CLINICAL AND BIOCHEMICAL STUDIES ON THE GLUTATHIONE S-TRANSFERASES

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CLINICAL AND BIOCHEMICAL STUDIES ON THE HUMAN GLUTATHIONE S-TRANSFERASES

The glutathione S-transferases (ligandins) are a ubiquitous system of xenobiotic metabolising enzymes. In the rat liver they comprise up to 10% of soluble hepatic protein. Studies in the rat suggested that ligandin was an accurate and sensitive marker of hepatocellular necrosis, and of renal tubular necrosis. The first part of this thesis examines the release of ligandin from liver and kidney in human liver and renal disease in an attempt to determine whether the measurement of ligandin is clinically useful.

Ligandin was purified from human liver cytosol using a combination of anion exchange chromatography and gel filtration. The purified protein had similar physicochemical characteristics to ligandin purified by others. The protein was used to raise a monospecific antibody. Ligandin was iodinated by the Chloramine-T method, which yielded a labelled protein of high specific activity. A sensitive and specific radioimmunoassay for human ligandin was developed which had a low intra- and interassay variation.

The assay was applied to the study of human liver disease. In acute hepatitis ligandin is released from the liver into serum early in the illness. High serum ligandin levels are seen in the first week of acute hepatitis. The rapid return to normal suggests that ligandin may provide an early indication of recovery.

In chronic hepatitis ligandin levels correlated significantly with histological severity of disease, whereas SGOT showed no such cor-

relation. Ligandin may be a better index of severity of disease and for treatment than SGOT.

Ligandin was released from the kidney in severe renal ischaemia and in acute tubular necrosis, but was not a reliable predictor or indicator of acute tubular necrosis.

Part two examines the distribution of of GSH-T activity in organs and in hepatocellular carcinoma. Ligandin was shown to be immunologically similar in all tissues studied. Isoelectric focusing of cytosol separated the three groups of GSH-T activity. Considerable variety in the distribution and activity of GSH-T's was shown in different organs from a single donor, and in the same organs from different donors. Anionic transferase activity was shown to contribute a significant proportion of activity in organs other than the liver, and to be the major source of activity in ovary and lung. In hepatocellular carcinoma cationic GSH-T activity was present in amounts varying from near normal to absent. The anionic and neutral GSH-T's were present in amounts similar to that seen in normal liver. Immunohistochemical studies using a peroxidase-antiperoxidase method showed a rough correlation between tumour differentiation and the amount of ligandin in the tumour.

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PREFACE

The world in which we live is a hostile, inimical place, in which we are daily assaulted and abused by hundreds of noxious stimuli. Many are of natural origin; sun, wind, extremes of temperature, but many are man-made. We are faced with the problem of explaining how it is that man, and other animals, have developed over millenia, through ages when chemical industries were non-existent, protective systems of enzymes, which seem to have man made chemicals as their major substrates. Such is the diversity of drug metabolising systems, that of the vast array of substrates tested, only a relatively small proportion can be said to occur naturally. Should one consider cooked food with its polycyclic aromatic hydrocarbon carcinogens as a "natural product". If cooked food is 'natural' are petrol and diesel fuel less natural, since the fractional distillation of crude oil to yield various grades of fuel is merely a sophisticated type of cooking. Rather than try to separate 'natural' from man-made toxins, all exogenous substances which the organism can metabolise can be considered to be 'natural' substrates. After all evolution could not predict the advent of the plastics industry.

Even before man appeared on the scene to enormously increase the likelihood of being exposed to toxins, many environmental pollutants must have been present. Fire and smoke produce their fair share of carcinogens. Fungi produce aflotoxins. Various foods contain toxic alkaloids, and man, in his perversity, will insist in using the leaves of various plants, which are nutritionally useless, to produce potions

and essences containing toxic substances e.g. tea, coffee, tobacco and marijuana. Even oxygen, the most basic requirement for life, may be toxic.

It is not therefore surprising that a highly efficient enzyme system of very broad specificity exists to deal with these toxins. Selective pressures would have put organisms with these enzymes at an advantage.

Furthermore, an organism's metabolism produces its own poisons - the unknown toxins of uraemia and liver failure for instance. The kidney and the liver have systems for handling endogenously produced toxins and it should not come as too much of a surprise if metabolising systems for endogenous and exogenous toxins overlap.

This thesis deals with one such group of drug metabolising enzymes, the glutathione S-transferases.

The work developed out of time spent in the Liver Research Group at the University of Cape Town. This group is concerned with clinical research in liver diseases and basic scientific research in liver metabolism and pathophysiology. In parallel with the interest of the group, this thesis covers both clinical and biochemical work.

The clinical side of this thesis arose out of a realisation that an adequate test to estimate the extent of hepatic necrosis does not exist. Initial work carried out in the Group suggested that assay of these proteins (also known as ligandin) in serum may be just such a test. This work also suggested that the study of human urinary ligandin might be of use in the diagnosis of renal disease.

Because the subject of this thesis is so broad, encompassing as it does both clinical and biochemical work, it has been necessary to split

the thesis into two parts. Part one describes the purification of ligandin, the development of a radioimmunoassay for ligandin, and the application of the RIA to the study of human disease. The experimental section is preceded by a review of the literature on ligandin, together with a consideration of the role of ligandin as a diagnostic tool.

Part 2, which deals with the biochemical investigations into the distribution and structure of the human glutathione S-transferases (GSH-T), is preceded by a review of the chemistry of the GSH-T's. In addition since the physiological functions of the glutathione S-transferases are in some measure dependant on their anatomical localisation, this review will include a consideration of the evidence for and against the various physiological functions that have been ascribed to the GSH-T's.

The subject of glutathione conjugation has been reviewed many times (1,2,3), and therefore will not be included in this thesis.

PART ONE

LIGANDIN

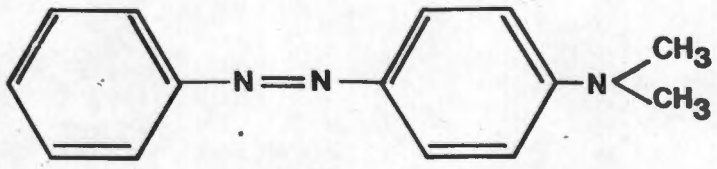
CHAPTER I

HISTORICAL ASPECTS

In 1967 Dr. Ketterer at the Courtauld Institute of Biochemistry working with carcinogen-binding proteins showed that the aminoazodye carcinogen 4-dimethylaminoazobenzene (fig. 1.1) when injected into rats was bound to three hepatic cytosolic proteins (4). They were able to purify the most basic of these proteins, which they found to have a pI of 8.4 (measured by electrophoresis), a sedimentation coefficient of 3.5S, and a molecular size (measured by Sephadex chromatography) of 45,000 daltons (4).

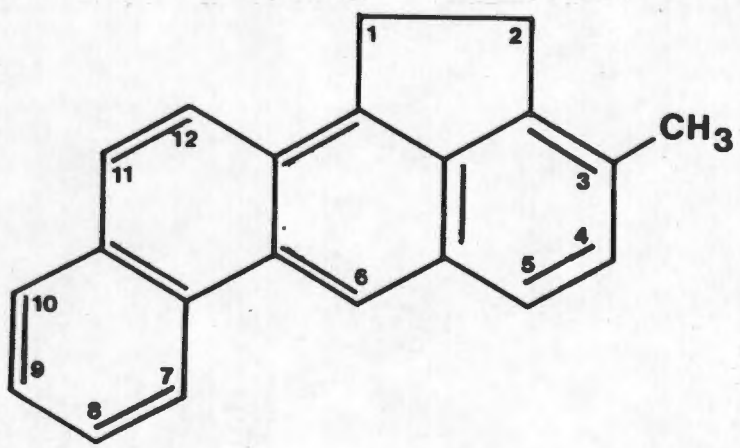
In 1969 at the Fels Institute in Philadelphia Dr Litwak and co-workers, who were studying cortisol binding in the liver cell, found labelled cortisol was taken up by rat liver bound to four cytosol proteins (5), which they called cortisol metabolite binders I, II, III and IV. Binder I was purified to homogeneity, and was found to have a pI of 8.9 (by isoelectric focusing), a sedimentation coefficient of 3.47S, and a molecular weight of 37,000 daltons using amino acid analysis, and 50-60,000 daltons by Sephadex chromatography (5). Binder I was shown to be identical to the aminoazodye binding protein described by Ketterer et al (6), and to the protein binding the polycyclic hydrocarbon 3-methylcholanthrene (3-MC)(fig. 1.2) in rat liver cytosol (7).

Also in 1969 at the Liver Research Centre of the Albert Einstein College of Medicine, Dr. Arias and his colleagues, studying the uptake of bilirubin and organic anions by the liver produced the now classical description of the X, Y, and Z fractions of liver cytosol. When brom-



DIMETHYLAMINOAZOBENZENE

Fig. 1.1. Structural formula of 4-Dimethylaminoazobenzene.



3-METHYL CHOLANTHRENE

Fig. 1.2. Structural formula of 3-Methylcholanthrene.

sulphophthalein (BSP)(fig. 1.3) or labelled bilirubin (fig. 1.4) is added to the cytosol fraction of rat liver and chromatographed on Sephadex G-75, the ligand elutes in three peaks (8). The X peak elutes at the void volume, the Y peak at a molecular size of ±47,000 daltons and Z at a molecular size of ±14,000 daltons.

Y protein was purified to homogeneity and found to have a pI of 8.9, and a molecular weight of 46,000 daltons by Sephadex chromatography (84,214). The physicochemical similarities between Y protein, cortisol metabolite binder I and the aminoazodye binding protein were soon recognised and these three proteins were shown to be immunologically identical. The term ligandin was proposed, on the basis of the wide spectrum of ligands known to bind to this protein, to replace the older terms, which were too restrictive (9).

Identity between GSH-T B and ligandin was suspected when it was found that there was a significant formation of BSP-GSH conjugate when BSP was added as a marker during Y protein purification (10). When GSH was added to cytosol it eluted from G-75 Sephadex at the same elution volume as BSP bound to Y protein. In addition GSH-T activity with 3,4-dichloronitrobenzene (DCNB) and BSP eluted at the same elution volume with superimposable curves (10,11). Thus it was proposed that Y protein and GSH-aryl transferase were identical. Some doubt was cast on this finding when it was shown that rat liver cytosol subjected to isoelectric focusing resulted in clear separation of GSH-T activity towards DCNB and ligandin immunoreactivity (12). In retrospect this finding was due to the fact that DCNB is a poor enzyme substrate for ligandin and thus failed to detect activity due to this protein. The DCNB conjugating activity being measured was due to another GSH-T sep-

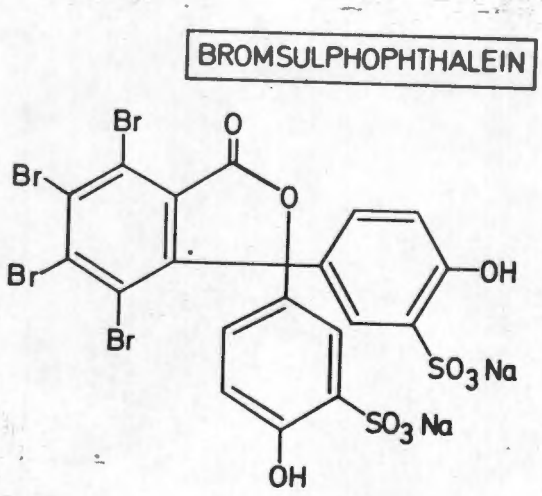


Fig. 1.3. Structural formula of Bromsulphophthalein.

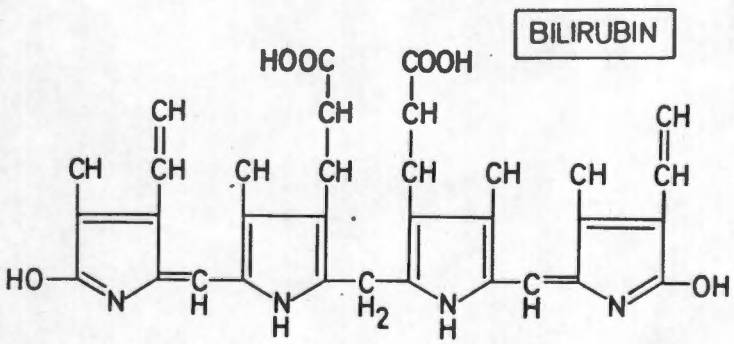


Fig. 1.4. Structural formula of Bilirubin.

arated from ligandin by the isoeletrofocusing procedure. On gel filtration all the GSH-T's eluted at the same volume because they all have approximately the same molecular size (approximately 46,000 daltons). This confusion was resolved when it was shown unequivocally that purified ligandin and GSH-T B were identical with respect to substrate specificity, kinetics, enzyme inhibition kinetics, binding activity, physical properties, and antigenicity (13).

Jakoby (14) suggested the use of ligandin as a generic term, used interchangably with GSH-T. Arias (15) defined ligandin as a basic dimeric cytoplasmic protein, which binds various electrophiles non-covalently and some carcinogens covalently. In addition it has glutathione S-transferase (GSH-T) activity with several substrates. By this definition all cationic GSH-T's from any organ, from any species, are ligandins. However generally the use of the term is more restricted, and in the rat is used interchangeably with GSH-T B, to denote either of the two dimeric forms in which the molecule may exist in the liver (see table 1.1). This is the sense in which the term will be used in this thesis. This terminology conforms to common usage in the literature. However since there is plethora of other terms which have also been used these are summarised in table 1.1.

In contrast to the rat, in man the cationic GSH-T's are immunologically identical (16,17) and have similar enzyme and binding activity. These proteins have also been called ligandin, and it is in this sense that the term will be used in reference to man. Other human GSH-T's will not be refered to as ligandin.

Ligandin:- In the rat this term refers to either of two immunologically similar dimers YaYa or YaYc. These dimers are also known as GSH-T B. In man this term refers to all GSH-T's with isoelectric points above 7.5, and which are immunologically similar.

Y-protein:- 40-50,000 molecular weight peak on gel filtration following incubation of cytosol and BSP or bilirubin, which on PAGE-SDS has three dominant bands, Ya, Yb, and Yc:-

Ya:- 22,000 molecular weight monomer.

Yb:- 23,500 molecular weight monomer.

Yc:- 25,000 molecular weight monomer.

Glutathione S-transferase B:- used synonymously with ligandin.

Glutathione S-transferase A:- GSH-T from rat liver consisting of YbYb subunits. Enzymatically and immunologically similar to GSH-T C.

Glutathione S-transferase C:- GSH-T from rat liver consisting of YbYb subunits. See also GSH-T A.

Glutathione S-transferase AA:- GSH-T from rat liver consisting of YcYc subunits.

Cationic human GSH-T's:- GSH-T α, β, γ, δ, ϵ. Also known as human ligandin

Neutral human GSH-T's:- GSH-T with isoelectric 6-6.5. Also known as GSH-T υ.

Anionic human GSH-T's:- GSH-T with isoelectric point 4.5-5.4. In the erythrocyte known as GSH-T ρ, and in liver as GSH-T ω.

Table 1.1. Definition of terms used in this thesis. This usage conforms to that commonly seen in the literature. Terms refer to species and subunits of rat and human glutathione S-transferases.

CHAPTER II

PHYSIOCHEMICAL CHARACTERISTICS

Rat liver ligandin:-

Discontinuous SDS-PAGE analysis of the pooled rat liver Y protein peak shows approximately equal concentrations of three bands in the 22-24,000 dalton range. These have been named Ya, Yb, and Yc in increasing order of molecular size by Bass et al (18). Purified rat liver GSH-T B and ligandin have two bands, corresponding to Ya and Yc and called YA and YB by Bhargava et al (19,20a). I have adopted the Bass nomenclature, as have most other workers in the field since it permits consideration of Yb which has recently become of relevance.

From studies with circular dichroism it is known that ligandin has a structure, consisting of 40% α-helix, 15% β- pleated structure, and 45% random coil (20). Ligandin purified as originally described consisted of two non-identical monomers, molecular weight 22,000 and 24,000 daltons (18,20a). If however the anion exchange steps of the ligandin purification are performed at a higher pH i.e. 9.1, then only one band, Ya, is seen on SDS-PAGE (18). The resulting dimer has characteristics very similar to YaYc ligandin (table 2.1).

Bass et al (18) showed that phenobarbitone induction increased the concentration of Ya and Yb but not Yc, and suggested that the native ligandin consisted of two dimers YaYa, and YcYc. Bhargava et al (20a) using dimethyl suberimidate as a cross linking reagent, could only show one band in the 47,000 dalton range on SDS-PAGE and suggested that the native protein was a YaYc dimer. Carne et al were able to

Table 2.1.

COMPARISON OF YaYc LIGANDIN WITH YaYa LIGANDIN

	YaYc	YaYa
pI	8.7-9.1	8.7-9.1
Mol. size	±46,000	±46,000
Subunit size	22,000 + 25,000	2x22,000
Specific activity (μmol/min/mg)		
CDNB	11	25
DCNB	0.003	0.027
Ka with bilirubin (M^{-1})	$1x10^6$	$1.1x10^6$
BSP (M^{-1})	$6x10^6$	$4.5x10^6$

separate a YaYa dimer from a YaYc dimer on CM-cellulose (21). Subsequently Bhargava et al (19) showed that fresh preparations of ligandin contained equal amounts of Ya and Yc, but on storage the Ya subunit increased without loss of catalytic activity. Isoelectric focusing of stored ligandin gave two bands. A more basic band contained Ya only but the other had equal amounts of Ya and Yc. When a stored sample of ligandin was applied to thiol Sepharose no YaYc appeared in the wash. Only Ya subunits were seen on PAGE-SDS of the wash (Yc has most of the cysteine residues in ligandin). When a fresh preparation of ligandin was applied to the same column no ligandin (YaYa or YaYc) appeared in the wash. This evidence suggested that native ligandin existed as a YaYc heterodimer.

Hayes et al (22) using anion exchange chromatography of phenobarbitone induced rat liver cytosol found two adjacent protein peaks with GSH-T activity with aryl substrates. SDS-PAGE analysis of these peaks showed the first to consist mainly of Ya (indicating a YaYa dimer). The early fractions of the second peak consisted of YaYc with a trace of Yb, but the later fractions consisted predominantly of Yb. These workers also showed that GSH-T B purified according to the method of Jakoby eluted from CM-Sephadex at the same ionic strength as their YaYc dimer, and purified ligandin prepared according to Bass et al eluted at the same ionic strength as their YaYa dimer. These two dimers had similar enzyme activity (22). The evidence therefore suggests that in the native state both species, YaYa and YaYc exist, but whether they have a precursor-product relationship, or are the products of different genes is not clear.

Of the GSH-T's, the structure/function relationships of ligandin have been best studied. The two subunits are not disulphide linked (20a). The two C-terminal amino acids are argenine and phenylalanine. N-terminal analysis could not be done. Amino acid analysis of the two subunits was similar, except that Yc has most of the cysteine molecules of native ligandin (19). YaYc ligandin binds bilirubin at a stoichiometric ratio of 1:1 at a high affinity site (Ka- 5x107 M-1)(20), but there is also a low affinity site. YaYa ligandin binds bilirubin with a ratio of 2:1 (bilirubin:ligandin)(19).

Covalent binding occurs largely through the cysteine residues (23) which are mainly on Yc (19). Labelled aminoazodye and 3-methyl cholanthrene have been shown to be covalently bound. This binding is to Yc and occurs in vivo and in cell culture but not with homogenate (26) suggesting that microsomal activation is required prior to binding.

Ligandin can be chemically modified so that bilirubin binding at the high affinity site is lost, but GSH-T activity is retained (20a). Concentrations of bilirubin sufficient to saturate the high affinity site do not inhibit enzyme activity, but at higher concentrations there is binding to a second low affinity site and GSH-T activity is inhibited. This suggests that the GSH-T substrate binding site is the same site as the low affinity binding site. This site is present on both Ya and Yc, accounting for the presence of similar GSH-T activity in the YaYa and YaYc dimers (19).

Human Glutathione S-Transferases:-

Originally five immunologically identical cationic GSH-T's (ligandins) with pI's ranging from 7.8 to 8.5 were described in human liver tissue. All five have similar amino acid composition and are thought to represent post-translational modification, eg. deamidation, of a single gene product (16). More recently several new GSH-T's have been described. A GSH-T with a pI of 6 - 6.5 is present in some specimens of human liver. Its presence corresponded with the ability to catalyse conjugation of 4-phenyl-3-buten-2-one. This was the only peak of activity with this substrate (27,28). This GSH-T has a higher specific activity with benzo(a)pyrene-4,5-oxide than the basic GSH-T's, but does not react with cumene hydroperoxide or Δ^5-androstenedione (see chapter XV) (27). In addition in some specimens there is at least one major GSH-T with a pI of 4.6 (GSH-T ω) and two minor GSH-T's with pI's about 5.4 (29,30). GSH-T ω is slightly smaller than the cationic GSH-T's with a molecular weight of 46,000 daltons. It too consists of two subunits, molecular weight 22,500 daltons (30). Antibodies to the cationic GSH-T's have been shown to cross react with the anionic GSH-T. However the published amino acid analysis of GSH-T ω is sufficiently different to that of the cationic GSH-T's to cast doubt on claims that they are related. In fact the amino acid analysis of GSH-T ω closely resembles that of GSH-T ρ in the red cell, which is immunologically different from the cationic liver GSH-T's (table 2.2). Like GSH-T ρ, GSH-T ω is only active with CDNB as substrate.

GSH-T ρ, the major GSH-T present in red blood cells, has a pI of 4.51 (31). The enzyme has also been demonstrated in lymphocytes, but others with partially pure enzymes from different lines of white cells

Table 2.2. CHARACTERISTICS OF THE TRANSFERASES ISOLATED FROM HUMAN ORGANS

SOURCE	GSH-T	MOL. WT. daltons	pI	SUBUNIT SIZE daltons	REFS.
Human liver	α	49,000	7.8	25,000	16,199
	β	49,000	8.25	25,000	16,199
	γ	49,000	8.55	25,000	16,199
	δ	49,000	8.75	25,000	16,199
	ε	48,000	8.5	25,000	16,199
	υ		6-6.5		28
	ω	46,000	4.4-4.7	24,000	29,30
Human red cells	ρ	47,500	4.51	24,000	31
Human placenta		60,000	4.65-4.8		34,35
Human lung			4.62		29

have failed to show any difference from the liver enzymes (32). The red cell enzyme is possibly not as homogenous as first thought. An electrophoretic system has shown two charge species, which varied in concentration from sample to sample (33). These differences could be artefacts rather than due to true differences in structure.

