C2, C4 and C5) and 3 minor fractions.

3. The anionic material was not purified in this way because only 50% of the activity bound to the *S*-hexylglutathione column.

4. The major cationic enzymes had similar half-saturation concentrations for GSH (0.2 mM) and 1-chloro-2,4-dinitrobenzene (0.4 mM); those of the anionic material were higher (0.7 mM, 1.9 mM respectively).

5. The substrate specificities of the cationic enzymes C1 and C2 were similar (e.g., conjugation of bromosulphophthalein) as were those of C4 and C5 (e.g., conjugation of 1,2-epoxy-3-(*p*-nitrophenoxy) propane). The anionic material had a different specificity (e.g., rapid conjugation of *p*-nitrobenzyl chloride).

6. SDS-polyacrylamide gel electrophoresis showed C1 and C2 to be homodimers of subunit M_r 22,400, C4 to be a heterodimer (M_r's 22,400 and 24,500), and C5 predominantly an M_r 22,400 homodimer.

INTRODUCTION

The glutathione *S*-transferases (EC 2.5.1.18) are a group of detoxification enzymes with a widespread distribution in the animal kingdom. The enzymes in rat and human tissues have been partially characterized (Jakoby, 1978; Habig *et al.*, 1974). They are dimeric proteins, and in the rat appear to comprise two of at least 4 subunits, termed Ya (M_r 22,000) Yb (M_r 23,500), Yb′ (M_r 23,500) and Yc (M_r 25,000) (Bass *et al.*, 1977; Mannervik and Jensson, 1982).

In mammals the enzymes are thought to act in at least three different ways, the best-documented being the catalysis of the reaction between a wide range of xenobiotics and reduced glutathione (GSH) to form a conjugate which is further metabolized (Meister, 1981) and then excreted (Boyland and Chasseaud, 1969). Other enzymic functions include the activation of glutathione as a nucleophile in the reaction with *p*-nitrophenyl acetate (Keen and Jakoby, 1978) and in the isomerization of delta^5-3-ketosteroids to delta^4-3-ketosteroids (Benson *et al.*, 1977). The other two functions of the mammalian glutathione *S*-transferases are not catalytic. They bind non-covalently to bile salts such as lithocholate (Hayes *et al.*, 1980) and other hydrophobic ligands such as bromosulphophthalein (Jakoby and Keen, 1977), although the physiological significance of this is a matter of debate (Gregus and Klaassen, 1982). Their third function is to bind covalently to compounds such as the azo-dyes (Litwack *et al.*, 1971) in a so-called 'sacrificial' reaction (Jakoby and Keen, 1977).

Mannervik and Jensson (1982) were able to establish some relationship between the catalytic activities and subunit compositions of the rat liver enzymes. A difference in catalytic activities was used to distinguish between the two forms of the M_r 23,500 (Yb) subunit. Previously, Bhargava *et al.* (1980) and Hayes *et al.* (1981) had shown that the binding of bromosulphophthalein and of lithocholate is a property of the Ya subunit.

Although the glutathione *S*-transferases have been studied extensively in rat and human tissues, little information is available on their roles and distribution in non-mammalian species. It is known that in earthworms conjugation is limited to the substrates 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene (Stenersen *et al.*, 1979). Fish, because of their ecological niche, are subject to xenobiotic loads (Hawkes, 1980). Nevertheless, the northern pike (*Esox lucius*) has detectable activity only towards 1-chloro-2,4-dinitrobenzene, despite its being a rather stationary inhabitant of some of the more polluted European waters (Balk *et al.*, 1980). The rainbow trout (*Salmo gairdneri*) and other salmonids are commercially-important species that are particularly sensitive to environmental pollutants although they possess NADPH-linked microsomal hydroxylases (Buhler, 1966), glutathione *S*-transferase activity with 1-chloro-2,4-dinitrobenzene and some of the related enzymes of mercapturic acid biosynthesis (Bauermeister *et al.*, 1983)

To help establish the role of the glutathione *S*-transferases in rainbow trout we have used chromatofocusing (Sluyterman and Elgersma, 1978) to resolve the hepatic enzymes and have investigated their substrate specificities and subunit compositions.