There is a single GSH-T in human placenta which resembles the red cell enzyme in substrate specifity (only active with CDNB), pI (4.65), pH optimum and low interaction with bilirubin (34,35).

CHAPTER III

NON-SUBSTRATE BINDING

Pure hepatic ligandin (YaYc) has a single high affinity binding site and a second, low affinity, binding site (36,37). Several authors have noted that ligandin appears to lose binding sites with purification (28,29,40). The binding affinity may also be decreased. These findings are partly explained by the presence of other contaminating GSH-T's in cytosol, but addition of GSH increases the binding ability of ligandin (36,38). Others have failed to show that bilirubin binding is increased by the addition of GSH (39). On adding albumin to cytosol, bilirubin remains bound to ligandin (39), despite the fact that the Ka of albumin exceeds that of ligandin. However addition of albumin to purified ligandin resulted in the formation of bilirubin-albumin complexes at the expense of bilirubin-ligandin complexes. Low concentrations of serum could remove bilirubin from pure ligandin. These data suggest that the affinity of ligandin for bilirubin is higher intracellularly than when ligandin is removed from its intracellular environment (39).

The ligandin YaYa dimer has two high affinity binding sites for bilirubin (19). The high affinity binding site therefore is thought to reside on the Ya peptide. BSP is normally non-covalently bound, but by photoactivation can be made to bind covalently. Labelled BSP under these conditions binds virtually exclusively to the Ya subunit of a YaYc dimer, confirming that Ya carries the high affinity binding site (19). Chemical modification of ligandin shows that enzyme activity re-

quires the presence of the low affinity binding site, which is probably also the enzyme substrate binding site (20a,40). Non-substrate ligands can competitively inhibit GSH-T activity once the primary site is fully saturated (36). Not all binding is that simple however, as instances of noncompetitive and uncompetitive inhibition of binding have been described (41,42).

Non-covalent binding of ligands by the other GSH-T's and covalent binding of molecules by ligandin and the other GSH-T's will be discussed later.

CHAPTER IV

LIGANDINAEMIA IN LIVER DISEASE

Introduction:-

Clinical enzymology had its origins in 1908 when serum and urine from patients with pancreatitis were shown to have α-amylase activity (43). Clinical use of enzyme tests was slow to develop and only became wide spread when Ladue et al (44) and DeRitis et al (45) simultaneously described the clinical use of transaminase reactions in liver disease.

Since then a host of different enzyme activities have been shown in serum in disease states. However only a mere handful have survived into modern clinical practice.

In hepatology commonly used measurements include glutamic oxaloacetic transaminase (SGOT), also known as aspartate amino transferase (AST), glutamate pyruvate transaminase (SGPT), also known as alanine amino transferase (ALT), alkaline phosphatase (AP), lactic dehydrogenase (LDH), and γ-glutamyl transpeptidase (GGT). Since this study examines the measurement of ligandin in serum as an index of hepatocellular necrosis only those enzyme activities which reflect necrosis i.e. SGOT and SGPT, will be discussed.

The Transaminases:-

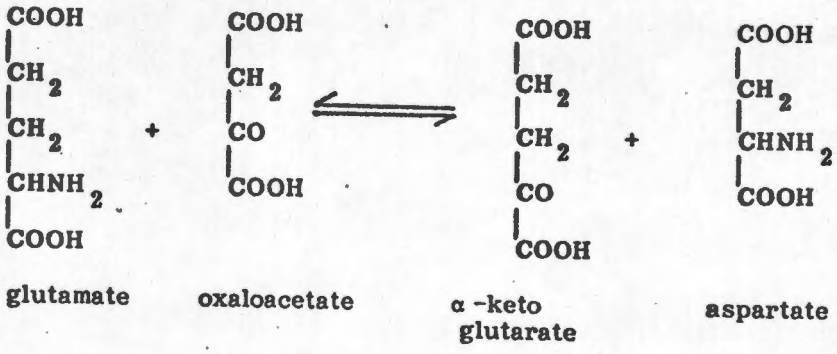
The transaminases catalyse the intermolecular transfer of an amino group from an α-amino acid to an α-keto acid. This is an oxidative deamination, and does not involve decarboxylation. Ammonia is not form-

ed. Enzyme catalysed transamination was first described in pigeon breast muscle (47) in 1937.

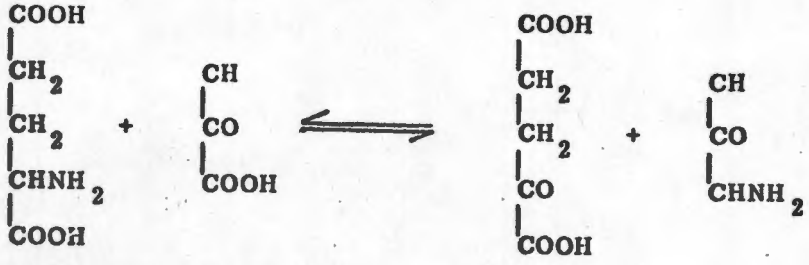
Although the term transaminase is in common usage it is perhaps more correct to use the term aminotransferase and specify the particular enzyme by product e.g. alanine aminotransferase.

There are a variety of transamination reactions recorded in plants, animals and man, but only two, SGOT and SGPT are clinically useful (48).

SGOT catalyses the reaction (3.1);



SGPT catalyses the reaction (3.2);



glutamate pyruvate α-keto glutarate alanine

Both enzymes require pyridoxal phosphate as a cofactor. SGOT has been separated from SGPT and has been purified (49,50).

The transmainase activities of different tissues vary, with distinct species differences. In any one tissue however the SGOT activity exceeds the SGPT activity. SGOT activity is highest in skeletal muscle, diaphragm, heart muscle and liver, but is also present in brain, red cells, and kidney. SGPT is more restricted in its distribution, being found mainly in the liver, and to a lesser extent in skeletal muscle (48,51). Different isoenzymes of SGOT are present in cytoplasm and mitochondria, but SGPT is purely a cytoplasmic enzyme. SGOT has a molecular weight of 100,000 daltons, and SGPT 110,000 daltons (52).

Measurement of the transaminases:-

Many techniques have been described to measure activity of these two enzymes, most of which are modifications of three basic techniques, spectrophotometric, flourimetric, and colorimetric.

With the spectrophotometric method NADH, and an excess of malic dehydrogenase (SGOT) or lactic dehydrogernase (SGPT), are added to the reaction mixture. Oxaloacetate is converted to malic acid, and pyruvate is converted to lactate. NADH is oxidised in both these reactions, and this can be followed by the change in absorbance at 340 nm. This technique is suitable for micromethodology, and is commonly used in clinical laboratories.

The fluorometric method (53,54) utilises the oxidation of NADH as before, but measures the fluorescence of the condensation product of the NAD which is generated, with methyl-ethyl ketone. The colorimetric method measures the amount of pyruvate formed. For SGOT oxaloacetate is converted to pyruvate first, which then reacts with dinitrophenylhydrazine, which can be detected on addition of alcoholic potassium hydroxide. For SGPT pyruvate is formed directly (55). The rate of transamination is very sensitive to temperature (56,57), which has led to difficulties with standardization. These have largely been overcome with automation. Haemolysis is probably the major physiological factor which affects the reaction rate, causing an increased reaction rate, possibly due to release of enzyme from red cells (56,58).

Distribution of transaminases in extracellular fluid:-

There is a considerable gradient in transaminase concentration between the cell and the extracellular fluid. The maintainance of this

gradient depends up on the integrity of the cell membrane. Although it has long been known that leakage of potassium and other small molecules into serum was an early indication of cell damage it became more recently apparent that larger molecules, such as enzymes are also released in the early stages of cell injury (59,60).

The concentration of a tissue derived enzyme in serum depends on many factors, e.g. rate of release from cells, volume and rate of distribution, and clearance from the extracellular space. These factors will be discussed in turn.

The rate of release from cells depends on the intracellular concentration of enzyme, the size of the organ involved, and the extent of the injury to the cells (61). It is important to realise that release does not only occur from necrotic cells, but that even minimally damaged cells may release enzymes. Evidence for this has come from work with cell suspensions, isolated muscle, and experiments with isolated perfused livers (59,60,62,63).

In the isolated perfused rat liver it has been shown that a relative or absolute oxygen lack causes release of enzymes into the perfusate (64). The perfused livers of fasted rats release more enzyme than the livers of fed rats. Following perfusion these livers were histologically normal. This and other evidence suggests that enzymes can leak out of intact cells (64).

Work in isolated perfused livers has also shown that the rate of release of enzymes depends on their intracellular location. Mitochondrial enzymes are released in smaller quantities and more slowly than cytoplasmic enzymes (63,65,66). The release of mitochondrial enzymes has been correlated with cell death (67), whereas the release of

cytoplasmic enzymes precedes necrosis. Similarly, the release of smaller enzymes is more rapid than larger molecules (67).

The blood disappearance curves for most enzymes are biphasic, with an initial rapid clearance, followed by a slower clearance phase. Once in serum the enzymes are rapidly redistributed throughout the extracellular space (68,69). This redistribution is responsible for the the initial rapid clearance. The second slower disapearance curve may be due to catabolism and uptake by tissues. It has been shown that loss of activity does not contribute much to plasma disappearance (70).

It is not known how SGOT and SGPT or most other enzymes are cleared from the extravascular compartment. Clearance is not affected by hepatectomy, nephrectomy, or intestinal evisceration (69,71,72). Most tissues will take up circulating enzymes, usually by the parenchymal cells, and not the reticulo-endothelial cells. Very little intact enzyme is excreted in urine or bile (70).

Other enzymes have been used for the study of liver damage, e.g. ornithine carbamyl transferase and isocitric dehydrogenase are liver specific, but do not give any more information than transaminases (73,74). Sorbitol dehydrogenase is also liver specific but is relatively unstable. Activity of this enzyme tends to follow SGPT activity (75).

Alcohol preferentially damages the centrilobular zone and is also a mitochondrial toxin. Therefore glutamate dehydrogenase which is present in a centrilobular distribution and is a mitochondrial enzyme is possibly a more sensitive index of hepatocellular damage in alcoholic liver disease (76). SGPT is more periportally distributed.

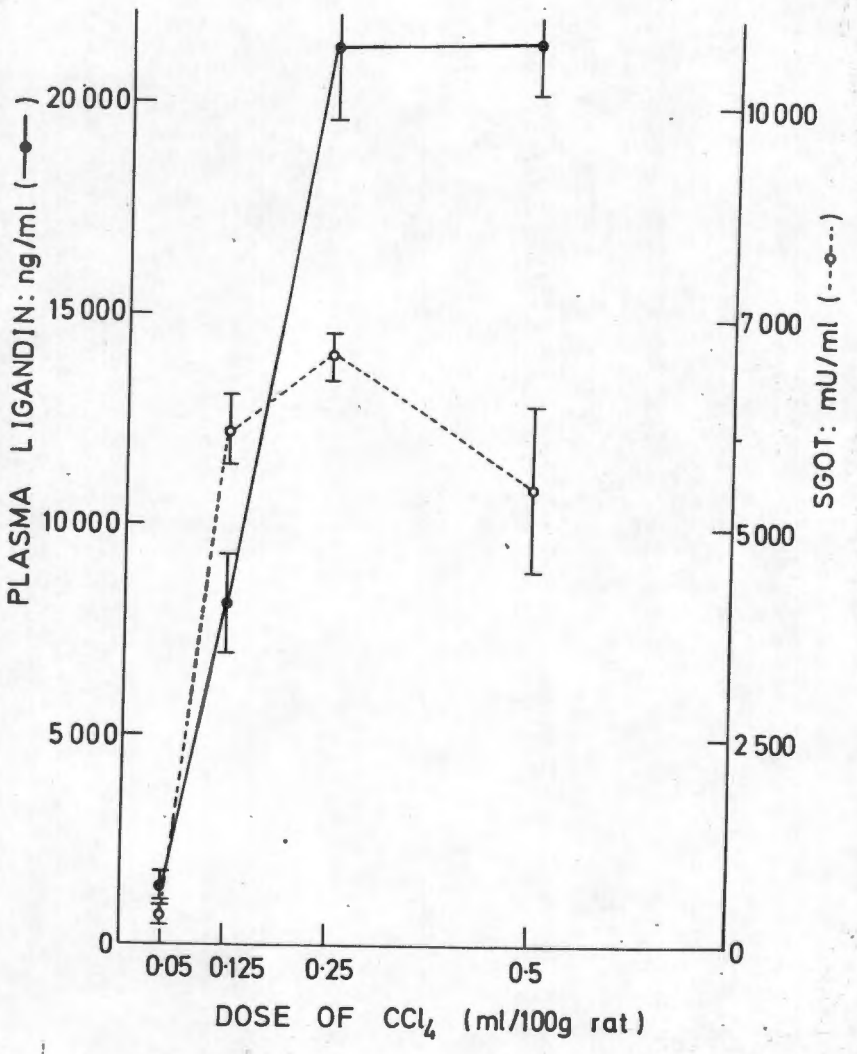


Fig. 4.1. Response of plasma ligandin and SGOT 24 hrs after CCl_4. After Bass et al (84).

Non-enzymatic markers such as serum ferritin (78), serum iron (79), and bile salts (80) have also been investigated as markers of hepatocellular damage but have not received universal acceptance.

Ligandin and GSH-T activity in experimental liver disease:-

The use of the GSH-T's in the diagnosis of liver disease has been little studied. GSH-T activity with BSP (81,82) and CDNB (83) has been studied in rats given carbon tetrachloride. BSP proved a rather insensitive substrate, but activity with CDNB was found to be elevated early, peaking 12 hours before SGOT activity. GSH-T activity was also cleared before SGOT and SGPT activity. Ligandin has also been measured immunologically by radioimmunoassay (RIA) in CCl_4 treated rats (84). At three hours ligandin was more than ten times the upper limit of normal, but SGOT was not significantly raised. Peak ligandin concentration was nearly 400x normal levels, whereas peak SGOT was 150x normal levels. The magnitude of release of ligandin was dose dependent up to a dose of 0.25 ml/100 gm body weight, after which the dose response curve flattened out (fig. 4.1). The SGOT response curve correlated less well with dose than did the ligandin response.

The finding that ligandin was released early, and that the magnitude of ligandin release was so great, led Bass et al (84) to conclude that in the rat the measurement of serum ligandin was a more sensitive index of hepatocellular necrosis than SGOT.

Bass (85) also studied the disappearance of ligandin and showed that its half life was less that one hour.

The question then arises as to which of the two, RIA or enzyme assay, is preferable. Since the first introduction of the radioimmuno-

assay (RIA) (86), its use in clinical medicine has increased dramatically. RIA has the advantage of being able to detect lower concentrations of protein (or other antigens) than enzyme activity or functional assays of the same protein. Functional activity is dependent on upon assay conditions and is subject to many exogenous inhibitors of activity. RIA in contrast, measures the mass of an antigen, irrespective of its functional state. A good example of the difference between these two techniques is the assay of prostatic acid phosphatase in prostatic carcinoma. Use of RIA to detect prostatic carcinoma has increased detection rate considerably compared to the older enzyme assay (87). GSH-T enzyme assay in particular may be prone to exogenous inhibition, since many therapeutic agents which may be present in blood of patients may either be enzyme substrates, e.g. paracetamol, or may be competitive inhibitors of enzyme activity, e.g. penicillin.

Enzyme diagnosis in human liver disease:-

It is unfortunate that among enzymes used in clinical practice there are very few organ specific, and certainly no disease specific enzymes. Diagnostic enzymology flourishes best in hepatology, where the size of the organ, the lack of a basement membrane around sinusoids, and the diverse enzyme equipment of hepatocytes combine to be uniquely useful (61).

It is common cause that SGOT and SGPT rise dramatically in acute viral hepatitis. Clermont and Chalmers (57) analysed data from a number of groups and concluded that by the time that patients became jaundiced all had elevated transaminases, but that the onset of transaminase elevation occurred as many as 16 days prior to the onset of jaun-

dice. The peak transaminase elevation most frequently occurred about 1-2 days before the onset of jaundice (88) and reached more than 20x normal levels (57). At least 50% of patients will have levels exceeding 600 Karmen units, and only rarely will the admission SGOT be normal. SGOT returns to normal before SGPT. Up to 95% of patients may have residual elevation of SGPT when SGOT has fallen to within normal limits.

Data from the Willowbrook State School studies suggest that in hepatitis A transaminases rapidly return to normal, whereas in hepatitis B this may be delayed by months (89). It is common experience that SGOT and SGPT may remain elevated long after clinical remission has occurred. Up to 26% of patients may have persistently elevated transaminases at 6 months (90).

Ligandin was first studied in human disease in 1978 (91), but since our first communication only a handful of further publications have appeared.

Normal serum levels in man have varied from less than 4 ng/ml (92,93) to less than 8.5 ng/ml (91).

Bass et al reported that ligandin is elevated in acute viral hepatitis and chronic active hepatitis more frequently than the serum glutamic oxaloacetic transaminase (SGOT)(AST), whereas in non-hepatic diseases the SGOT is elevated more frequently than ligandin is (91).

Ohmi et al (92) studied ligandin measured by RIA in acute hepatitis and found that in fatal cases of fulminant viral hepatitis ligandin levels are markedly elevated in those patients in whom it was measured within two days of the onset of coma (range 58 to 780 ng/ml, mean 316 ng/ml). In patients who survived, the mean elevation of ligandin

measured at the same stage of illness was 47 ng/ml (range 8 to 82 ng/ml). In patients with acute drug induced hepatitis due to paracetamol or halothane the mean elevation was 2297 ng/ml (range 680 - 5350 ng/ml). Based on the assumption that the serum ligandin reflects necrosis, the authors interpreted these results as indicating different mechanisms of necrosis in the two conditions. In drug-induced hepatitis they felt necrosis occurs in a single massive burst following exposure to the drug, but in viral hepatitis necrosis occurs over a longer period of time and is intermittent or progressive, and less massive (92). A time course study of the release of ligandin in hepatitis was not performed.

Tsuru et al (93), also using a RIA for ligandin, showed correlation with serum glutamic pyruvic transaminase (SGPT)(ALT) in acute and chronic hepatitis, but not in cirrhosis. Levels of ligandin in acute hepatitis were reported to be 16.8 $\pm$ 11.1 ng/ml. The highest reported levels were 67 and 37 ng/ml. In chronic hepatitis the levels were 36.0 $\pm$ 19 ng/ml. In this study the authors concluded that measurement of ligandin was not clinically useful.

Serum GSH-T activity with CDNB as substrate has also been studied in human liver disease and shown to correlate with the SGOT in acute hepatitis, especially if the SGOT was markedly elevated (94). There was also a correlation between serum GSH-T and SGOT in fulminant hepatitis. Correlation with SGPT was generally better than with SGOT, except in those patients with acute hepatitis who had low levels of SGPT, and in patients with fulminant hepatitis. Over the course of the disease serum GSH-T activity more or less paralleled the transaminase levels but returned to normal well before the transaminases did (94).

Chronic active hepatitis (CAH):-

In chronic active hepatitis the diagnosis rests on clinical suspicion and liver biopsy. Currently used laboratory methods to assess disease activity include the transaminase activity, serum globulin concentration, and histology. Given the possibility of sampling error, biopsy appearance is the standard against which all other tests must be compared. Since biopsy cannot be repeated at weekly or monthly intervals the transaminases are commonly used for day to day monitoring.

It is well known that CAH can be asymptomatic (95), and that histologically active disease can be present in the absence of elevated transaminases (80). Summerskill et al (96) showed that clinical and biochemical remission occurred simultaneously, but that histological remission lagged, so that at 24 months less than 60% of those in clinical and biochemical remission were in histological remission. Czaja et al (97) investigated the relationship between serum transaminase and histological severity and showed that about 50% of patients with CAH will have transaminase levels less than 2x normal, and about 19% will have normal transaminases. Most of these patients will have histological changes that are not associated with an aggressive course. On the other hand when the transaminases were more than twice normal this was associated with more severe histological changes. Transaminases however, do not differentiate between CAH and chronic persistent hepatitis (98). Liver biopsy is required for this purpose.

Serum bile acids have been measured in CAH, and shown to reflect the histological status of the liver better than the transaminases (80). Although these have been used in specialist units, serial meas-

urements of bile acids have not achieved the popularity of the transaminases.

Ligandin has also been studied in CAH (91,93), but only superficially. Ligandin immunoreactivity was elevated in CAH (36 ± 19 ng/ml) (91,93), but was not found to be clinically useful. The authors were able to show that, as in acute hepatitis, ligandin concentration correlated with SGPT activity. However when GSH-T activity with CDNB as substrate was examined (94) no correlation with SGOT or SGPT could be demonstrated.

Other liver diseases:-

Ligandinaemia has also been studied in cirrhosis and hepatocellular carcinoma. In cirrhosis no correlation could be found between transaminase activity and ligandin immunoreactivity (93), or serum GSH-T activity (94).

Ligandin is absent or reduced in hepatic tumours in rat and man (99,100,101). In the rat well differentiated hepatocellular carcinomas retain ligandin better than poorly differentiated carcinomas do (99). Ligandin was present in 9 of 12 Morris hepatoma cell lines. These cells retained ligandin when transplanted into rats, but ligandin was absent from most metastases. Serum ligandin was elevated in all but one recipient of the tumour (100). The serum ligandin could have come either from the tumour or from the surrounding liver. This question was examined by transplanting hepatoma cells into athymic nude mice at a site distant from the liver. These animals do not accept normal hepatic tissue, but will accept hepatocellular carcinoma cells. As the tumour grew in size the serum ligandin rose, suggesting that the tumour

itself was secreting ligandin, since the mouse liver ligandin was shown not to cross react with the antibody used.

Ligandin was present in 7 of 11 specimens of human hepatocellular carcinoma (5.2 $\pm$ 2.1 ug/ml range 0 - 17.4 ug/ml). Elevated levels were found in the serum of some these patients (251 $\pm$ 93 ng/ml). Serum ligandin was elevated in only 2 of 9 patients with hepatic metastases from primary neoplasms elsewhere (100). Tsuru et al (93) were able to show a correlation between serum GSH-T activity with CDNB and SGOT in patients with hepatocellular carcinoma.

The usefullness of ligandin as an index of hepatic disease is still not clear. Questions remain about the release of ligandin in hepatic disease, particularly in acute viral hepatitis. There is thus a need for a prospective study over a period of weeks, months or years, depending on the disease, which will determine whether serum ligandin levels represent a better index of necrosis or prognosis than currently available tests.

CHAPTER V

LIGANDINURIA IN RENAL DISEASE

The use of urinary enzymology in the diagnosis of renal disease has not met with wide acceptance. Several tubular brush border enzymes e.g alkaline phosphatase (102), maltase (102) and α-fucosidase (103), as well as lysosomal enzymes such as β-galactosidase, β-glucosidase and N-acetyl-β-D-glucosaminidase (NAG), have been investigated (104-106). NAG and β-glucosidase have been shown to be elevated in rejection of the transplanted kidney. Enzymuria may be the first manifestation of rejection, appearing before any clinical or other biochemical manifestations (107,108).

Experimental tubular necrosis, induced with mercuric chloride or potassium dichromate causes enzymuria, but not in advance of other indicators of renal dysfunction (102). In gentamicin treated rats urinary NAG and α-fucosidase are increased before other early manifestations, such as proteinuria and changes in osmolality (103). This difference may be due to the degree of necrosis, i.e. the sudden massive necrosis caused by $HgCl_2$ and $K_2Cr_2O_7$, which may result in release of tubular enzymes, concomitant with a deterioration in renal function, whereas gentamycin toxicity results in a gradual loss of cells, and thus enzymuria can occur for some time prior to the onset of renal dysfunction, which occurs only after considerable loss of tubular cells.

NAG excretion has been studied in various types of human renal disease (105,108). In tubular necrosis following hypotension urinary NAG levels are elevated up to 1200x normal levels. In acute and

chronic glomerulonephritis NAG levels of up to 10x normal are seen, while in the nephrotic syndrome levels of up to 40x normal commonly occur. However in some patients in whom an episode of hypotension is associated with a period of oliguria, but who do not develop acute tubular necrosis, urinary NAG levels in the acute tubular necrosis range were found (105).

It is because of this extreme sensitivity and lack of specifity that the use of urinary enzymes for diagnosis has not become widespread. Thus urinary enzyme excretion increases in renal disease, but does not distinguish one form from another, with the possible exception that acute tubular necrosis is associated with massive enzyme excretion. This can be considered to be the renal equivalent of massive SGOT release in acute viral hepatitis. However unlike liver disease, the massive release of enzymes is also seen in patients who do not have clinically significant renal disease. In addition, unlike liver disease there are other easily performed tests to detect the presence of renal disease, e.g. serum creatinine.

The localisation of ligandin to the proximal renal tubules has prompted the study of the release of ligandin into the urine. The experimental model of proximal tubular necrosis is mercuric chloride ($HgCl_2$) or potassium dichromate ($K_2Cr_2O_7$) intoxication (109, 110). $HgCl_2$ produces necrosis limited to the pars recta of the proximal tubules. $K_2Cr_2O_7$ causes necrosis localised to the pars convoluta. Rats treated with $HgCl_2$ excrete ligandin in the urine and release ligandin into the blood within 12 hours (111). This is associated with a rise in the serum urea at 12 hours. Urine ligandin excretion ceases by 24 hours but ligandin is still detectable in the serum

at this time. The kidneys of animals sacrificed at 24 hours show a reduction of ligandin concentration to approximately 30% of control levels (111).

It had been reported that $K_2Cr_2O_7$ intoxication does not cause ligandinuria or measurable GSH-T in the urine (112,113). With the advent of the more sensitive RIA this was studied in more detail and it has now been shown that ligandin is indeed released into the urine in $K_2Cr_2O_7$ treated rats (111). However in contrast to $HgCl_2$ intoxication, ligandinuria is only detectable after 24 hours and persists to at least 72 hours. This release of ligandin has been shown to correlate with histological extension of $K_2Cr_2O_7$ induced necrosis into the pars recta (111). Most of the immunoreactive ligandin in the urine of nephrotoxin treated rats elutes from Sephadex G-100 at the same elution volume as purified ligandin (85), and has the same affinity for the antibody as purified ligandin, indicating that the immunoreactivity detected in the urine is due to native ligandin. This contrasts with the finding that serum ligandin is excreted as a larger molecule with reduced immunoreactivity, and suggests a possible means of determining the origin of urinary ligandin (85).

Rats given a toxic dose of gentamicin excrete ligandin (measured by immunodiffusion) and GSH-T activity in the urine, whereas these are undetectable in normal urine (114).

Ligandinuria in man:-

In man the measurement of ligandinuria may have an application in the diagnosis of acute tubular necrosis, a leading cause of acute renal

failure. At present the diagnosis rests on clinical grounds, and is supported by side room investigations and a few biochemical tests. Clinically in the setting in which acute tubular necrosis occurs, (excluding those cases caused by drugs), i.e. hypotension and septicaemia, the initial differential diagnosis lies between prerenal renal failure and acute tubular necrosis. The two conditions may be differentiated by several factors, detailed in table 5.1., but in many cases uncertainty exists, and any test which could differentiate between the two would obviously be clinically useful. It is possible that ligandinuria may be able to differentiate between these conditions.

Renal transplantation has become a common form of therapy. One of the major remaining problems is the elucidation renal dysfunction in the immediate post transplant period. A renal allograft may never function, may function initially but fail suddenly within a week, or after some months, or function may gradually deteriorate (119).

In the immediate post-transplant period the differential diagnosis of a non-functioning graft includes dehydration, ureteric or catheter obstruction, urinary leak, acute tubular necrosis or hyperacute rejection. In the later post-transplant period acute and chronic rejection, ureteric stenosis and septicaemia may cause decreasing renal function (119).

Despite inevitable ischeamia, kidneys from living related donors function well following transplantation (119). However cadaver kidneys, or kidneys which have been perfused fare less well. Twenty to 50% of these kidneys may develop acute tubular necrosis, which may occur immediately, or be preceded by a short period of urine formation (119).

Table 5.1.

DIFFERENTIATION BETWEEN ACUTE TUBULAR NECROSIS & PRERENAL RENAL FAILURE

Test	Prerenal failure	Acute tubular necrosis	refs
Urine sediment	moderate hyaline & granular casts	Brown granular casts epithelial cells ++	115
Urine sodium	less than 10 mEq/l	more than 25 mEq/l	115
Urine/plasma osmolality	increased	isosthenuria	116
Urine/plasma urea	more than 20:1	less than 3:1	117 116
Urine/plasma creatinine	more than 40:1	less than 15:1	117
$\frac{\text{U/P Na}}{\text{U/P creatinine}}$	less than 1	more than 1	118

Acute or cellular rejection can occur at any time after the first week post transplantation. This condition is characterised by a cellular infiltrate in the interstitium, but may be accompanied by varying degrees of humoral rejection - vasculitis and fibrin deposition (119).

Acute cellular rejection may be diagnosed clinically by the presence of fever, allograft tenderness, leucocytosis, and decreased renal function (119). These criteria however apply equally well to perinephric abcsess, haematoma, or urinary leak with infection. ^{131}I-hippurate renogram scans are helpful in the diagnosis of rejection, but these really only measure renal perfusion, which may be altered for reasons other than rejection (120).

Acute tubular necrosis is a diagnosis by exclusion in this situation. Urinalysis in the post transplant period is unreliable (121), especially in the presence of preexisting decreased renal function. The diagnosis can only be made with certainty in retrospect, when renal function returns without recourse to treatment for rejection, or on biopsy.

The differentiation between acute tubular necrosis and acute rejection is important since the former requires only maintainance dialysis until renal function returns, but acute rejection requires early diagnosis and prompt treatment if irrerversible renal damage is to be prevented. Despite the importance of this differentiation renal biopsy is not often performed in the first two weeks after transplantation, because of the fear of endangering the graft. On the other hand one cannot treat all patients as if the diagnosis is acute rejection as this involves the use of high doses of methyl prednisolone, with its attendant complications.

Therefore there is a definite need for a simple, safe and accurate test to diagnose either acute tubular necrosis or acute rejection, with certainty.

Studies of renal ligandin excretion in man are very limited. In a study of enzymuria following renal angiography some patients excreted GSH-T activity in the urine. Ligandin immunoreactivity was also detectable in the urine (122). This was not associated with any evidence of tubular necrosis or deteriorating renal function, and presumably represented sub-clinical tubular cell damage.

Ligandin immunoreactivity (by immunodiffusion, which has a limit of detection of 1 - 10 ug/ml) and GSH-T activity were measured in the perfusates of donor kidneys. All those kidneys which produced ligandin in the perfusate developed post-transplant oliguria, while those kidneys which did not produce ligandin and GSH-T activity functioned normally (123). In a further study (124,125) GSH-T enzyme activity was studied in the perfusates and ultrafiltrate from donor kidneys. The GSH-T activity in the perfusates from kidneys which developed post transplant oliguria was significantly higher than from those kidneys which functioned normally. The difference was such that 100% predictability could be achieved. Ligandin in ultrafiltrates did not have the same predictabilty, although there was a difference in the mean GSH-T activity between those kidneys that did, and those that did not develop oliguria (125).

Urine from normal subjects, patients with acute and chronic renal failure, and acute renal failure not associated with tubular necrosis were negative for ligandin, measured by immunodiffusion. In two patients with acute tubular necrosis in whom urine was studied within 24

hours of the onset of illness ligandin was detected in the urine. In a further 10 patients with acute tubular necrosis in whom urine was only available 96 hours after the precipitating insult, no ligandin was detectable in the urine (126).

It is clear from these studies that ligandinuria reflects tubular necrosis. However the specificity of the test has not been completely defined. Nor has it been shown that the assay of ligandinuria is clinically useful. Sequential temporal patterns of ligandinuria in patients with renal disease have also not been defined.

For these reasons further studies of ligandinuria were thought to be necessary, and these have been included in chapter XI of this thesis.

CHAPTER VI

PURIFICATION OF HUMAN LIGANDIN

INTRODUCTION

Several methods for purification of the human transferases have been reported, most involve either affinity chromatography or anion exchange chromatography, and are somewhat lengthy. Purification of rat liver ligandin requires a simple three step procedure without the use of gradient elution. Thus human GSH-T was purified utilising a scheme based on that used to purify ligandin from rat liver. The following description is representative of several purifications performed during the course of this study.

MATERIALS AND METHODS

Human liver was obtained within 12 hours of death from victims of trauma. The liver was macroscopically normal. Tissue was used immediately or frozen at -70°C until required.

Enzyme Activity:-

This was measured spectrophotometrically monitoring the formation of adducts of glutathione and 1-chloro-2,4-dinitrobenzene at 25°C (127). The light path was 1 cm and the cuvette volume was 3 ml. Final concentration of CDNB and GSH was 1mM. Δe was 9600 M^{-1} cm^{-1}. λ max was 340nm. The buffer was 0.1M potassium phosphate pH 7.5. Correction was made for non-enzymatic activity. Results were expressed as mmoles product formed per minute per ml. Specific activity is express-

ed as mmoles product/minute/mg protein. Protein was measured by the Lowry method (128).

Purification:-

All procedures were performed at 4°C. Approximately 100 gm of liver was cut fine by scissors and homogenised in 3 volumes of 0.01M Tris:HCl, 0.25M sucrose, pH 8.67, using a vortex homogeniser (Ultra Turrex). The suspension was homogenised further with a motor driven teflon-glass mortar and pestle. The resulting homogenate was centrifuged at 27,000 xg for 30 minutes and the pellets discarded. The supernatant was then centrifuged at 100,000 xg for 2 hours, and the pellet discarded. The supernatant was concentrated to 50 ml using positive pressure ultrafiltration in an Amicon stirred cell with a PM 10 membrane, and applied to a 32 x 3.5 cm column of triethylaminoethyl-cellulose (TEAE) equilibrated with 0.01M Tris:HCl, pH 8.67 (buffer A). The column was developed by gravity with the same buffer at a flow rate of 60 ml per hour. Fractions of 8.4 ml were collected and the eluate monitored spectrophotometrically at 280 nm. Fractions were tested for GSH-T activity with CDNB.

The active fractions were pooled, diafiltrated to 15 ml in 0.1M sodium phosphate buffer, 0.15M sodium chloride, pH 7.4 in an Amicon stirred cell using a PM 10 membrane, and applied to a 90 x 2.5 cm column of Sephadex G-100 previously equilibrated with the same buffer. The column was developed with the same buffer by pump driven upward flow at a rate of 30 ml per hour. Fractions of 4 ml were collected. The eluate was monitored at 280 nm and fractions were assayed for activity with CDNB. The active fractions were pooled, concentrated to 10

ml, re-applied to the same column, and eluted under the same conditions.

The active fractions from the second Sephadex G-100 column were pooled, dialyzed into buffer A and concentrated to 7 ml. The concentrate was applied to a 45 x 2.5 cm column of QAE-Sephadex equilibrated with the same buffer. The protein was eluted by gravity with the same buffer at a flow rate of 45 ml per hour. Fractions of 4 ml were collected. The eluate was monitored as before for enzyme activity and at 280 nm. The active fractions were pooled.

This procedure resulted in a protein which was only slightly contaminated. Final purification was achieved by either of the following methods.:-

1. Rechromatography on QAE-Sephadex:- This was performed under identical conditions to the previous step.

2. Isoelectric focusing (IEF):- This was performed in a sucrose density gradient in the pH range 3 - 10, using an LKB 4101 column. The sample was applied after 50% of the column volume had been filled. The column was developed at 600V for 16 hours and then drained by pump at a rate of 1-2 ml/min. Fractions of 2 ml were collected.

Polyacrylamide gel electrophoresis (PAGE):-

The discontinuous system of Maizel was used (129). The resolving gel was cast between 25 x 10 cm glass plates separated by a 2 mm perspex spacer. The resolving gel consisted of 11% polyacrylamide in 0.375M Tris:HCl, 0.1% SDS, pH 8.9. The stacking gel was 3% polyacrylamide in 0.0625M Tris:phosphate, 0.1% SDS, pH 6.7. Gels were polymerized by light in the presence of riboflavin. Electrophoresis was per-

formed for 16 hours at 100V. The electrode buffer was 0.005M Tris: glycine, O.1% SDS, pH 8.6.

Samples were prepared for electrophoresis by heating at 100°C for 2 minutes with 1% SDS, 1% 2-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue in 0.0625M Tris:phosphate pH 6.7. Five to 20 µl of each sample, containing 1 - 100 µg of protein were applied. After electrophoresis the gels were fixed and stained in 0.1% Coomassie Blue, 8% TCA in 33% methanol/67% water for 2 - 3 hours and destained in 5% methanol/7% acetic acid. The gels were either photographed wet or dried down by vacuum and heat between Whatman No. 3 chromatographic paper and cellophane, prior to photography.

Isoelectric focusing in polyacrylamide tube gels:-

This was performed in 5% acrylamide tube gels according to the method of Wrigley (130) in a water cooled MRA apparatus. The gels were cast in 10.5 x 0.3 cm rods and polymerized with 0.045% ammonium persulphate. The gels contained 5% ampholyte pH 3-10. After polymerization the gels were left to stand overnight at 4°C. After pre-running for 20 mins at 300 V to remove the ammonium persulphate, the sample was applied. The sample application solution consisted of 20 µ l of protein solution containing ±20 µ g protein, 50 µl glycerol and 100 µ l of 0.1% ampholyte. About 80-100 µ l were applied to each gel. Anolyte was 0.01M 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES) and catholyte was 0.01M ethanolamine. The sample was overlaid with 10 µ l of ampholyte. Focusing was initiated at 200 mV. Voltage was increased at 1/2-hourly intervals to 500 mV and then held constant for a further four hours. The gels were then fixed in 5% TCA and stained in 0.1%

Coomassie Blue in acetic acid:ethanol:water (2:9:9 v/v) and destained with acetic acid:ethanol:water (2:5:13 v/v). Alternatively the gels were cut into 2 mm sections which were shaken for two hours in 0.3 ml normal saline. Enzyme activity and pH were determined on these samples.

Molecular Weight Estimation:-

The molecular size of purified ligandin was estimated by gel filtration on a 90 X 2.5 cm column of Sephadex G-100 in 0.01M sodium phosphate, 0.1M sodium chloride, pH 7.4, run under identical conditions to that described above. Molecular weight markers were blue Dextran (100,000), bovine serum albumin (67,000), ovalbumin (43,000), and cytochrome C (13,000). The molecular size of ligandin subunits was determined by PAGE in SDS using phosphorylase b (94,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soy bean trypsin inhibitor (20,100) and α-lactalbumin (14,400) (Pharmacia). R_f values were calculated and straight line plots obtained by the method of least squares.

RESULTS

Purification:-

This purification procedure resulted in a 60 fold purification with a yield of 7% (table 6.1). The elution profiles from TEAE-cellulose, Sephadex G-100 and QAE-Sephadex chromatography steps are shown in fig. 6.1 A-E. Fifty nine percent of GSH-T activity applied to the TEAE-cellulose is not bound and elutes at the protein front as a single peak with a prominant trail. Only the peak was pooled. Chromato-

PURIFICATION OF HUMAN LIGANDIN

	Volume ml	Protein concen- mg/ml	Total activity υmol/min/ml	Specific activity υmol/mg	Purific- ation x-fold	% Yield of activity
Cytosol	235	28.40	4605	0.69	———	100
TEAE	103	6.50	2735	4.10	5.9	59
G-100'	75	4.37	2000	6.10	8.8	43
G-100''	72	2.79	1962	9.76	14.1	43
QAE'	13	0.93	432	35.75	51.8	9
QAE''	17	0.47	327	41.10	59.6	7

Table 6.1. Summary of purification of human ligandin using 1-chloro-2,4-dinitrobenzene as substrate.

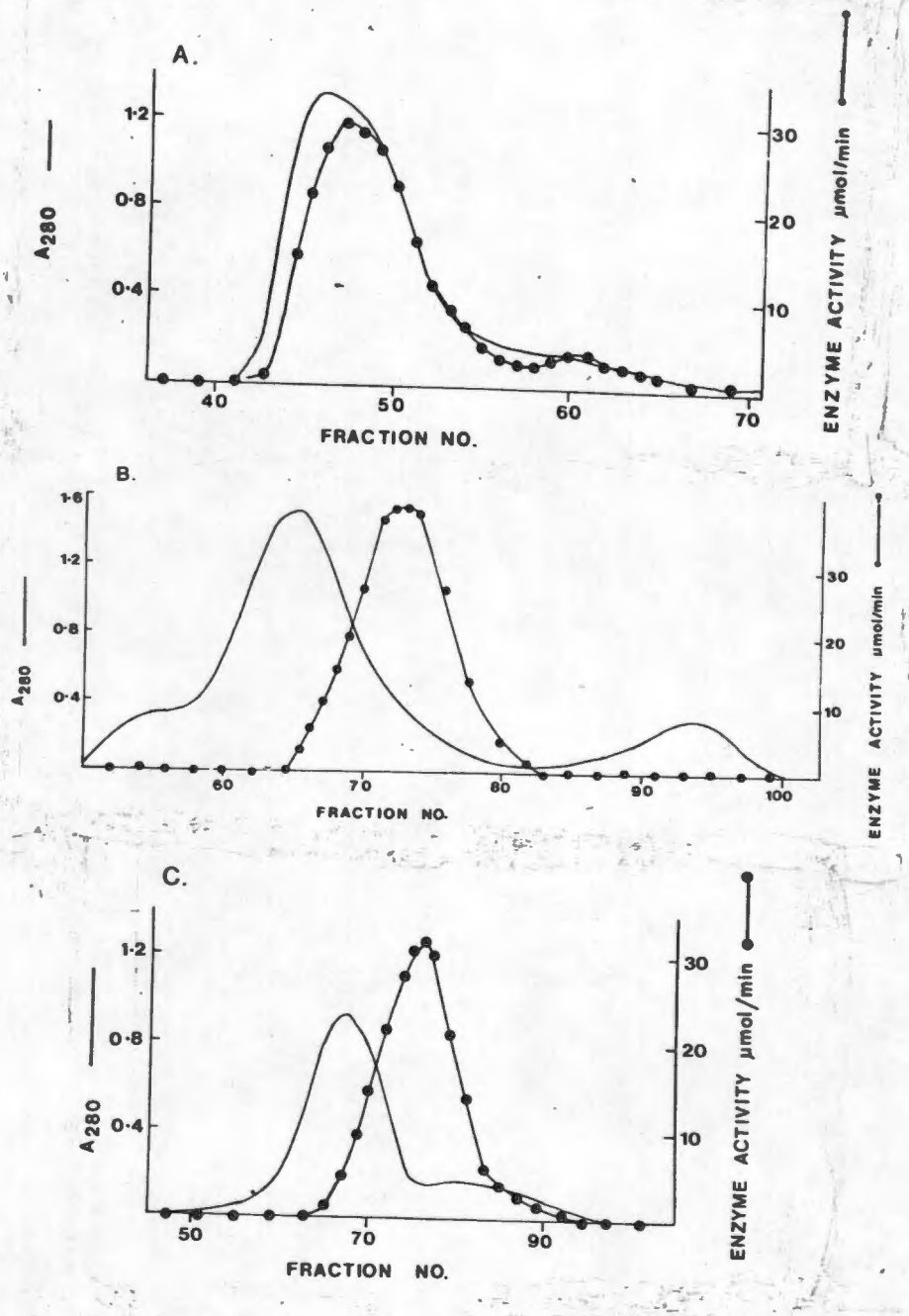


Fig. 6.1. Purification of human ligandin. (A), Elution of protein and GSH-T enzyme activity from TEAE-cellulose in 0.01M Tris HCl pH 8.67; (B), Elution of protein and enzyme activity from Sephadex G-100' in 0.01M phosphate buffer, 0,15M NaCl, pH 7.4; (C), Elution of protein and enzyme activity from G-100'' in phosphate buffer; (D), Elution of protein and enzyme activity from QAE-Sephadex A-50 in 0.01M Tris HCl pH 8.67; (E) Elution of protein and enzyme acitivity from the second QAE-Sephadex A-50 column in Tris buffer. (See Methods and Results for details).