MATERIALS AND METHODS

Materials

1-Chloro-2,4-dinitrobenzene was purchased from BDH Chemicals, Poole, Dorset, UK. 1,2-Dichloro-4-nitrobenzene and trans-4-phenyl-3-buten-2-one were from Aldrich Chemical Co. Ltd, Gillingham, Dorset, UK. 1,2-Epoxy-3-(*p*-nitrophenoxy)propane, *p*-nitrophenyl acetate, *p*-nitrobenzyl chloride, bromosulphophthalein, ethacrynic acid, reduced glutathione, DL-dithiothreitol, dehydroisoandrosterone, MW-SDS-70L molecular weight markers and epoxy-activated Sepharose 6B were all from Sigma London Chemical Co., Poole, Dorset, UK. Polybuffer exchanger PBE 94 and Polybuffers 94 and 96 were from Pharmacia (Great Britain) Ltd., Hounslow, Middlesex, UK.

Delta^5-androstene-3,17-dione was synthesized from dehydroisoandrosterone using the method of Djerassi *et al.* (1956). *S*-Hexylglutathione was synthesized and then coupled to epoxy-activated Sepharose 6B as described by Mannervik and Guthenberg (1981).

Fish and preparation of cytosol

Rainbow trout (100–300 g) (*Salmo gairdneri*) were from a local trout farm (Penicuik Trout Farm, Penicuik, Midlothian) and liver cytosol was prepared as described previously (Ramage and Nimmo, 1983).

Analytical methods

Glutathione *S*-transferase activity was assayed with delta^5-androstene-3,17-dione using the method of Benson and Talalay (1976), with *p*-nitrophenyl acetate using that of Keen and Jakoby (1978) and with the remaining substrates according to Habig *et al.* (1974).

S-*Hexylglutathione affinity chromatography*

Affinity chromatography using *S*-hexylglutathione-Sepharose 6B was carried out as described previously (Ramage and Nimmo, 1983).

Chromatofocusing

Columns (1 × 60 cm) were packed with PBE 94, equilibrated and used according to the manufacturer's instructions. For chromatofocusing in the range pH 9–7, cytosol was partially purified by *S*-hexylglutathione affinity chromatography, ultrafiltered using an Amicon model 52 ultrafiltration cell equipped with a YM 30 membrane (mol. wt. cut-off 30,000) and then dialysed overnight against a column start buffer (100 vol.; 2 changes) whose composition depended on the pH gradient chosen (see manufacturer's instructions). When cytosol was chromatofocused in the range pH 8–5 the affinity chromatography and ultrafiltration steps were omitted. In all cases the flow rate was 24 ml/hr and the fraction vol 2.5 ml.

Enzyme kinetics

Determination of empirical half-saturation concentrations. Initial velocities (v_1, v_2) were determined in quadruplicate at two concentrations (S_1, S_2) of the substrate under consideration and a fixed concentration of the second substrate. The empirical half-saturation concentration (K_{emp}) was calculated from the Michaelis–Menten equation (see Atkins and Nimmo, 1981):

$$K_{emp} = \frac{v_2 - v_1}{v_1/S_1 - v_2/S_2}$$

and its SE by the jackknife technique (Miller, 1974).

Inhibition of the conjugation of 1-*chloro*-2,4-*dinitrobenzene.* The reaction mixture (final vol. 3.1 ml) contained 1 mM 1-chloro-2,4-dinitrobenzene, 1 mM GSH and either inhibitor (dissolved in 100 μl ethanol) or ethanol (100 μl, control). The initial velocity observed in the presence of the inhibitor was expressed as a percentage of that of the control.

SDS–polyacrylamide gel electrophoresis

Protein was precipitated from pooled fractions by adding an equal vol of 20% (w/v) trichloracetic acid. After mixing, the samples were left on ice for 30 min and then centrifuged (1000 *g*; 10 min). The precipitated protein was redissolved in sample incubation buffer [SDS 1% (w/v); Tris 0.3% (w/v); 2-mercaptoethanol 2% (w/v); adjusted to pH 6.8 with HCl] to give a concentration of about 2 mg/ml. Bromophenol Blue (0.0025% w/v) in glycerol (40% w/v) was added followed by 1 M Tris until the colour of the sample changed from orange to blue.

SDS–polyacrylamide gel electrophoresis was carried out using 15% (w/v) acrylamide gels modified from the method of Laemmli (1970); they were calibrated using MW-SDS-70L mol. wt. markers.

RESULTS

Chromatofocusing

When freshly-prepared trout liver cytosol was chromatofocused in the range pH 8–5, the glutathione *S*-transferases were separated into cationic and anionic forms. The former were eluted by the pH gradient as a broad peak and represented over 97% of the total activity recovered, whilst the latter were eluted as a single peak by 1 M NaCl applied to the column when the pH had fallen to 5.0 (data not shown).

The cationic glutathione *S*-transferases were partially-purified from cytosol from 8 livers by *S*-hexylglutathione affinity chromatography and ultrafiltration followed by chromatofocusing (pH 9–7). This resolved them into at least 7 different forms (Fig. 1), of which peaks C1, C2, C4 and C5 were the major ones. An additional peak, C1a can be seen in Fig. 1, but because it did not appear consistently in elution profiles and because its properties were identical with those of C1 it has been excluded from this work. The anionic glutathione *S*-transferases were not purified in this way because approximately 50% of the total activity with 1-chloro-2,4-dinitrobenzene did not to bind to *S*-hexylglutathione–Sepharose 6B. The anionic material used in the experiments described below was the peak of activity eluted from the chromatofocusing column at pH 5 by 1.0 M NaCl. It had a total activity (8 livers; 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM GSH as substrates) of 0.8 μmol/min.

Kinetic properties and substrate specificities

To investigate the kinetic properties of the cationic and anionic forms, initial velocities were determined at different concentrations of GSH and 1-chloro-2,4-dinitrobenzene. The concentration of GSH was varied from 1–10 mM (with 1 mM 1-chloro-2,4-dinitrobenzene) and the concentration of 1-chloro-2,4-dinitrobenzene from 0.1–1 mM (with 1 mM GSH). In both instances double-reciprocal plots were curvilinear, so that no simple kinetic interpretation was possible. Instead, an empirical

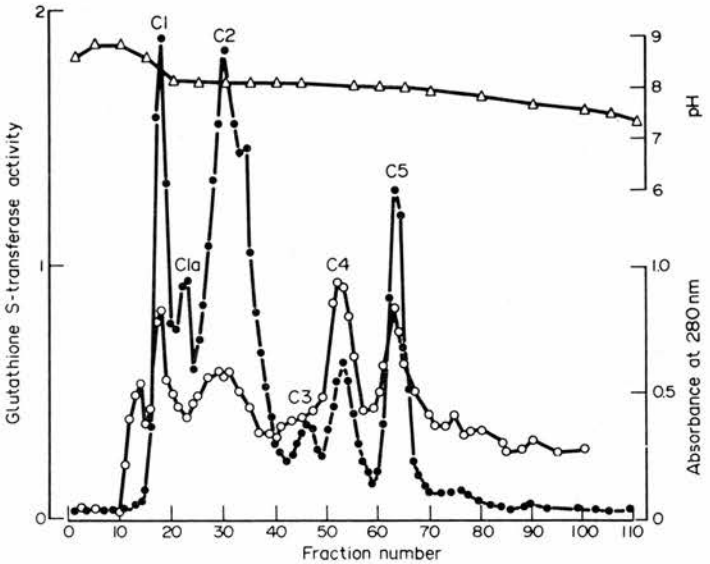

Fig. 1. Elution profile on chromatofocusing of partially-purified trout liver cytosol. Cytosol was partially-purified by *S*-hexylglutathione affinity chromatography and ultrafiltration (see Materials and Methods). The resulting material (10 ml) was applied to and eluted from a column (1 × 60 cm) packed with PBE 94, according to the manufacturer's instructions. Flow rate: 24 ml/hr; fraction vol: 2.5 ml. ● Glutathione *S*-transferase activity (μmol substrate conjugated/min ml of fraction), assayed with 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene; ○, A_{280}, Δ, pH.