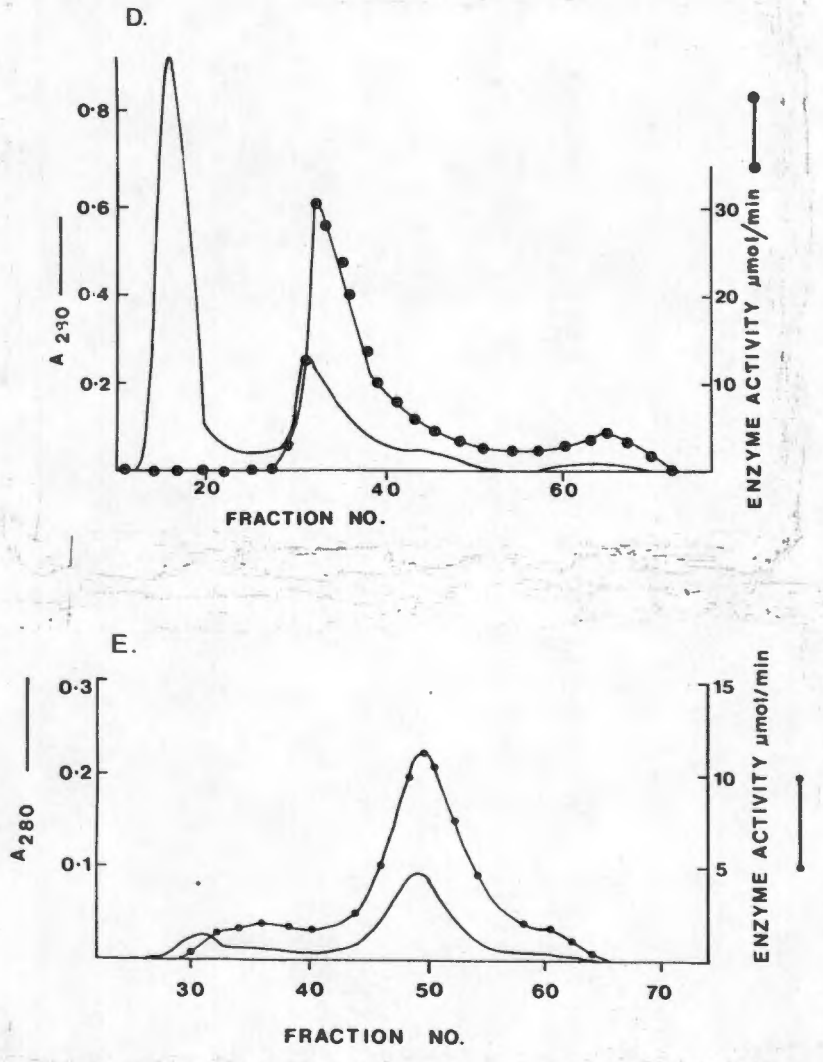


Fig. 6.1. Purification of human ligandin. (A), Elution of protein and GSH-T enzyme activity from TEAE-cellulose in 0.01M Tris HCl pH 8.67; (B), Elution of protein and enzyme activity from Sephadex G-100' in 0.01M phosphate buffer, 0,15M NaCl, pH 7.4; (C), Elution of protein and enzyme activity from G-100'' in phosphate buffer; (D), Elution of protein and enzyme activity from QAE-Sephadex A-50 in 0.01M Tris HCl pH 8.67; (E) Elution of protein and enzyme acitivity from the second QAE-Sephadex A-50 column in Tris buffer. (See Methods and Results for details).

graphy on Sephadex G-100 yielded a single symmetrical peak of GSH-T activity eluting at 1.5x the void volume. The first QAE-Sephadex step resulted in two peaks. GSH-T activity was confined to the second peak.

Further purification was achieved by one of the following procedures:-

1. Isoelectric focusing, which yielded a single peak of enzyme activity. Since this method required a further step to remove ampholytes and sucrose this method was not routinely used.

2. Repeat QAE-Sephadex chromatography, which yielded two protein peaks, both of which had enzyme activity. The second peak contained most of the GSH-T activity, and contained a protein homogenous by SDS-PAGE. This method was simpler than 1, but was associated with considerable loss of activity.

The second peak from the QAE-Sephadex column was pooled and had the following characteristics:-

1. On discontinuous SDS-PAGE the protein migrated as a single band in the 26600 dalton molecular weight region (fig. 6.2).

2. The protein eluted as a single homogenous peak with symmetrical enzyme activity when chromatographed on Sephadex G-100. The molecular size of the peak was estimated by comparison with chromatographic standards to be 47,500 daltons, with the entire peak eluting between 43,000 and 54,000 daltons (fig. 6.3).

3. Isoelectric focusing in gels yielded two adjacent catalytically active fractions focusing at pH 7.84 and 8.06 (fig. 6.4). Stained gels

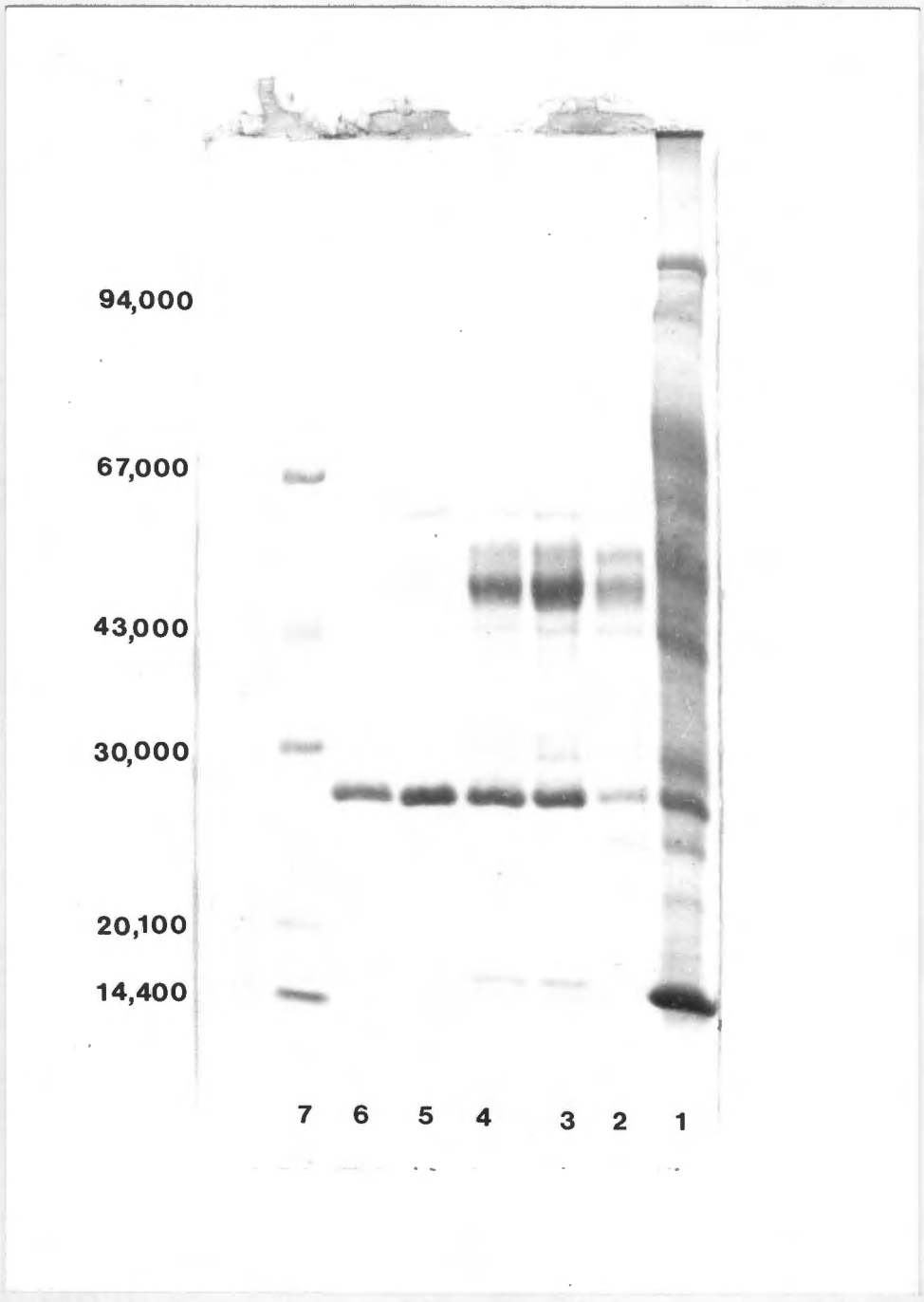


Fig. 6.2. Discontinuous slab PAGE in SDS (11% polyacrylamide) of; (1) 100,000 xg supernatant of human liver; (2) pooled protein peak from TEAE-cellulose; (3) pooled peak of enzyme activity from G-100'; (4) pooled peak of enzyme activity from G-100''; (5) pooled peak of enzyme activity from QAE'; (6) pooled peak of enzyme activity from QAE''; (7) molecular weight markers.

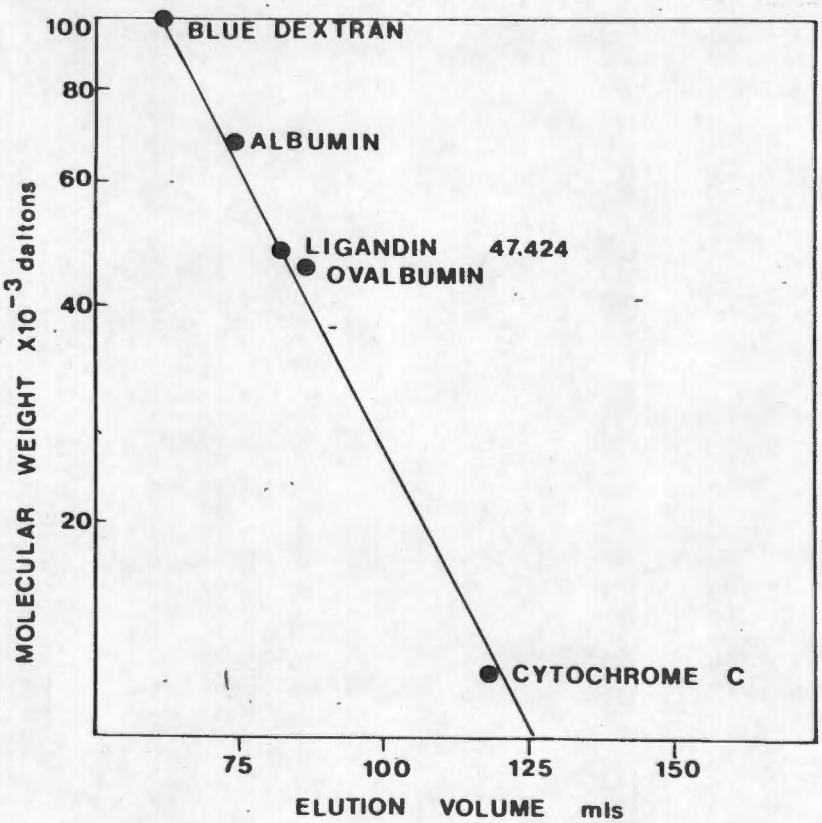


Fig. 6.3. Determination of comparative molecular weights of purified human hepatic ligandin on Sephadex G-100 chromatography. Details of the procedures used and of the molecular weights of the standard proteins are given in the text.

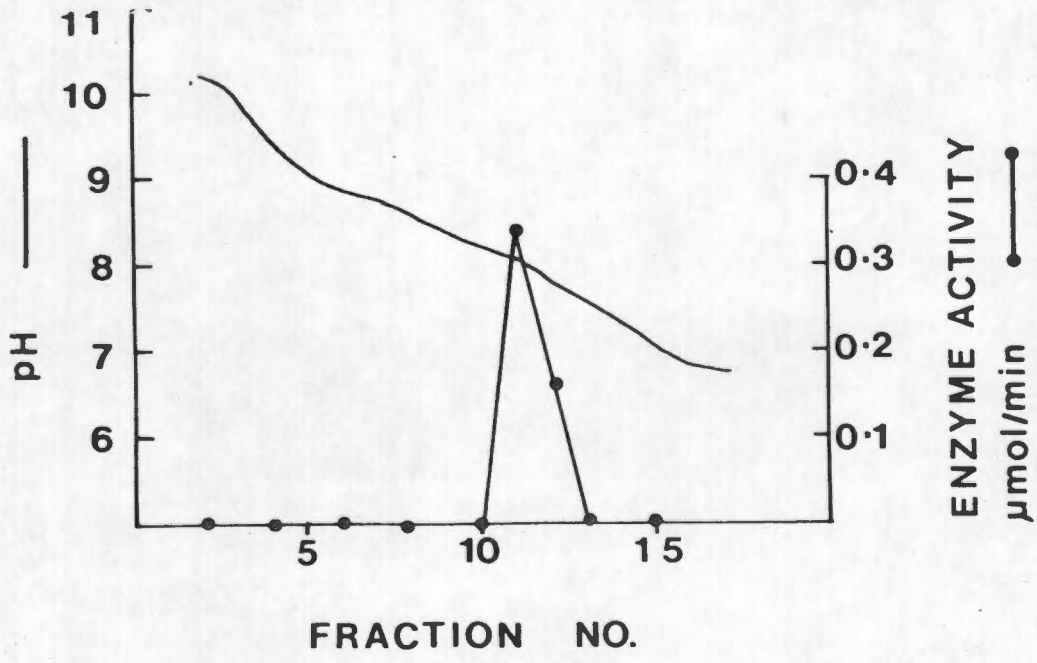


Fig. 6.4. Tube gel iscelectric focusing of purified human ligandin in the pH range 3 - 10. The gel was sliced into 2 mm sections. Enzyme activity was recoverable in two adjacent slices corresponding to pH 7.84 and 8.06.

showed two closely focused strongly staining bands corresponding to the two active fractions. In addition there were several very faint bands visible which focused at lower pH (fig. 6.5).

4. Specific activity with CDNB as substrate was 27.56 υmol/min/mg protein. Km and Vmax estimated by the Lineweaver-Burke double reciprocal plot were 0.35 and 579 υmol/min/mol enzyme respectively (fig. 6.6).

DISCUSSION

Purification:-

The purification procedure described here results in the purification of two catalytically active charge isomers of GSH-T.

The protein isolated here is pure by several accepted criteria. The protein eluted from Sephadex G-100 as a single symmetrical peak, consisted of a single band on PAGE in SDS, and could be used to raise an antiserum in rabbits which resulted in a single line on immunodiffusion and immunoelectrophoresis against soluble liver proteins (chapter V).

The 2 bands seen on IEF may represent two immunologically identical GSH-T's of identical size or may be an example of 'microheterogeneity'. Comparison of pI estimated by gel electrofocusing with those of previously published results suggests that these may correspond to GSH-T α and β. GSH-Tα has a pI of 7.8, and GSH-Tβ 8.25 (16). The faintly seen bands of lower pI may indicate inhomogeneity of the preparation, but since these extra bands are not seen on PAGE, they may represent other GSH-T's, the protein concentration of which may have been

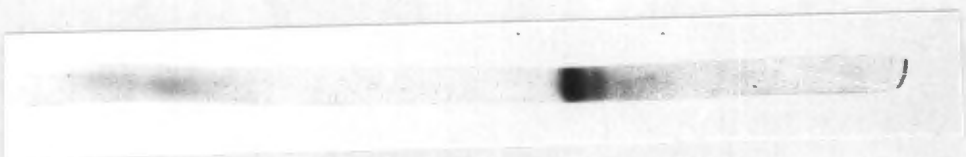


Fig. 6.5. Tube gel isoelectric focusing of purified human ligandin in the pH range 3 - 10 of purified human hepatic ligandin. The gel was stained as described in the Methods.

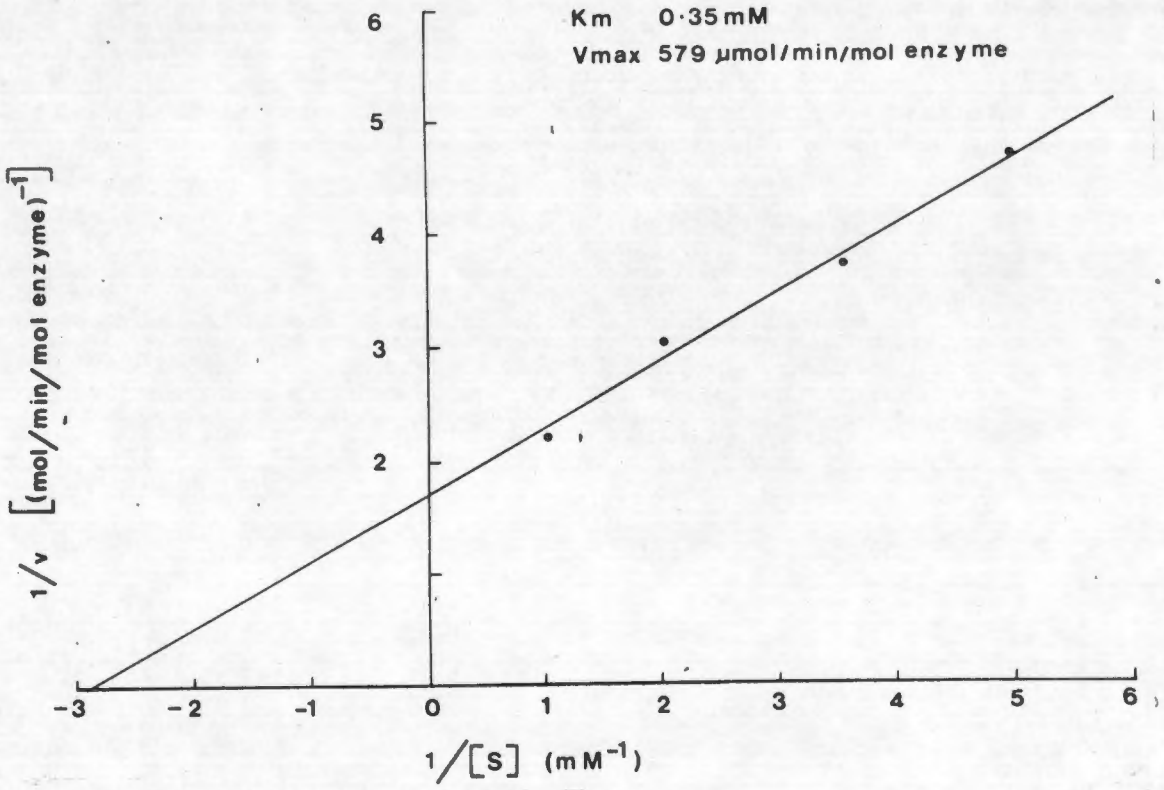


Fig. 6.6. Double reciprocal plot of reaction velocity (v) and substrate concentration [S] for 1-chloro-2,4-dinitrobenzene. Each point represents the mean of three determinations.

too low to detect cactalytic activity. The specific activity recorded here however is closer to that of GSH-Tδ, which is given as 34 υmol/min/mg protein (16). The molecular size of the dimer and the monomer are in agreement with previously published values for the cationic human GSH-T's. Unlike the rat, the human hepatic cationic GSH-T's have two identical subunits. Density gradient polyacrylamide gel electrophoresis did not succeed in separating the monomers.

The Km and Vmax found in this study are somewhat lower than those quoted by Kamisaka et al (16). This may be due to the presence of preservatives, glycerol, GSH and EDTA, which were used in that study, but which were not employed here.

CHAPTER VII

ANTISERUM PRODUCTION

This chapter describes the production of an antiserum and testing thereof for specifity.

METHODS

Inoculation:-

Three month old male albino rabbits were immunized with purified ligandin according to the following schedule:-

1. 15 ug of protein in 1 ml of 0.01M sodium phosphate buffer pH 7.4, 0.15M NaCl was emulsified with 1.5 ml of complete Freund's adjuvant by expulsion via a 21 gauge syringe needle.
2. The emulsion was injected into multiple subcutaneous sites on the back of each rabbit at 3-week intervals.
3. Animals were bled at 10-day intervals following each booster innoculation.

Processing of antisera:-

Blood obtained from immunized rabbits was allowed to clot at room temperature for 1 hour then stored at 4°C for 24 hours. Serum was separated by centrifugation at 4,000 xg for 30 minutes at 4°C (131). Aliquots of separated sera were stored at -20°C with 0.1% sodium azide added as a preservative.

Testing of antisera:-

The antibody was tested by immunodiffusion and immunoelectrophoresis in agar against purified ligandin and human liver cytosol. Immune precipitates were stained with amido black (132). Non-immune rabbit sera (NIRS) were used as antiserum controls. Normal saline, human serum and other human proteins were antigen controls.

Immunodiffusion (133):-

This was performed in 1.2% agar in 0.05M sodium phosphate buffer pH 7.4, 0.15M NaCl with 100 mg of sodium azide added. Wells of 4 mm diameter (10 ul capacity) were routinely employed. Antigen samples were placed in peripheral wells and antiserum in the central well. The plate was allowed to develop at room temperature for 24 hours and at 4°C for a further 24 to 48 hours.

Immunoelectrophoresis(134):-

This was performed in 1% Agar in 0.14M Veronal buffer pH 8.7/0.2% sodium azide. Electrophoresis was performed at 12mA/plate for 1.5-2 hours. Antiserum was then added to the troughs and the plates were allowed to develop as for immunodiffusion.

Cross Absorption:-

50 ul of human liver cytosol and 400 ul of rabbit antihuman ligandin antiserum were incubated for 48 hours at 4°C. Liver cytosol was also incubated with non-immune rabbit serum and 0.9M saline. After 48 hours the mixture was centrifuged at 18,000 xg for 30 minutes and the supernatants tested by immunodiffusion.

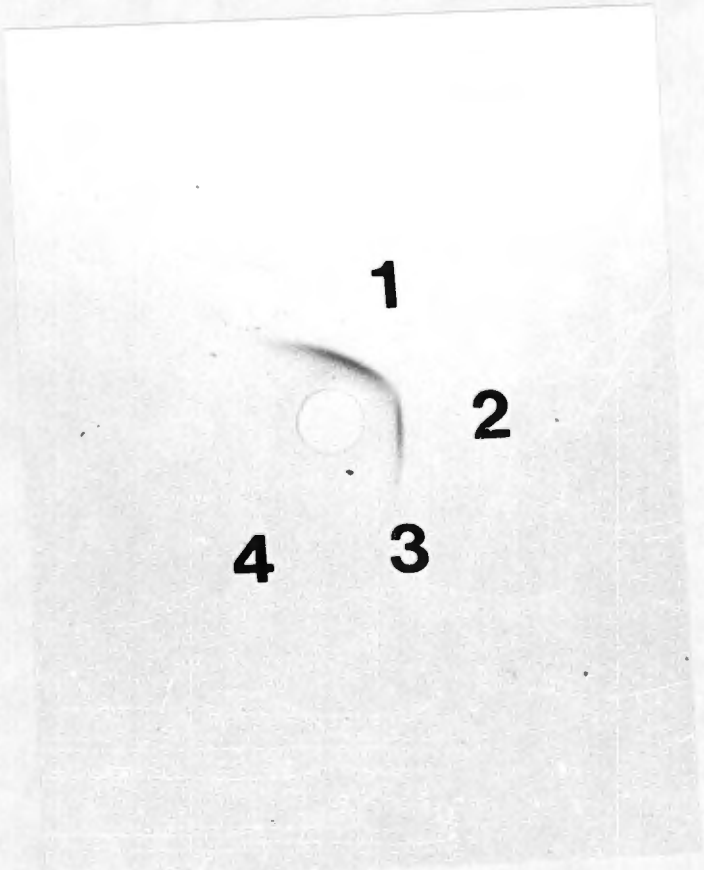


Fig. 7.1. Immunodiffusion in agar gel. The centre well contains rabbit anti-human ligandin antiserum. Peripheral wells contain; (1) human hepatic cytosol; (2) purified human ligandin; (3) human albumin; (4) horse heart myoglobin.

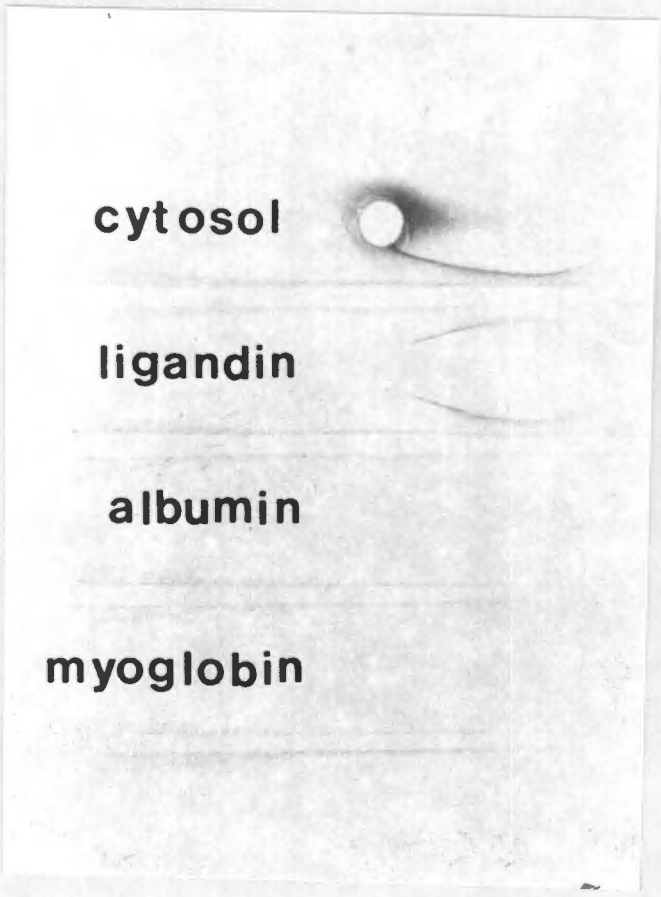


Fig. 7.2. Immunoelectrophoresis in agar gel. The troughs contain rabbit anti-human ligandin antiserum.

RESULTS

Rabbit antiserum to human ligandin produced a single precipitin line on immunodiffusion and immunoelectrophoresis against purified ligandin and hepatic cytosol (figs. 7.1, 7.2). No precipitin lines were seen with nonimmune rabbit serum in the central well or with proteins other than ligandin in the peripheral wells. Purified ligandin gave a single precipitin line against a polyvalent rabbit antiserum prepared against human hepatic cytosol proteins. Following absorption with hepatic cytosol the antiserum no longer produced precipitin lines with ligandin but formed precipitin lines with control cytosol which had been preincubated with non-immune rabbit serum or saline.

DISCUSSION

The rabbit anti-human ligandin antiserum raised in the present study was shown to be monospecific in that it gave single precipitin lines on immunodiffusion and immunoelectrophoresis with purified human hepatic ligandin and with human hepatic cytosol. Multivalant rabbit anti-human hepatic cytosol also gave a single precipitin line with purified ligandin.. Other human proteins tested against rabbit anti-human ligandin antiserum did not show any cross-reactivity on immunodiffusion or immunoelectrophoresis.

CHAPTER VIII

IODINATION

INTRODUCTION

Several methods exist for the production of labelled proteins. All have disadvantages. Iodination is usually chosen for in vitro labelling because of ease of counting, and the ease of the labelling procedure. ^{125}Iodine is usually prefered to ^{131}iodine because of the longer half life of the former. Direct iodination has the advantage of a high specific activity, however the labelling procedure may alter the immunogenicity of the antigen. Since labelling involves the introduction of an atom not usually present in the molecule, this too may alter the immunogenicity of the protein, or may alter physicochemical characteristics such as the pI. Finally labelled proteins are subject to degradation or aggregation on storage, or to loss of iodine from the molecule. All these factors have to be taken into account when selecting and testing a method of iodination. A further step is required to separate intact labelled protein from damaged products of iodination and from free iodine, both present in the reaction mixture.

Since rat ligandin had been successfully iodinated by the chloramine-T method and separation of intact labelled ligandin from damaged protein and free iodine easily achieved, a similar system was used for the iodination of human ligandin.

METHODS

All counting was performed in standard tubes in a Packard Auto-gamma Scintillation Spectrometer connected to a Teletype printer. Background radioactivity was automatically subtracted from all samples. For the RIA counts were recorded on paper tape for use on a computer.

Iodination:-

A modification of the method of Greenwood et al (135) was used to iodinate ligandin. Reagents were mixed at room temperature in a small glass test tube using constriction pipettes. Reagents were added in the following order.

Carrier free ^{125}I, 1 mCi	10 µl
0.5M sodium phosphate buffer pH 7.4 (PBS)	25 µl

The following reagents were then added with mixing:-

Ligandin, 15 µg	25 µl
Chloramine-T, 10 µg in 10 ml PBS	25 µl

The reaction was allowed to proceed for 30 seconds and was terminated by the addition of:-

Sodium metabisulphite, 50 µg in 10 ml PBS	100 µl
Potassium iodide, 2 mg in 10 ml PBS	200 µl

Purification of the reaction mixture was achieved using a combination of gel filtration and ion exchange chromatography. The entire reaction mixture mixed with 0.5 ml 0.1% BSA in 0.03M Tris/HCl buffer, pH 8.8 was applied to a 30 x 0.7 cm glass Biorad disposable column packed to a height of 4 cms with TEAE-cellulose and to an additional 25 cms above the cellulose, with Sephadex G-25 in the same buffer. The

reaction mixture was eluted by gravity with the same buffer at a flow rate of 18-20 ml/hour. Fractions of 0.4-0.6 ml were collected.

Assessment of iodination:-

The incorporation of ^{125}I into ligandin, the nature and extent of iodination damage, the efficiency of the procedures used to purify the label, and the stability of ^{125}I-labelled ligandin were assessed by the following methods:

1. Trichloroacetic acid (TCA) precipitation was performed using 5 µl of reaction mixture or 20 µl of the purification column fractions to which were added 1 ml 0.05M sodium phosphate buffer pH 7.4, 0.1% bovine serum albumin. The mixture was incubated at 4°C for 30 minutes and then centrifuged for 30 minutes at 2,000 xg. Radioactivity in the precipitate and in 0.5 ml of the supernatant was counted separately. Results were expressed as the percentage precipitable counts vs total radioactivity in the original sample.

2. Chromatelectrophoresis (136) was performed on 50 X 3.5 cm strips of Toyo 514 paper using 5 µl of human albumin as a carrier, and bromophenol blue as a marker for damaged protein products of iodination. The system was validated by digesting a 0.5 ml aliquot of ^{125}I-ligandin with 0.5 ml of trypsin (1.8 mg/ml) and applying the digest to the same chromatoelectrophoresis system. The chromatelectrophoresis was run in 0.05M veronal buffer, pH 8.6, for 1 hour at 400V. The strips were then dried and cut into 1 cm portions for determination of radioactivity. Once the characteristic migration of the "intact", "damaged"

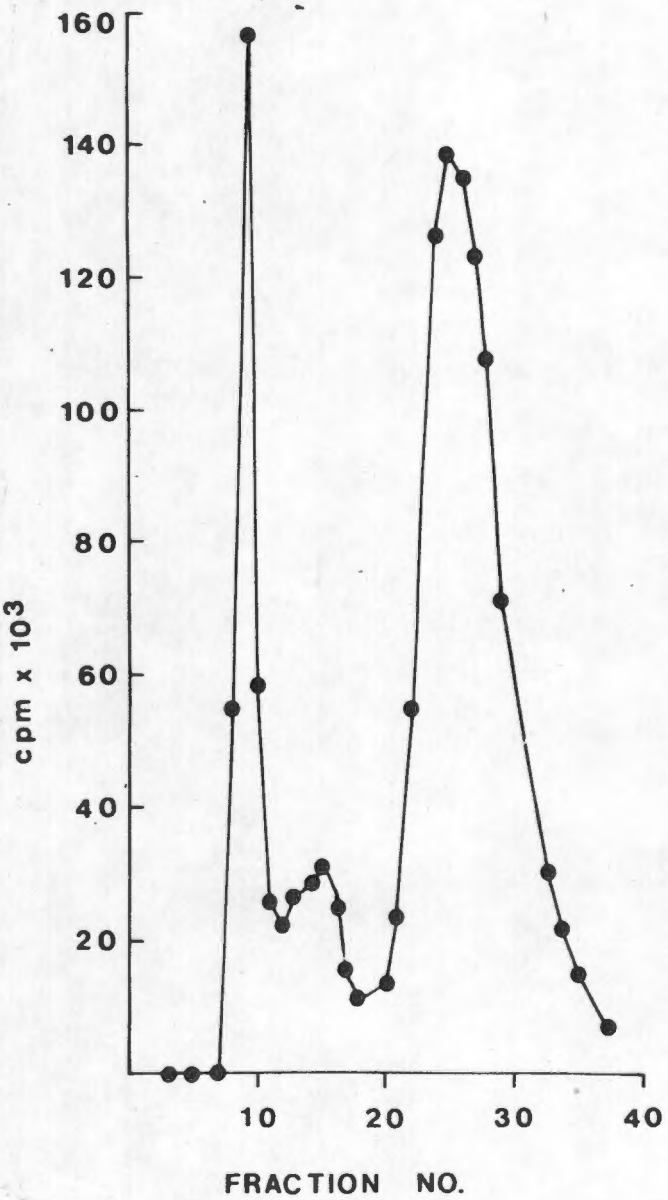


Fig. 8.1. Purification of 125I-ligandin by Sephadex G-25/TEAE-cellulose chromatography. The iodination reaction mixture was added to 0.5 ml 0.1% BSA in 0.03M Tris HCl and applied to the column, which was run in the same buffer.

and "free" fractions were established the strips were cut into 3 portions for determination of radioactivity. Results were expressed as percentage of total radioactivity per strip.

3. Gel filtration was performed using Sephadex G-100 in 0.1M sodium phosphate buffer, 0.15M NaCl on a 1.5 x 60 cm column. 10 µl of the peak fraction from peak 1 of the purification column was added to 5 ml of unlabelled ligandin (0.2 mg/ml), and applied to the column. The column was developed with the same buffer at a flow rate of 4ml/min. The eluate was monitored at 280 nm and radioactivity determined.

4. Isoelectric focusing (IEF):- 10 µl of labelled protein was added to 20 ml of unlabelled protein (0.2 mg/ml). IEF of the labelled protein was performed in tube gels under identical running conditions as before, except that ampholytes in the pH range 6.5 - 9 were used. Anolyte and catholyte were 0.01M HEPES and 0.01M ethanolamine respectively.

RESULTS

Elution of the iodination reaction mixture from the combination gel filtration/anion exchange column resulted in three peaks (fig 8.1). The iodination reaction mixture and an aliquot from each peak tube was assessed by TCA precipitation and chromatelectrophoresis.

Validation of the chromatelectrophoresis:-

An aliquot from peak 1 subjected to chromatelectrophoresis remained at the origin. Following tryptic digestion this aliquot migrated

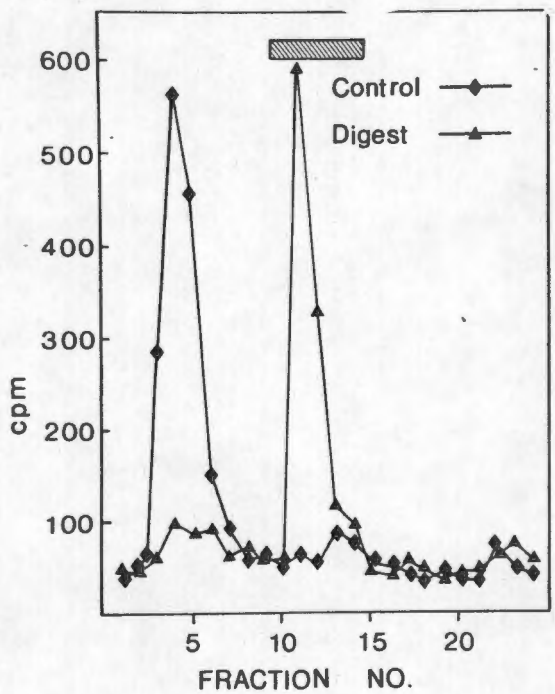


Fig. 8.2. Validation of chromatelectrophoresis. An aliquot from the peak tube from the iodination purification column remained at the origin. Another aliquot from the same tube was subjected to tryptic digestion and migrated with the blue front (shown by the hatched bar). Free ^{125}I migrated ahead of the blue front (not shown).

PURIFICATION OF LABELLED LIGANDIN

	TCA %	Chromatelectorphoresis %		
		origin	blue front	fast running
reaction mixture	52.5 ± 6.56	40.4 ± 9.37	26.4 ± 7.9	33.2 ± 8.0
Peak I	93.1 ± 1.98	94.7 ± 2.90	4.2 ± 3.2	1.1 ± 1.1
Peak II	93.2 ± 5.94	15.2 ± 12.95	73.9 ± 10.2	10.8 ± 9.8
Peak III	6.3 ± 1.83	1.8 ± 1.62	4.2 ± 3.9	94.3 ± 5.0

Table 8.1. Assessment of purification of labelled ligandin by chromatelectrophoresis and TCA precipitation. An aliquot from the peak tube was assessed. Radioactivity remaining at the origin represents intact labelled protein, radioactivity migrating with the blue front indicates damaged labelled protein, and fast migrating radioactivity represents free labelled iodine.

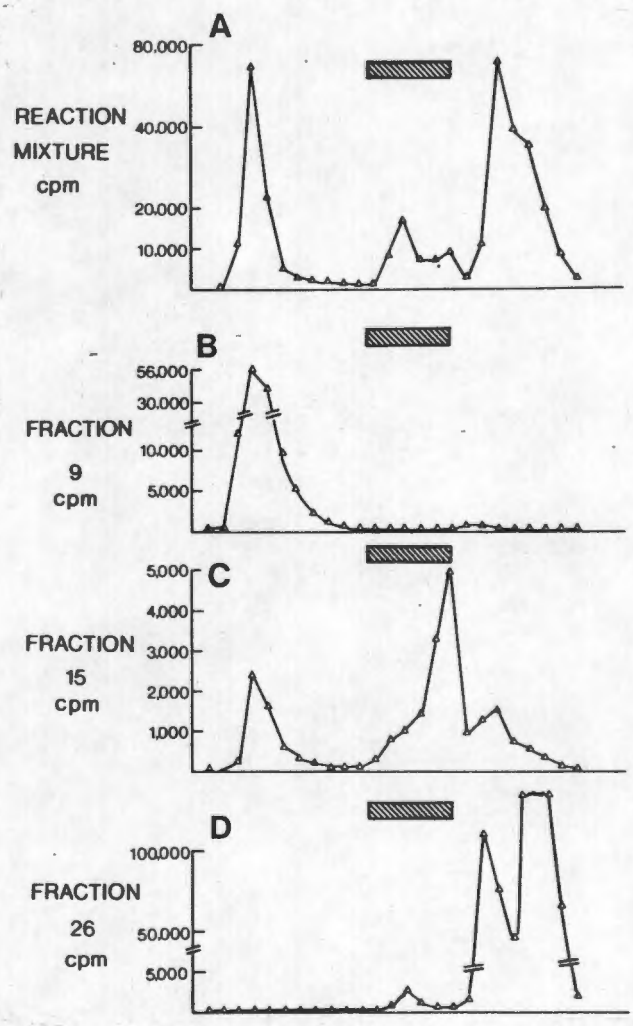


Fig. 8.3. Chromatelectrophoresis of (A), iodination reaction mixture; (B), fraction 9 from the purification column; (C), fraction 15 (D) fraction 26. The blue front is represented by the hatched bar.

with the bromophenol blue front (fig. 8.2). Free iodine migrated ahead of the blue front (data not shown).

Separation of intact labelled protein from free iodine and damaged products of iodination:-

An aliquot from peak 1 was more than 90 % TCA precipitable (table 8.1) and on chromatelectrophoresis remained largely at the origin (fig. 8.3). These criteria, plus the result of the tryptic digest suggest that this peak contains mainly intact ligandin. Peak 2 was 80 - 90% TCA precipitable and migrated with the blue front (fig. 8.3)(table 8.1), suggesting that this peak contained mainly damaged products of iodination. Peak three was barely precipitable with TCA and migrated ahead of the blue front (fig. 8.3))(table 8.1), and consisted of free iodine.

Characterisation of ^{125}I-ligandin:-

On gel filtration an aliquot from peak 1 eluted with unlabelled ligandin. On IEF the labelled protein focused at the same pI as GSH-T enzyme activity and purified ligandin, i.e. between pH 7.8 and 8.1 (fig. 8.4).

Optimization of iodination conditions:-

Optimum iodination conditions were determined using chromat-electrophoresis. Conditions were chosen so that between 30 and 50% of ^{125}I was incorporated into ligandin. The technique was highly reproducible and mean values for TCA precipitation and chromatelectro-

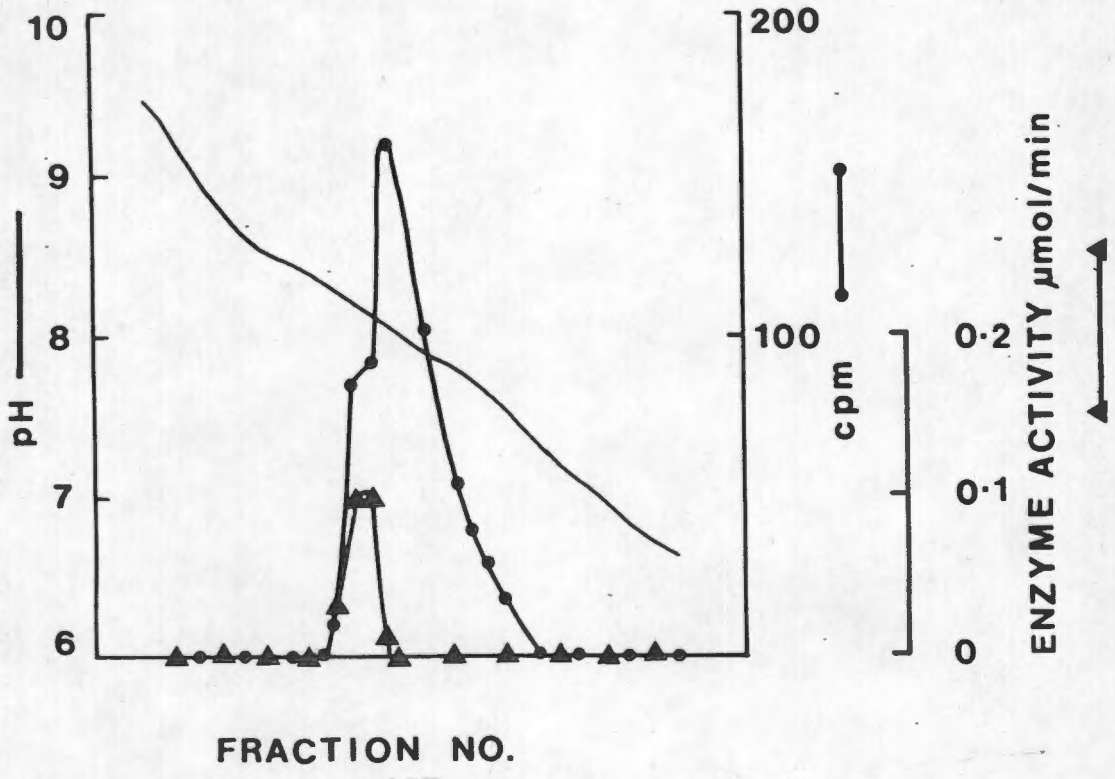


Fig. 8.4. Isoelectric focusing of ^{125}I-ligandin. 10 ul of fraction 9 and 100 ul of purified ligandin were mixed and subjected to isoelectrric focusing. Enzyme activity and radioactivity both focused at between pH 7.8 and 8.1.

phoresis for 10 consecutive iodination/purification procedures performed over several months are given in table 6.1.

The average incorporation of ^{125}I into ligandin (derived from both TCA and chromatoelectrophoretic data) was 35%. The approximate specific activity of the labelled protein was calculated from the formula -

$$\text{Specific Activity (SA)} = \frac{\text{incorporation of } ^{125}I \text{ } 1{,}000\,\upsilon Ci}{\text{mass of iodinated protein}}$$

and was 44.87 υCi/υg ligandin. The number of iodine atoms incorporated per molecule was derived from the formula -

$$\text{mmole } ^{125}I/\text{mmole ligandin} = \frac{\text{ligandin SA x ligandin mol wt}}{^{125}I \text{ SA x } ^{125}I \text{ atomic wt}}$$

Using the quoted specific activity of ^{125}I of 13 - 17 mCi/υg I, and assuming a molecular weight of 47,500 for ligandin, the incorporation was 0.99 - 1.33 mmole ^{123}I/mmole ligandin. This calculation assumes that the specific activity of labelled ligandin was not altered by the purification procedure, in other words, the procedure did not differentiate between unlabelled, monoiodinated intact, or polyiodinated intact ligandin.

Storage of labelled ligandin:-

This was assessed by chromatelectrophoresis (fig. 8.5). On storage labelled ligandin deteriorated with loss of iodine from the molecule. This is in contrast to iodinated rat ligandin, where an increase in the damaged fraction occurs with storage. The rate of loss of ligandin was sufficiently slow that specific activity remained high at 4 weeks.