half-saturation constant (K_{emp}) was calculated for GSH from initial velocities observed at 1 mM 1-chloro-2,4-dinitrobenzene and either 0.2 or 5.0 mM GSH (see Materials and Methods). The corresponding concentration for 1-chloro-2,4-dinitrobenzene was derived from the initial velocities observed at 1 mM GSH and either 0.2 or 1.0 mM 1-chloro-2,4-dinitrobenzene. Table 1 shows that the 4 cationic forms examined had similar half-saturation concentrations and that these were less than those of the anionic material.

Table 1. Empirical half-saturation concentrations for GSH and 1-chloro-2,4-dinitrobenzene of the major forms of the glutathione *S*-transferases separated by chromatofocusing

Peak	GSH (mM)	K_{emp} 1-Chloro-2,4-dinitrobenzene (mM)
C1	0.2	0.5
C2	0.2	0.4
C4	0.3	0.4
C5	0.2	0.4
A	0.7	1.9

Cytosol was subjected to affinity chromatography, ultrafiltration and chromatofocusing in the pH range 9–7 to give peaks C1–C5. The anionic peak (A) was prepared by chromatofocusing cytosol in the pH range 8–5 and then eluting the column with 1.0 M NaCl. Empirical half-saturation concentrations (K_{emp}) were determined as in Materials and Methods. Their standard errors, calculated by the jack-knife technique (Miller, 1974), were always less than 15% of the values themselves.

The specificities of the main forms of the glutathione *S*-transferases for a number of substrates are in Table 2. The anionic material (A) differs from the others in that it is the only one with appreciable activity towards *p*-nitrophenyl acetate, *p*-nitrobenzyl chloride and delta^5-androstene-3,17-dione. The conjugation of 1,2-epoxy-3-(*p*-nitrophenoxy)propane is associated with cationic enzymes C4 and C5 whilst that of bromosulphophthalein is a characteristic of peaks C1 and C2. The conjugation of trans-4-phenyl-3-buten-2-one and ethacrynic acid appears to be a property of the cationic but not of the anionic forms.

To find out if these differences in specificity were due to differences in the affinities of the enzymes for the substrates, the effect of some of the substrates on the rate of conjugation of 1-chloro-2,4-dinitrobenzene with GSH was tested (Table 3). It can be seen that although the anionic material does not

Table 2. Substrate specificities of the glutathione *S*-transferases

Peak	Substrate ENPP	NPA	BSP	NBC	TBO	Δ^5A	ETHA
C1	0	0.2	0.3	1.3	0.2	0	4.7
C2	0	0	1.0	0.5	0.3	0	6.7
C4	40	0	0	0	0.3	0	9.4
C5	34	0.9	0	0.2	0.3	0	0
A	0	10.7	0	318	0	7.1	0

Cytosol was fractionated as described in the legend to Table 1. The activities of the peaks were determined as in Materials and Methods, and expressed as percentages of those with 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM GSH.

Abbreviations: ENPP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; NPA, *p*-nitrophenyl acetate; BSP, bromosulphophthalein; NBC, *p*-nitrobenzyl chloride; TBO, trans-4-phenyl-3-buten-2-one; Δ^5A, delta^5-androstene-3,17-dione; ETHA, ethacrynic acid.