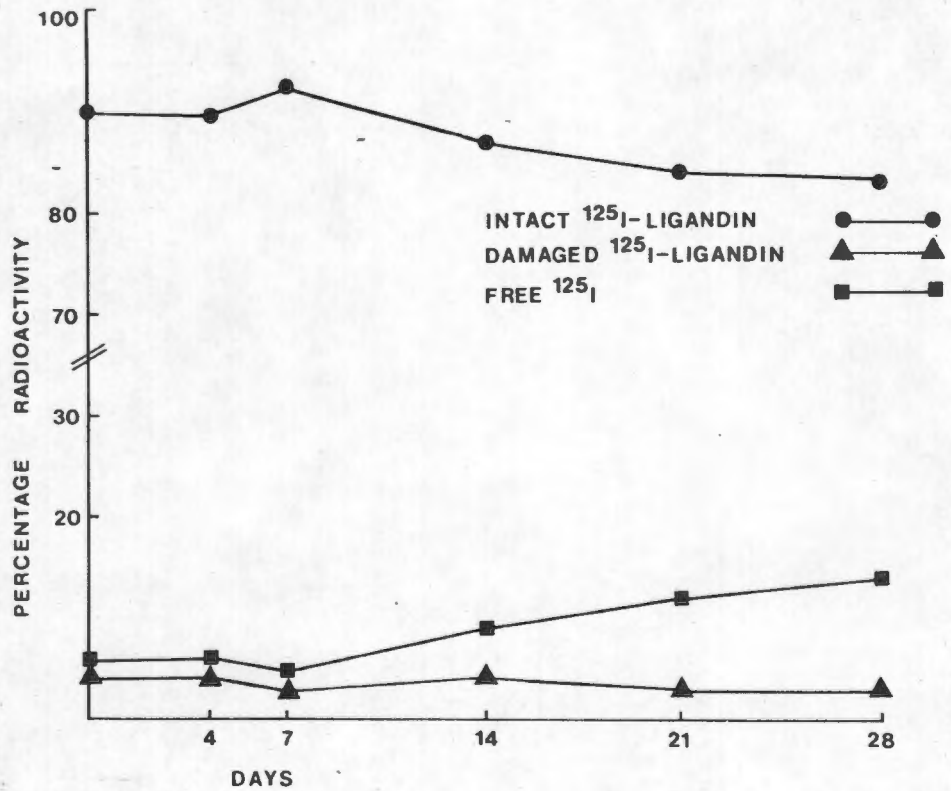


Fig. 8.5. Storage of ^{125}I-ligandin. The change in the percentage of label migrating in the chromatelectrophoresis system as intact, damaged or free freactions is plotted against time.

DISCUSSION

Using the method used by of Bass et al (18) to iodinate rat ligandin a successful iodination of human ligandin was achieved. The iodination yielded more than 90% pure labelled ligandin and was sufficient to allow dilution of up to 200 times prior to use in the RIA.

Immunoreactivity of labelled ligandin was not directly tested, but iodination did not appear to change the physicochemical characteristics greatly. Elution of labelled protein from gel filtration and from IEF tube gels was identical to elution of unlabelled ligandin. Overiodination. which is associated with a loss of immunoreactivity did not occur since the average incorporation of iodine was 1:1 iodine:ligandin.

The iodination was associated with a considerable amount of damaged products, much of which was TCA precipitable. This is born out by the fact that the second peak of the purification column migrated with the products of tryptic digestion of the labelled protein. Unlike the damaged products of iodination of rat ligandin, which were mainly fragments and small peptides, the damaged iodinated human ligandin seems to consist of mainly larger fragments which are TCA precipitable.

Labelled rat ligandin decays with an increase in the damaged fraction, whereas labelled human ligandin decays through loss of iodide. Loss of iodide per se does not result in a reduction of immunoreactivity, but causes a decrease in specific activity. There is however a general loss of immunoreactivity of most labelled stored proteins, probably reflecting simple degradation of the protein. To avoid this, labelled ligandin was discarded after two weeks.

CHAPTER IX

THE RADIOIMMUNOASSAY

INTRODUCTION

Having obtained purified ligandin, purified labelled ligandin and a monospecific antiserum to ligandin, all the necessary ingredients for the radioimmunoassay were now available. This section discusses the development of the RIA, the method of separating bound from free labelled antigen, assessment of assay performance, and of antibody-antigen affinity.

METHODS

Determination of radioactivity:-

The double antibody method of Hunter (137) was used to separate antibody-bound from free labelled antigen.

All counting was performed as previously described. A minimum of 10,000 counts per tube were accumulated. Data was captured on paper tape for transfer to a computer file.

All determinations were performed in standard plastic tubes (Falcon plastics No.2052). All dilutions of tracer, standards, unknowns, NIRS and antiserum were made in a diluent buffer comprising 0.05M sodium phosphate buffer pH 7.4, 0.15M NaCl, 0.01M EDTA, 0.5% bovine serum albumin (BSA), 0.1% sodium azide.

Tube protocol:-

The double antibody method of Hunter (137) was used to separate antibody bound from free labelled ligandin.

The components in appropriate dilutions were added to each standard and unknown sample tube in the following order:-

diluent buffer	200 ul.
standard/unknown	200 ul.
rabbit anti-ligandin antiserum (first antibody)	200 ul.
NIRS	100 ul.
^{125}I-ligandin	200 ul.

This comprised the first incubation mixture. Whenever a particular component was omitted the volume deficit was made up with diluent buffer. The tubes were incubated for 24 hours at 4°C. The second antibody in the form of donkey anti-rabbit globulin (DARG)(100 ul) was added to give a final incubation volume of 1 ml. Following a second 24 hour incubation at 4°C the tubes were centrifuged at 2,000 xg for 30 minutes. The supernatants were decanted and after drainage onto absorbent paper towelling the tubes were counted.

Assay protocol:-

Each assay included:-

4 Tubes containing only labelled ligandin (total counts).

4 Tubes containing only second antibody to estimate non-specific binding of label (NSB).

4 Tubes containing label and both antibodies, but no standard or unknown to assess maximum binding of label in the absence of unlabelled antigen (B_0).

22 Tubes containing duplicate standard concentrations of unlabelled ligandin ranging from 0.3 to 500-600 nanograms per tube. This was the standard curve.

Duplicate dilutions of a large pool of ligandin which had been aliquotted and frozen were used as internal standards. Two or three dilutions were used. Each aliquot was used once only.

A variable number of unknowns in duplicate and in dilutions were assayed so that measurements in the sensitive part of the curve could be made.

Determination of first antibody titre:-

This was determined by incubation of serial dilutions of rabbit anti-ligandin antiserum with constant amounts of ^{125}I-ligandin according to the standard assay protocol. The dilution selected for the assay was that dilution which demonstrated 50% of maximum binding of the tracer in the absence of unlabelled antigen. This dilution was called the antibody titre. Serial bleeds 10 days after each booster were tested.

Optimization of the second antibody precipitation:-

This was achieved using serial dilutions from a large pooled stock of donkey anti-rabbit globulin (DARG) which were incubated with serial dilutions of NIRS in the presence of a constant amount of ^{125}I-ligandin and a 1/1,000 initial dilution of first antibody according to the assay protocol outlined above.

Optimization of assay incubation times:-

This was achieved by keeping the first incubation time constant and varying the second incubation time between 8 and 48 hours, and keeping the second incubation time constant at 24 hours and varying the first incubation time between 2 and 48 hours.

Cross reactivity with other proteins:-

Standard dilutions (1 mg/ml) of human albumin, myoglobin, cytochrome C and rat ligandin were assayed by RIA. Results were expressed as percentage cross reactivity, and are calculated by the formula:-

$$\frac{\text{measured concentration} \times 100}{\text{actual concentration}}$$

Calculation of binding data:-

This was performed by an adaptation of the RIARUN Fortran programme devised by Rodbard and Lewald (138) which was run on a Univac series 1100 system computer. This programme fits the standard curve of the assay to a linear function by the logit-log transform using the relationship;-

$$\text{logit } (Y) = a + b \log_e(X).$$

$$Y = B/B_0, \qquad \text{(both corrected for NSB)}$$

$$\text{logit } (Y) = \log_e (Y/1-Y)$$

X is the dose of unlabelled antigen

a and b are the intercept and slope respectively of the linearised curve.

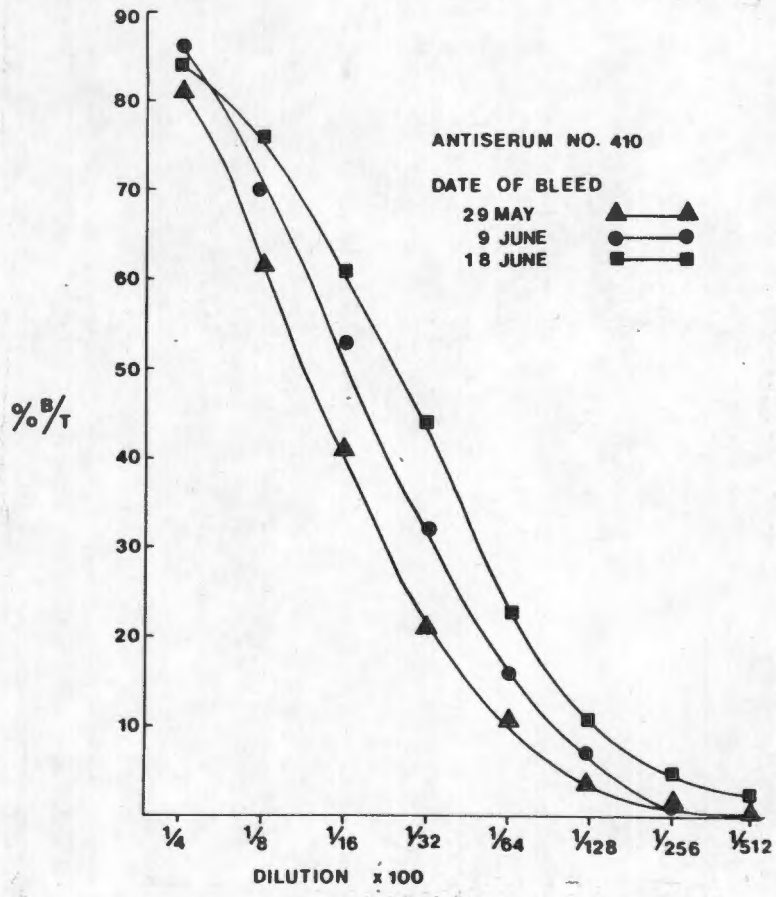


Fig. 9.1. Antiserum dilution curves of rabbit anti-human ligandin antiserum. On the ordinate is percentage ratio of antibody bound radioactivity to total radioactivity. On the abscissa is the dilution of antiserum. Serial dilutions of consecutive bleeds from rabbit 410 were incubated with fixed amounts of ^{125}I-ligandin according to standard assay protocol. With each bleed the 'titre', i.e. the dilution of antiserum which exhibits 50% of maximum binding, rises.

The affinity of the antiserum for the antibody was determined by Scatchard plot analysis (139), also provided by the RIARUN programme.

Assessment of assay efficiency:-

This was performed by statistical analysis of several criteria of assay performance, values for which were provided by the RIARUN programme. Assays with criteria falling more than two standard deviations from the mean were repeated.

RESULTS AND DISCUSSION

Determination of the first antibody titre:-

The rising titre of antibody is shown for successive bleeds from a single rabbit is shown in fig. 9.1. The antiserum chosen (no. 410/18/6) exhibited 50% of maximum binding of the tracer at an initial dilution of 1:3600 and was used in this concentration in subsequent assays.

Optimization of second antibody titre:-

Optimal binding was achieved when NIRS was used in dilutions of 1:200 to 1:400, and DARG was used in dilutions of 1:12 to 1:20 (fig. 9.2).

Optimization of incubation times (fig. 9.3):-

Twenty four hours was chosen for first and second antibody incubations. These times provided the optimum between %B/T and convenience. Although after 48 hours of second incubation %B/T was approximately 10% higher than at 24 hours, this time period was considered impractical.

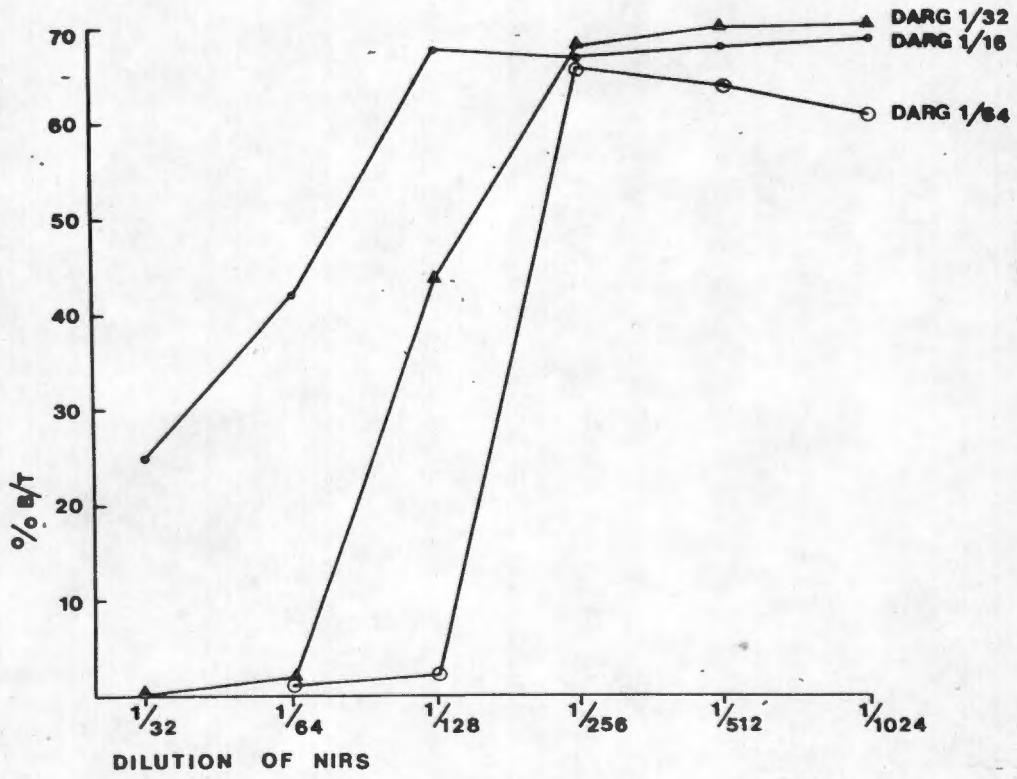


Fig. 9.2. Optimization of second antibody precipitation. Ordinate and abscissa are as for fig. 9.1. The dilutions of DARG and NIRS which gave maximum binding were determined by holding the concentration of the one constant and varying the concentration of the other using standard assay protocol. First antibody dilution was 1:1000.

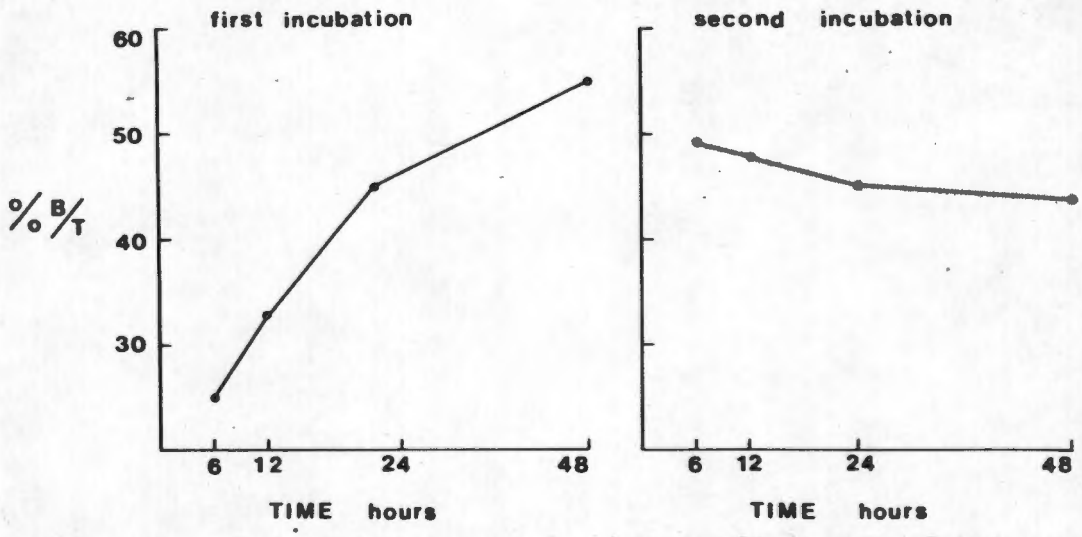


Fig. 9.3 Optimization of incubation times. Ordinate and abscissa are as for fig. 9.1. The maximum binding of ^{125}I-ligandin under standard assay conditions was assessed in the absence of standard or unknown. Each incubation period was held constant at 24 hours while the other was varied.

A typical standard curve obtained by the addition of increasing amounts of unlabelled ligandin to tubes containing constant amounts of anti-ligandin antiserum and ^{125}I-labelled ligandin is shown in fig. 9.4. The curve is sensitive in the low nanogram range.

Assessment of assay performance:-

Precision can be defined as the reproducibility of an assay and is a measure of the variation observed between repeated determinations of the same sample within and between assays. This is different from accuracy, which is the degree to which the measured value approximates the true value. Accuracy is related to the specifity of an assay and is considered later.

Precision is affected by many factors, e.g. errors in dilutions, pipetting errors and counting errors. Dilution errors were minimised by avoiding doubling dilutions for the standard curve and unknowns. Pipetting errors were allowed for in the calculation of binding data by assigning a coefficient of variation of 0.01 to pipetting volumes. This was performed automatically by the RIARUN programme. Counting errors were minimised by allowing at least 10,000 counts to accumulate minimised by allowing at least 10,000 counts to accumulate per tube. Counting errors are equal to the square root of the counts, and therefore for a count of 10,000 is 1%. Only the NSB tubes had less than 10,000 counts, but since NSB accounted for less than 2% of the counts this error was insignificant.

Precision of a competitive binding assay such as RIA also varies according to the dose level being measured. Precision is best in the mid-range of the curve, and falls off at the extremes.

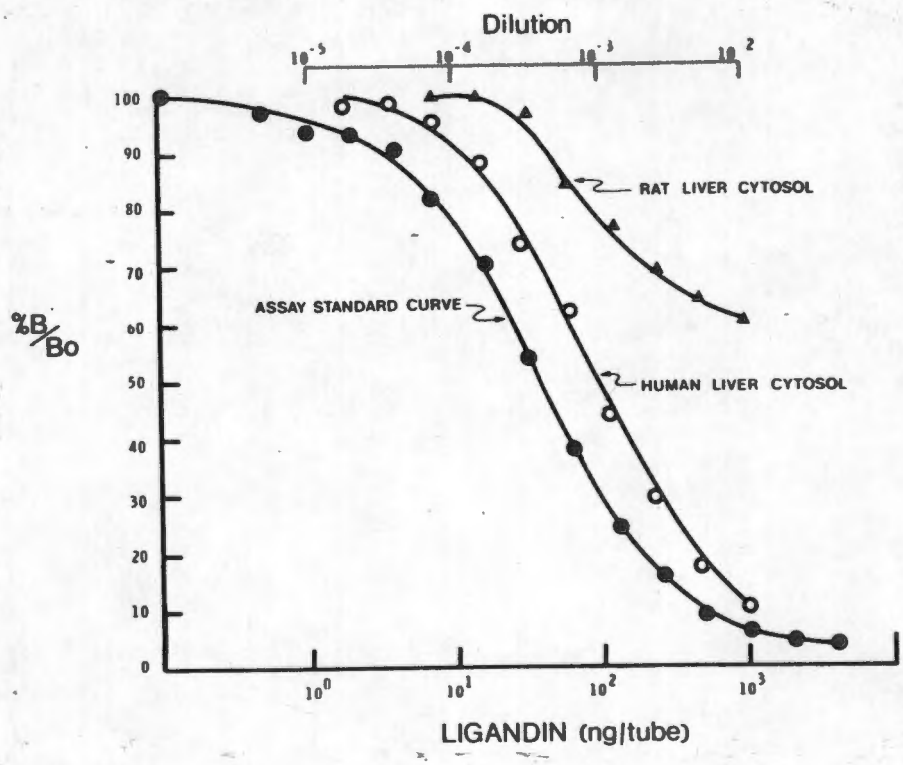


Fig. 9.4. Standard curve of the ligandin RIA. On the ordinate is the percentage ratio of antibody bound radioactivity to radioactivity bound in the absence of unlabelled ligandin. Also shown are dilution curves of human and rat hepatic cytosol. The dilution curve of rat hepatic cytosol is not parallel to the assay standard curve.

Precision was monitored by use of internal standards. Between assay variability was assessed by measuring two dilutions of a stock solution of ligandin (internal standards). Two dilutions were used to give an estimate of precision at two points on the curve. The mean (± S.E.M.) of a 1:2000 dilution of the stock solution was 10.0 (± 0.2) ng/tube (CV = 0.08), and for a 1:1000 dilution mean (± S.E.M.) was 23.7 (± 0.12) ng/tube (CV = 0.3).

Within assay variability was assessed by repeated measurement of a single sample in a single assay. Mean for 20 replicates was 1.05 (± 0.06) ng/tube (CV = 0.3).

Other indices upon which precision depends were:-

B_0	40.6 ± 0.94 % (mean ± SEM).
LD_{50} (dose at 50 % response)	17.3 ± 0.48 (mean ± SEM).
Slope of the standard curve	-1.13 ± 0.02 (mean ± SEM).

Sensitivity can be defined as the minimal detection limits of the assay, i.e. the least concentration of unlabelled ligand which can be differentiated from a sample containing no unlabelled ligand. This depends on the confidence limits of both estimates, and is best described by a statistical function, e.g. standard deviation or standard error. It is obvious then that sensitivity depends to a large extent on precision. Also important is the fact that the definition of sensitivity is most useful when it refers to the original sample rather than the amount of ligand in the assay tube. If an assay can detect 1 ng for an incubation volume of 200 µl of sample then assay sensitivity for the biological fluid from which the sample was drawn is 5 ng/ml.

Table 9.1.

CROSS REACTION OF PROTEINS WITH HUMAN LIGANDIN RIA

protein	concentration used in assay	immunoreactivity
Rat ligandin	1 ug/ml	235 ng/ml
Human albumin	1 mg/ml	12 ng/ml
Myoglobin	1 mg/ml	undetectable
Cytochrome C	1 mg/ml	undetectable

For this assay the minimal detectable concentration over 20 assays was 2.9 ± 0.3 ng/ml (mean ± S.E.M.), i.e less than 1 ng/tube.

Specifity is defined as the degree to which an assay will detect substances other than that for which the assay was designed. Lack of specificity can be considered under the headings of immunological cross reactivity, and non-specific binding.

Immunolgical cross reactivity can occur for three reasons. A homogenous antibody population may react with identical sites on proteins other than those intended, e.g. the -subunit of all the glycoprotein hormones is identical. The antiserum may contain populations of antibodies directed against similar but not identical sites on other proteins. Finally the purified protein used for inoculation may contain minor contaminants which may nevertheless be highly immunogenic. Immunological cross reactivity may be tested for by measuring possible cross reacting substances in known concentration in the assay in the absence of unlabelled antigen. Human albumin, myoglobin and cytochrome C have negligible cross reactivity in the assay (table 9.1). Rat liver cytosol exhibits partial cross reactivity at a non-identical antigenic site. This results in non-parallel dilution curves, i.e. curves of different slopes, representing antigens of different affinity for the antibody (fig. 9.3)(see below)

Non-specific binding refers to non-immunological binding of the tracer, and is caused by adsorption to surfaces e.g. tubes, especially if these are made of glass, or to non-specific binding to DARG, NIRS or carrier protein. Non-specific binding in this assay was assessed by incorporation of the NSB tubes containing tracer, but no antibody or

unlabelled ligand. Over 20 assays NSB was 1.5 $\pm$ 0.1 % (mean $\pm$ S.E.M.).

The sensitivity of a binding assay such as RIA is affected by many factors described above. However these are all secondary. The prime factor affecting a binding assay is the association constant of binding - the Ka, which is a reflection of the affinity of antibody for antigen. Binding affinity is assessed by Scatchard plot analysis (139), which is the graphic relationship of the ratio of bound ligand:free ligand vs concentration of bound ligand. The affinity constant, Ka, is given by the slope of the curve. Scatchard plot analysis of the ligandin-antiligandin reaction shows at least two populations of binding sites, represented by an exponential decay curve, which can be fitted to two linear curves, each of different slope. Antisera although monospecific are not monoclonal, and contain several populations of antibodies directed against several sites on the antigen, each with its own Ka. Under these circumstances the Scatchard plot is not linear.

Scatchard plot analysis is provided by the RIARUN programme. Calcluated values for 10 consecutive assays are Ka = 0.72 $\pm$ 0.04 x 10^{11} L/Mol (mean $\pm$ S.E.M.).

Antibody combining sites, designated as Q, can also be determined from the Scatchard plot and is given by the Y- intercept. For 10 consecutive assays Q = 0.12 $\pm$ 0.01 x 10^{-10} Mol/L (mean $\pm$ S.E.M.). It is important to note that the Scatchard plot calculated here is subject to several assumptions, not the least of which is that the mass of labelled antigen is known with accuracy. Because of these assumptions the data derived from the plot are at best only approximations.

Based on the successful RIA for rat ligandin a RIA for the cationic human GSH-T's has been developed. The double antibody technique used here gives good separation of antibody-bound from free antigen. The standard curve developed allows measurement of concentrations of less than 1ng/tube, and in addition is adequately precise. There was negligible cross reaction with other human proteins. Data concerning other possible interfering substances will be presented later. Unlike many other RIA procedures this assay is characterised by low non-specific binding, perhaps attributable in part to the presence of bovine serum albumin in the assay diluent.

The nature of the antigen-antibody reaction is such that at the extremes of the dose-response curve the standard deviation of the response is greater than in the mid-range. Therefore the dose response curve is most sensitive in its midrange. For this assay the sensitive part of the curve was roughly between 0.5 ng/tube and 100 ng/tube.

CHAPTER X

LIGANDINAEMIA IN LIVER DISEASE

INTRODUCTION

The value of diagnostic enzymology in liver disease has been discussed (see chapt. IV).

Some difficulty arises when one attempts to correlate the results of new tests with liver cell necrosis, because there is no "gold standard". There is no absolute indicator of necrosis, short of liver biopsy, which is not ethically justified in acute hepatitis, and cannot be performed done as frequently as would be required in chronic hepatitis. Even liver biopsy may yield false information because of sampling error. Therefore the results of new tests can only be compared, in a prospective study, to existing tests, or to clinical parameters, and the question asked, 'is this test of use in predicting; fulminant hepatitis; chronic hepatitis; or recovery from acute hepatitis; at an earlier stage than those currently available.

There are several reasons for investigating ligandin in the blood as a test for hepatic necrosis. In experimental animals the measurement of ligandin by RIA has been shown to be more sensitive than SGOT in detecting necrosis (84). Secondly, since ligandin is a smaller molecule than either SGPT or SGOT, and since it is largely cytoplasmic in location, its rate of release might expected to be more rapid than SGOT or SGPT. Finally since ligandin is present in such large amounts in liver the magnitude of the rise in serum may be very large. All of these factors would tend to increase sensitivity. Concomitant with the increase in sensitivity there might be a loss of specifity as release

might be caused by a variety of minor stimuli. The advantages of RIA over measurement of enzyme activity have been discussed, but in this instance the particular advantage is that RIA is not affected by haemolysis, which interferes markedly with determination of SGOT. In addition the RIA may be performed on stored serum, which is not possible for SGOT.

This study therefore was undertaken to examine serum ligandin in human liver disease and to determine if measurement of serum ligandin was an index of necrosis or had diagnostic or prognostic value.

METHODS

Validation of measurement of ligandin in serum:-

1. Recovery from serum:- Unlabelled ligandin in concentrations of 10 and 100 ng/ml were added to serum from normal volunteers and from patients with acute viral hepatitis, with and without hepatic encephalopathy. Serum was assayed for ligandin by RIA before and after addition of purified ligandin. Results were expressed as percentage recovery of ligandin.

2. Interference by drugs:- Penicillin, digoxin, gentamycin and furosemide in concentrations of 1, 5, and 10 ug/ml were added to unlabelled ligandin (35 and 24 ng/ml). The samples were assayed for ligandin by RIA and results expressed as percentage recovery.

3. Molecular size of immunoreactive ligandin in serum:- Three ml of serum from normal volunteers and from patients with acute hepatitis was chromatographed on Sephadex G-100, column size 1.5 x 90 cm developed in 0.01M sodium phosphate buffer, 0.15M sodium chloride, pH 7.4, by pump-driven upward flow at 30 ml/min. Two ml fractions were collected. The

fractions were assayed for ligandin by RIA. Molecular weight markers were used as before to calibrate the column.

4. Immunoreactivity:- This was assessed by serial dilution of serum from normal volunteers and patients with acute hepatitis. The diluted samples were assayed for ligandin by RIA, and the slope of the dilution curves compared to the slope of the standard curve using the Rodbard programme (91).

5. Storage of serum:- Aliquots of serum from patients with acute hepatitis were allowed to stand at room temperature, at 4°C, and at -20°C for 6 hours, 12 hours, 24 hours, and 2 weeks. The samples were assayed before and after storage. Results were expressed as % recovery.

6. Normal concentration in serum:- Serum from 34 normal volunteers was assayed for ligandin by RIA. Results were expressed as ng/ml.

Virology:-

Hepatitis A virus infection was detected by measuring anti HAV IgM antibodies (HAVAB, Abbott). Hepatitis B virus infection was determined by measuring HBsAg (AusRIA II, Abbott). Some patients contracted hepatitis during an outbreak of hepatitis A in a military camp. Anti HAV IgM was not determined in all of these patients but for the purposes of analysis they were regarded as having had hepatitis A.

Glutamic oxaloacetic transaminase:-

This was assayed using a Centrifuchem rotary spectrophotometer. The reagents were α-ketoglutarate, aspartate, reduced NADH, malate dehydrogenase and lactic dehydrogenase. The reaction was allowed to proceed for 3 minutes during which time lactic dehydrogenase converted en-

dogenous pyruvate to lactate. Any change in absorbance after this time was assumed to be due to NADH oxidation associated with the SGOT catalysed conversion of oxaloacetate to malate. Results were expressed as U/L, and the normal range was less than 12 U/L.

Patient Selection:-

1. Acute Viral Hepatitis:- All patients who were referred to Groote Schuur Hospital Liver Clinic over a two year period with a diagnosis of hepatitis were included. In addition sera from a further 13 patients attending the Infectious Diseases Section of Harari Hospital in Salisbury, Zimbabwe were examined.

Diagnosis was made on clinical grounds, and confirmed by finding elevated transaminase activity in the serum. Patients who had symptoms suggestive of acute hepatitis, but who did not have elevated transaminases or serum bilirubin served as symptomatic controls.

Serial blood samples were taken during the illness and during convalescence. These were analysed for SGOT and for ligandin by radioimmunoassay. Correlation between ligandin and SGOT was established by the Spearman rank correlation coefficient (140).

2. Chronic active hepatitis:- Patients with suspected chronic active hepatitis were subjected to needle biopsy of the liver for diagnostic purposes. Patients with established cirrhosis were excluded from the study. At the time of biopsy and before therapy was started, blood was taken for SGOT and ligandin estimation. Serial ligandin and SGOT estimations were performed during the course of the disease. Some patients underwent second or third biopsies when SGOT and ligandin estimations were again performed in the immediate pre-biopsy period.

RECOVERY OF LIGANDIN FROM SERUM

	added ligandin	mean % recovery	range	number
normal serum	27 ng	95%	87-110%	4
jaundiced serum	27 ng	96%	81-104%	6

Table 10.1. Recovery of ligandin from serum of normals and patients with jaundice due to acute hepatitis. Serum was assayed by RIA before and after the addition of a fixed amount of ligandin.

Biopsy appearances were graded by two observers, without knowledge of the patients clinical or biochemical status. The severity of necrosis and the degree of inflammation were scored separately on a scale from 0 - 3. The separate scores were then added together. A score of 0 indicated no disease, a score of 1 - 2 indicated mild disease, 3 - 4 indicated moderate disease, and 5 - 6 indicated severe disease. This system was adopted in an attempt to separate out the effect that necrosis per se might have on serum ligandin. Correlation between histological severity and serum ligandin and SGOT was assessed with the Spearman rank correlation coefficient.

3. Other Diseases:- Patients referred routinely to the Groote Schuur Liver Clinic and who required a liver biopsy, had blood withdrawn at the time of biopsy for ligandin and SGOT estimation. Patients with non-hepatic diseases, with no evidence of coincidental liver disease were also studied, and served as non-hepatic controls.

RESULTS

Validation of measurement of serum ligandin:-

1. Recovery of exogenously added ligandin:- These results are shown in table 10.1.' More than 95% of added ligandin was recoverable from serum of normal volunteers, and patients with acute hepatitis.

2. Interference by drugs:- Recovery of ligandin was not influenced by addition of four commonly used drugs in pharmacological concentrations. Results are shown in table 10.2.

Drug	Concentration	Recovery mean ± S.E.M.	% Recovery
Penicillin	1 ug/ml	4.83 ± 0.25	98
	5 ug/ml	4.63 ± 0.12	94
	10 ug/ml	4.50 ± 0.10	92
Gentamycin	1 ug/ml	4.90 ± 0.20	100
	5 ug/ml	4.83 ± 0.12	98
	10 ug/ml	4.87 ± 0.15	100
Digoxin	1 ug/ml	4.77 ± 0.12	98
	5 ug/ml	4.90 ± 0.10	100
	10 ug/ml	4.77 ± 0.23	98
Furosemide	1 ug/ml	4.73 ± 0.15	96
	5 ug/ml	4.70 ± 0.10	96
	10 ug/ml	4.83 ± 0.06	98

Table 10.2. Recovery of ligandin from serum after the addition of four commonly used drugs. Ligandin recovery was complete in all instances.

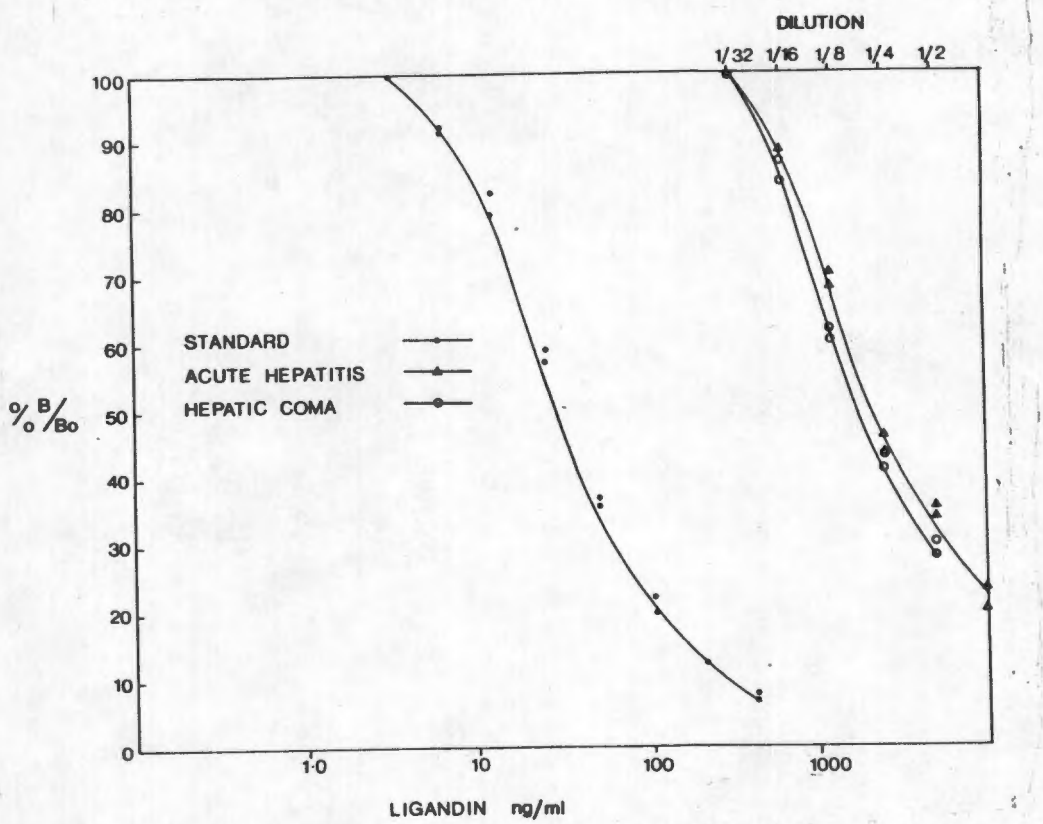


Fig. 10.1. Comparison of immunochemical discplacement curves of ligandin in the serum of patients and the standard curve of the ligandin RIA. On the ordinate is the percentage ratio of antibody bound radioactivity to radioactivity bound in the absence of unlabelled ligandin. On the abscissa (log scale) is the amount of unlabelled ligandin or dilution of sample.

3. Molecular size of immunoreactive ligandin:- Following gel filtration of normal serum, or serum from patients with acute hepatitis no immunoreactivity was detectable in the eluant fractions. The concentration of ligandin in normal serum is too low to permit detection after the dilution which accompanies gel filtration, but even high concentrations were not detected.

4. Immunoreactivity:- Ligandin concentration in normal serum was too low to permit serial dilution. Dilution curves, constructed by assaying serial dilutions of serum from patients with acute hepatitis with, and without hepatic encephalopathy, were parallel to the assay standard curve (fig. 10.1).

5. Serum ligandin in normal volunteers:- This was 3.78 $\pm$ 3.57 ng/ml (mean $\pm$ S.D.)(range 0 - 12 ng/ml). Since 12 ng/ml was the highest recorded value in normal volunteers, this was taken as the upper limit of normal, being slightly more than 2 standard deviations from the mean.

6. Storage of serum:- Serum could be stored at room temperature for 12 hours without loss of activity. At twenty four hours activity was reduced by 30 - 50%. At -20°C serum could be stored for at least two weeks with out loss of activity.

Acute Viral Hepatitis:-

Eighty patients were included in this study. Sixty eight patients fulfilled the criteria for the diagnosis of acute viral hep-

UNIVARIATE STATISTICS

	Statistic	Group 1	Group 2
Ligandin	Mean	65.1	36.7
	S.D.	235.0	59.7
	Skewness	7.4	2.5
	Kurtosis	64.4	5.9
	No of obs.	158	101
SGOT	Mean	134.0	219.1
	S.D.	271.0	320.0
	Skewness	3.9	1.8
	Kurtosis	18.1	2.4
	No of obs	135	87

Table 10.3. Univariate statistics for ligandin concentration and SGOT activity in acute hepatitis. All values of ligandin and SGOT during the illness were used to obtain these statistics.

atitis. All except 3 of these recovered clinically within 6 weeks. These 3 had a prolonged course and are considered separately. One patient had a relapse or a second infection two months after the initial episode of hepatitis. Virological studies were not available for the second illness.

There were 24 episodes of hepatitis A and 20 episodes of hepatitis B. In the remainder hepatitis B was excluded although a definitive diagnosis was not made because the appropriate serological tests were not available. Most of these patients were suspected of having hepatitis A because they contracted the disease during an epidemic of hepatitis A. Univariate statistics (table 10.3) suggested that the hepatitis B group was different to the rest of the patients and so this group was analysed separately. The non-B group was called group I, and the HBsAg +ve group was called group II.

Frequency of elevated values of ligandin and SGOT over the duration of the study in each group was assessed by establishing a ratio of the number of elevated to normal ligandin values, and a similar ratio for SGOT for each patient. A sign (+ or -) was assigned to the difference between the ligandin and SGOT ratio in each patient, and the sign test was applied to these results. This showed that in group I SGOT was elevated more frequently than ligandin ($p < 0.01$) during the period of study, but in group II there was no difference between the number of days that ligandin was elevated, and the number of days that SGOT was elevated. These results were confirmed by the Wilcoxon matched pairs sign test.

A further 12 patients were referred for suspected hepatitis but serum transaminase activity did not rise and the diagnosis was exclud-

	Group 1		Group 2	
	Ligandin ng/ml	SGOT U/L	Ligandin ng/ml	SGOT U/L
WEEK 1 mean	339	384	103	524
range	0–2398	13–1880	0–300	114–1103
WEEK 2 mean	53	159	42	391
range	0–352	17–810	0–160	6–1332
WEEK 3 mean	17	84	43	393
range	0–99	4–974	0–166	12–1044
WEEK 4 mean	6	36	27	100
range	0–17	5–171	4–108	8–360
WEEK 5 mean	5	21	23	47
range	0–12	1–50	0–97	2–126
WEEK 6 mean	8	13	11	60
range	0–17	1–25	0–27	1–94
WEEK 7 mean	4	16	10	25
range	0–7	1–22	0–22	12–42
WEEK 8 mean	—	12	6	14
range	—	—	1–11	6–30
WEEK 9 mean	—	—	6	—
range	—	—	0–17	—
WEEK 10 mean	—	—	7	—
range	—	—	0–16	—

Table 10.4. Acute viral hepatitis. This table shows the mean ligandin concentration measured by RIA, and mean SGOT activity during each week of illness. The range of values for ligandin and SGOT is also given.

	Group 1				Group 2			
	Ligandin %		SGOT %		Ligandin %		SGOT %	
WEEK 1	80	(n=20)	100	(n=10)	75	(n=16)	100	(n=15)
WEEK 2	47	(n=47)	100	(n=39)	73	(n=11)	80	(n=10)
WEEK 3	35	(n=46)	85	(n=40)	62	(n=13)	92	(n=13)
WEEK 4	18	(n=22)	90	(n=20)	83	(n=12)	90	(n=10)
WEEK 5	0	(n= 8)	62	(n= 8)	40	(n=15)	60	(n=15)
WEEK 6	—	—	—	—	50	(n= 8)	50	(n= 4)
WEEK 7	—	—	—	—	38	(n= 8)	80	(n= 5)
WEEK 8	—	—	—	—	0	(n= 5)	20	(n= 5)

Table 10.5. Percentage of all values of ligandin and SGOT which are elevated during each week of illness. The number of estimations of ligandin and SGOT during each week is given in parenthesis.

ACUTE VIRAL HEPATITIS MEDIAN LIGANDIN AND SGOT

	Group 1		Group 2	
	Ligandin ng/ml	SGOT U/L	Ligandin ng/ml	SGOT U/L
WEEK 1	123	96	38	440
WEEK 2	11	75	17	338
WEEK 3	5	34	19	120
WEEK 4	5	21	19	70
WEEK 5	6	20	9	24
WEEK 6	3	—	13	—

Table 10.6. Acute viral hepatitis. This table shows the median ligandin concentration measured by RIA, and median SGOT activity during each week of illness.

ed.

The correlation between ligandin and SGOT was assessed in each group. In group I r_s (Spearman rank correlation coefficient) = 0.62 ($p < 0.01$). In group II $r_s = 0.51$ ($p < 0.01$). If only values of ligandin above 96 ng/ml, i.e more than 8x normal, are considered then in group I for ligandin and SGOT $r_s = 0.86$, ($p < 0.01$), but in group II there is no correlation between ligandin and SGOT, $r_s = 0.04$, ($p > 0.10$).

Thirteen patients in group I had sufficient data to correlate SGOT and ligandin. In 3 of the thirteen ligandin was positively and significantly correlated with SGOT ($r_s = 0.89$ to $r_s = 0.96$, $p < 0.05$). In the other 10 patients $r_s = 0.20$ to $r_s = 0.90$, ($p > 0.05$).

In group II in 9 patients there was sufficient data for ligandin and SGOT to be correlated. In only one patient was ligandin significantly correlated with SGOT ($r_s = 0.75$, $p < 0.05$). In the other 8 patients $r_s = -0.60$ to $r_s = 0.85$ ($p > 0.05$). Both in group I and group II small numbers of paired measurments in some patients prevented significance from being reached, rather than lack of correlation, since with the Spearman rank correlation if n=5 then r_s must equal 1.0 for significance to be achieved.

The mean, range and percentage of elevated values per week are shown in tables 10.4 and 10.5. Since neither ligandin nor SGOT were normally distributed the medians given in table 10.6.

In group I in the first week of illness 80% of all ligandin values were elevated (mean 339 ng/ml, range 0 - 2398 ng/ml). All SGOT values in the first week were elevated (mean 384 U/L, range 13 - 1880 U/L).

In the first week of illness, in the first sample taken, only one patient did not have an elevated ligandin concentration.