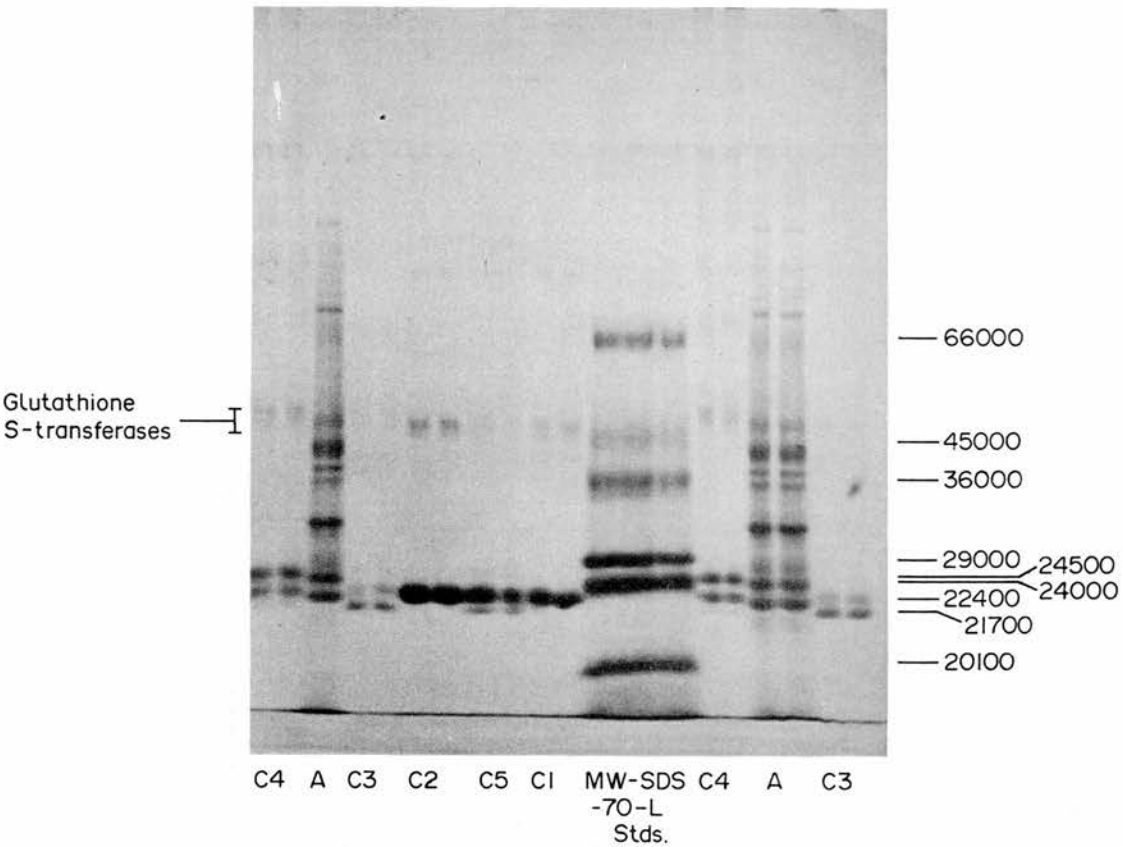

Fig. 2. SDS–polyacrylamide gel electrophoresis of glutathione *S*-transferases from trout liver. Cationic enzymes C1–C5 were the fractions shown in Fig. 1. The anionic enzyme was prepared by chromatofocusing cytosol from pH 8–5 and eluting the column with 1.0 M NaCl. The gel was prepared and run as in Materials and Methods, and calibrated using MW-SDS-70L mol. wt. markers.

conjugate either bromosulphophthalein or ethacrynic acid, both compounds inhibit its ability to conjugate 1-chloro-2,4-dinitrobenzene. Similarly *p*-nitrobenzyl chloride and ethacrynic acid inhibited transferases C4 and C5 respectively although neither transferase conjugated these substrates.

SDS–polyacrylamide gel electrophoresis

The electrophoretogram of peaks C1–C5 and the anionic material (A) is shown in Fig. 2. C1 and C2 both gave one band of M_r 22,400, implying that these proteins are homodimers. C3 appears as two bands of M_r 21,700 and 22,400, the smaller predominating. This indicates that C3 is a homodimer of the M_r 21,700 subunit and that the band of M_r 22,400 is a contaminant from C2. C4 contains two bands of M_r 22,400 and 24,500 in roughly equal proportions and may therefore be a heterodimer of these subunits. C5 has three bands: a predominant one of M_r 22,400 and two less apparent bands of M_r 21,700 and 24,500 respectively. The very faint M_r 24,500 band could be a contaminant from C4, but the faint M_r 21,700 one cannot be explained in this way. Thus C5 could be a mixture of two enzymes, the predominant one being a homodimer of the M_r 22,400, subunit and the other either an M_r 21,700 homodimer or an M_r 22,400, M_r 21,700 heterodimer. The anionic material contained a number of bands and was therefore relatively impure: hence its subunit composition cannot be defined precisely.

Table 3. Inhibition of conjugation of GSH and 1-chloro-2,4-dinitrobenzene by potential substrates

Peak	Potential substrate BSP (0.03 mM)	NBC (0.5 mM)	ETHA (0.01 mM)
C1	66	3	90
C2	71	31	83
C4	52	19	81
C5	52	14	86
A	85	69	59

Cytosol was fractionated as described in the legend to Table 1, and the percentage inhibition of the conjugation of 1 mM 1-chloro-2,4-dinitrobenzene with 1 mM GSH caused by the addition of potential substrate (concentration in parentheses) determined. Abbreviations as for Table 2.

DISCUSSION

We have shown that there are at least seven different hepatic glutathione *S*-transferases in rainbow trout, which we have called either anionic or cationic on the basis of their behaviour on a PBE 96 chromatofocusing column.

The cationic forms comprise about 97% of the total 1-chloro-2,4-dinitrobenzene-conjugating activity, most of which appears in peaks C1, C2, C4 and C5. These four peaks all have a relatively low half-saturation concentration for both 1-chloro-2,4-dinitrobenzene and GSH (Table 1), but have different substrate specificities (Table 2). For example, peaks C1 and C2 alone conjugate bromosulphophthalein with GSH, and peaks C4 and C5 1,2-epoxy-3-(*p*-nitrophenoxy)propane. 1-Chloro-2,4-dinitrobenzene is conjugated far more rapidly by all the cationic enzymes than any of the other substrates tested.

It is known that the glutathione *S*-transferases of trout are dimeric proteins with an M_r of approx. 46,000 (Nimmo *et al.*, 1981), so it follows from the data in Fig. 2 that peaks C1 and C2 are homodimers with a monomeric M_r of 22,400. C3 is also a homodimer but with a monomeric M_r of 21,700, whereas C4 is a heterodimer of the M_r 22,400 and M_r 24,500 subunits. C5 appears to comprise more than one enzyme as it possesses two subunits of M_r 21,700 and M_r 22,400 with the latter predominating, implying that the bulk of the material is again an M_r 22,400 homodimer. If this is so, there must be at least two different sorts of M_r 22,400 subunit, because the catalytic properties of C1 and C2 are different from those of C5.

The anionic material comprises only 3% of the total 1-chloro-2,4-dinitrobenzene-conjugating activity, but its higher half-saturation concentration for both 1-chloro-2,4-dinitrobenzene and GSH means that its relative contribution to the total activity would increase with the concentration of these two substrates. It also behaved anomalously on affinity chromatography, for only some of the activity bound to the matrix of the column, the rest being eluted in the void volume. Thus the peak eluted from the chromatofocusing column at pH 5 by 1.0 M NaCl (peak A) and whose substrate specificity was tested presumably contains at least two different enzymes. Nevertheless, it has a different substrate specificity from its cationic counterparts, for example reacting with *p*-nitrophenyl acetate and delta^5-androstene-3,17-dione. It also conjugated *p*-nitrobenzyl chloride at a greater rate than 1-chloro-2,4-dinitrobenzene, in this respect resembling the glutathione *S*-transferase with a high K_m found in shark liver by Sugiyama *et al.* (1981).

The inhibition studies (Table 3) show that the differences in the specificities of the various enzymes are not necessarily due to their having different affinities for the substrates tested. Both bromosulphophthalein and ethacrynic acid strongly inhibit the ability of the anionic material to conjugate 1-chloro-2,4-dinitrobenzene with GSH, yet neither is itself conjugated. Similarly, cationic peaks C4 and C5 are inhibited by *p*-nitrobenzyl chloride and ethacrynic acid respectively, these compounds being inactive as substrates for conjugation. Thus the trout liver enzymes may also be able to bind a number of compounds without actually modifying them, as do the hepatic glutathione *S*-transferases of rat (Jakoby and Keen, 1977) and shark (Sugiyama *et al.*, 1981).

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