In week 2 100% of SGOT estimations, but only 47% of ligandin estimations were elevated (SGOT mean 159 U/L, range 17 - 810 U/L), ligandin mean 53 ng/ml, range 0 - 352 ng/ml). In week 3 SGOT was raised in 85% (mean 84 U/L, range 4 - 974 U/L). Ligandin was raised in 35% of samples (mean 17 ng/ml, range 0 - 99 ng/ml). By week 4 mean ligandin was within the normal limits, although 18% of values were still elevated. Mean SGOT in week 4 was 36 U/L (range 5 - 171 U/L), and 90% of values were still elevated. After week 4 the numbers of estimations within each time period are small, but mean ligandin remained within normal limits, while mean SGOT only fell to normal in week 6.

In group II during the first week of illness 75% of ligandin values, and 100% of SGOT values were elevated. All patients had at least one elevated ligandin level. Mean ligandin was 103 ng/ml (range 0 - 300 ng/ml), and mean SGOT was 524 U/L (range 114 - 1103 U/L). In week 2 mean ligandin was 42 ng/ml (range 0 - 160 ng/ml), Seventy three percent of values were elevated. Mean SGOT in week 2 was 391 U/L (range 6 - 1332 U/L). Eighty percent of values were elevated. Mean ligandin fell to normal by week 6, but mean SGOT was not within normal limits by week 8. In this group elevated values of ligandin and SGOT continued to be detected throughout the study period.

Graphs from six patients in group I are given in fig. 10.2. In this group in each patient ligandin concentration returned to normal before or with SGOT activity. Graphs from 4 patients in group II are shown in fig. 10.3. In Group II in three patients ligandin elevation was more prolonged than SGOT.

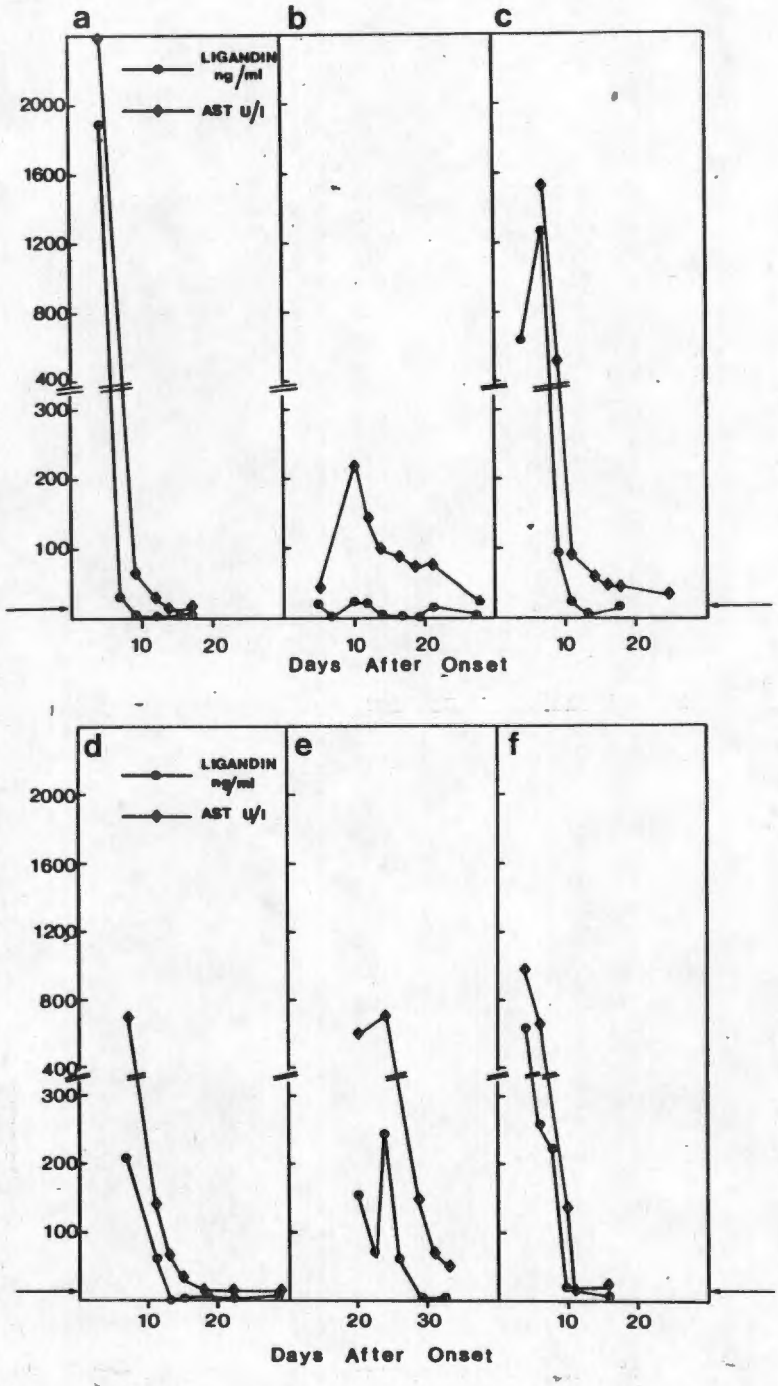


Fig. 10.2. Comparison of the disappearance curves of ligandin and AST (SGOT) in the serum of 6 patients with acute non-B hepatitis (group I). On the ordinate is the ligandin concentration and SGOT (AST) activity, and on the abcsissa is time in days after the onset of symptoms.

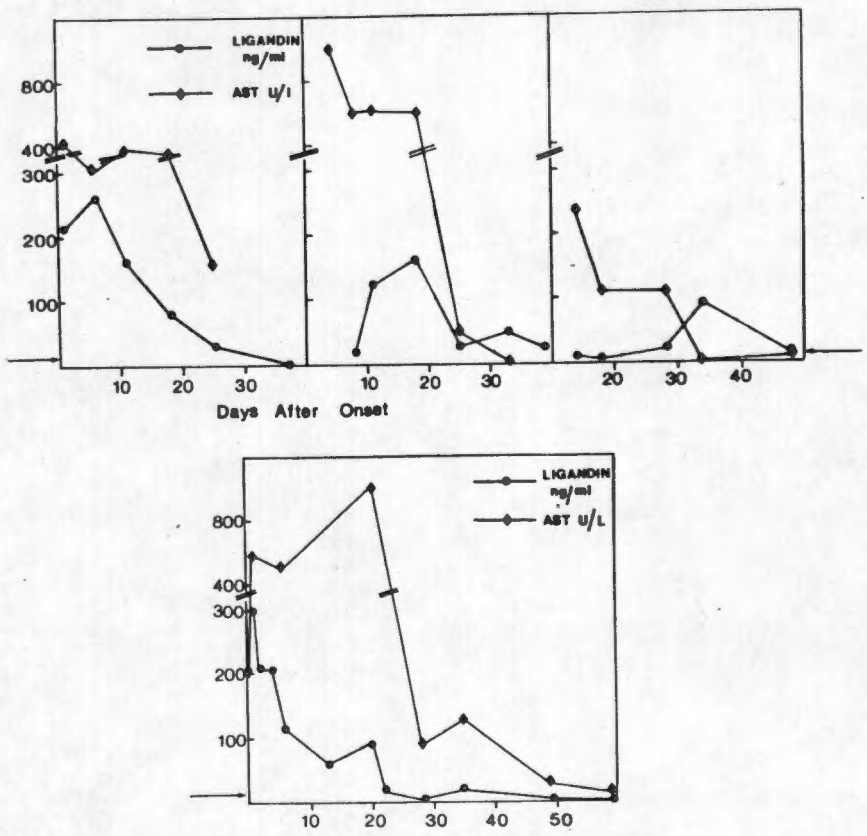


Fig. 10.3. Comparison of the disappearance curves of ligandin and SGOT (AST) in the serum of 4 patients with acute hepatitis B (group II). Ordinate and abscissa are as for fig. 10.3.

In two patients a very high ligandin, greater than 1000 ng/m was associated with a return to normal levels within 10 days (fig. 10.2a, 10.2c). Within two days ligandin concentration fell from greater than 1000 ng/ml to less than 100 ng/ml. The excretion curves in these patients suggest a biphasic clearance of ligandin, with a initial rapid and slower second phase of clearance. The curves could be fitted to the function $y = ax^b$, with $r = 0.76$ and $r = 0.98$ respectively. Corresponding SGOT clearance curves could be fitted to the same function with $r = 0.97$ and $r = 0.93$ respectively.

The serum half-life of ligandin and SGOT was estimated from these two curves (assuming that no further release of ligandin occurred between measurements). The half-life of ligandin for the initial rapid clearance phase was 8 hours, and 16 to 24 hours for the slower clearance phase. Calculated half-life of SGOT was 16 hours for the rapid clearance phase, and 24 to 48 hours for the slow clearance phase.

Frequency distributions of ligandin and SGOT with time were constructed using the week of illness as the unit of time because paucity of numbers did not permit analysis using days of illness as the time unit. Many patients had more than one estimation of ligandin and SGOT during a single week. The values at the beginning and the end of the week may have differed by several orders of magnitude. All estimations from all patients within the time unit are included in the frequency distributions, which were then constructed as if all values were independent. This method serves for descriptive purposes, i.e. gives information about the pattern of ligandin and SGOT values during the illness, but because the samples are not independently drawn, the fre-

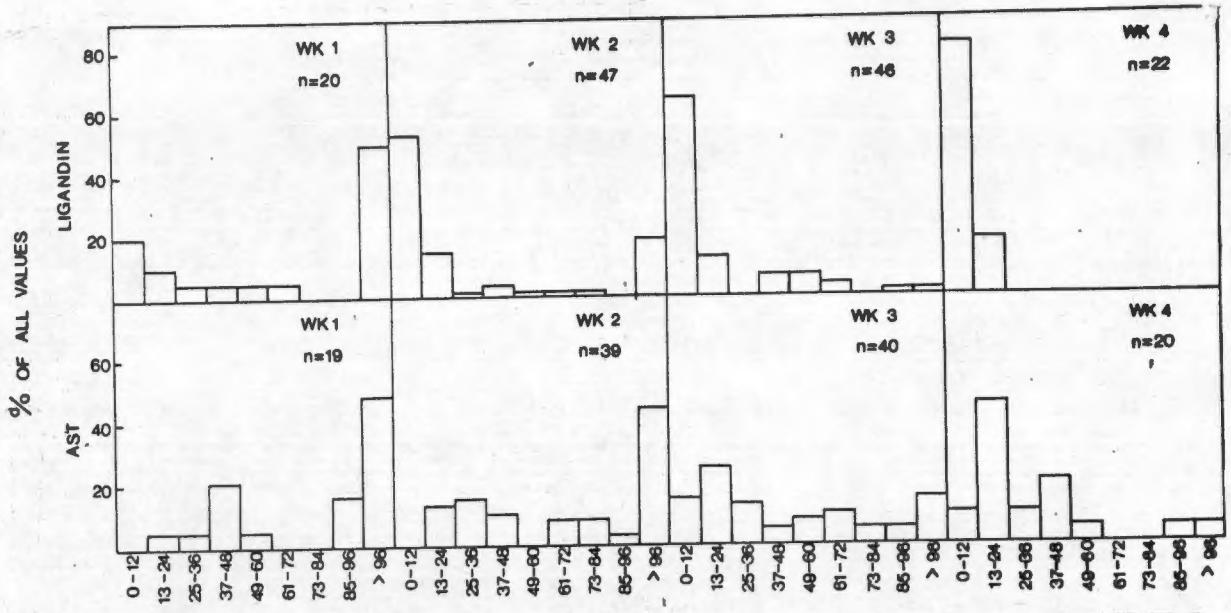


Fig. 10.4. Frequency distribution of ligandin and SGOT (AST) in patients with acute non-B hepatitis (group I). Ligandin and SGOT values were ranked into groups of 12 ng/ml (U/L). Eacn histogram represents all estimations taken within the stated time period. On the ordinate is the percentage of all values falling within a single rank, and on the abscissa is the rank of ligandin or SGOT values

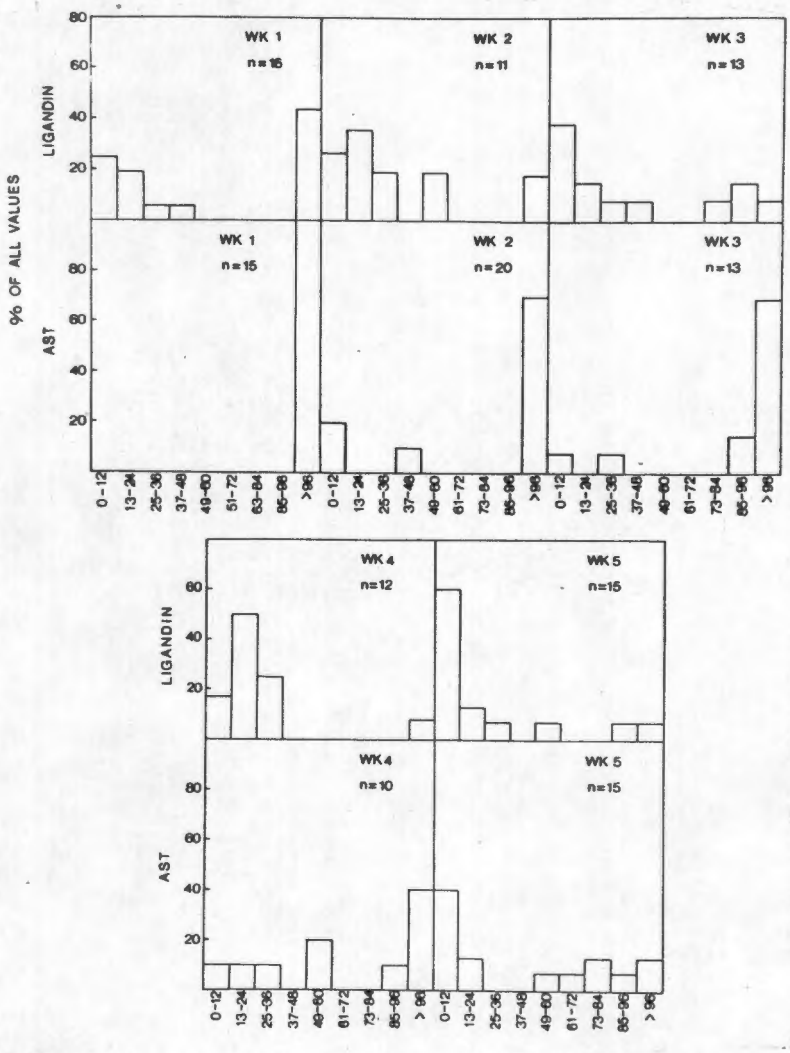


Fig. 10.5. Frequency distribution of ligandin and SGOT (AST) in patients with acute hepatitis B (group II). The histograms were constructed as before. Ordinate and abscissa are as in fig. 10.4.

quency distributions cannot be compared statistically, since the inclusion of non-independent samples leads to unacceptable bias.

Frequency distributions for ligandin and SGOT (AST) values for each week of illness are shown in fig. 10.4 & 10.5. In group I ligandin concentration showed a bimodal distribution, with a cluster of values at 0 - 24 ng/ml, a tail between 25 and 84 ng/ml, and then a second cluster above 96 ng/ml. In weeks 2 to 5 the cluster at 0 - 24 ng/ml became larger. The tail between the two clusters elongated in weeks 2 and 3 and then shortened and disappeared by week 6. The second cluster, above 96 ng/ml, decreased in week 2, formed part of the tail by weeks 3 and 4, and then disappeared. From week 6 onwards distribution was unimodal, clustered around a normal ligandin concentration.

In group II there was a similar frequency distribution, but the tail of intermediate values was less pronounced and the cluster of values above 96 ng/ml remained at least until week 5.

SGOT frequency distribution in group I also showed a bimodal distribution in week 1 and 2. However the lower cluster of values was at 24 - 48 U/L, compared to 0 - 24 ng/ml for ligandin. The tail between the two clusters persisted until week 5, and the cluster above 96 U/L was still present in weeks 6, 7 and 9. The cluster of low values did not centre around normal SGOT levels until week 5. After week 5 the numbers of values was too small for analysis.

In group II small numbers of SGOT estimations at each time peroid hamper analysis. In week 1 all values were above 96 ng/ml. In week 2 a cluster about 12 ng/ml and a smaller cluster at 37-48 ng/ml appeared. The cluster around 12 ng/ml persisted in week 4 and 5, but the cluster of higher values was still present and large in week 4, decreasing with

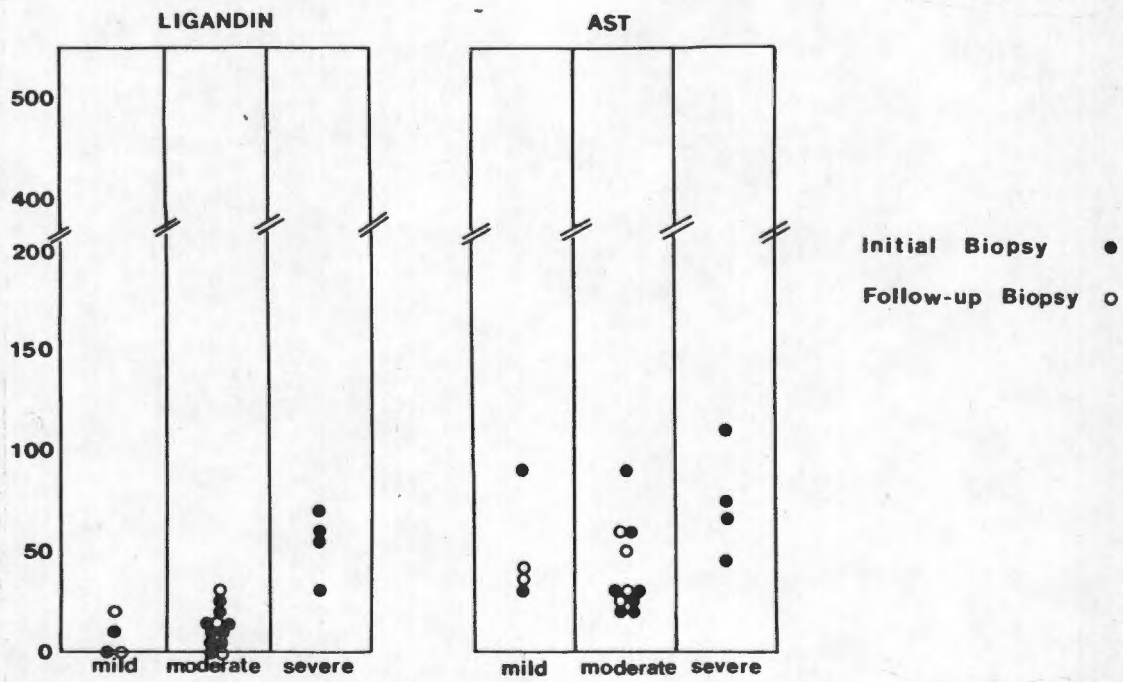


Fig. 10.6. Correlation of serum ligandin concentration and SGOT (AST) activity with histology in patients with chronic active hepatitis. Both initial and follow up biopsy data are included. On the ordinate is the ligandin concentration and SGOT activity, and on the abscissa is the severity of histological change.

evidence of a tail of intermediate values developing by week 5. Analysis after week 5 could be done because of insufficient numbers in each class.

All patients recovered without chronic sequelae. Of the 12 patients who did not develop hepatitis, none had elevated ligandin. Three patients with prolonged hepatitis, with an illness of more than two months duration were not included in the above analysis. In these patients ligandin remained elevated for the duration of illness but never rose above 4 times normal. In the single patient who had a second bout of hepatitis a single estimation of ligandin was available which was 506 ng/ml.

Chronic Active Hepatitis (CAH):-

Sixteen patients were included in this part of the study. Two biopsies showed mild disease. Serum ligandin in these patients was 0 and 10 ng/ml. SGOT was 31 and 88 U/L.

Ten patients had moderate disease activity. In this group 4 patients had elevated ligandin levels at the time of biopsy (mean for the group 10.4 ng/ml, range 0 - 23 ng/ml). All patients had elevated SGOT levels, mean 99 U/L, range 18 - 425 U/L.

Four patients had severe disease. All had elevated ligandin levels (mean 51.8 ng/ml, range 27 - 69 ng/ml). Mean SGOT in this group was 79 U/L. All patients had elevated SGOT levels (range 46-114 U/L).

Serum ligandin levels were correlated with disease activity ($r = 0.78$, $p < 0.01$), while there was no correlation between SGOT levels and disease activity ($r_s = 0.29$, $p > 0.10$)(fig. 10.6).

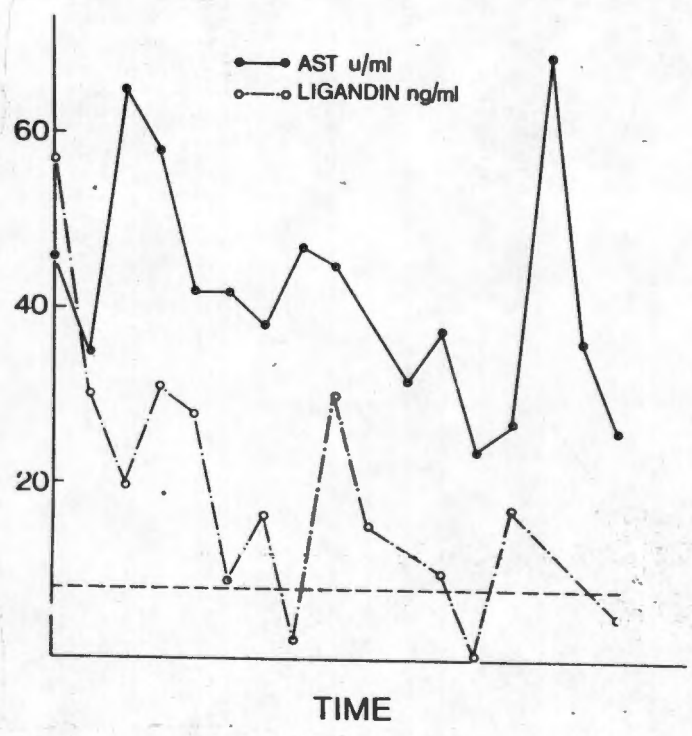


Fig. 10.7. Graph of the course of a single patient with chronic acive hepatitis over three years. Three biopsies taken over the course of the disease showed progressive improvement in the histological score

Seven patients underwent repeat liver biopsy at which time blood was again taken for SGOT and ligandin. There were too few patients in this group to make statistical comparisons, but if these biopsies were added to the original 16 then for the correlation between ligandin and histological severity is still significant ($r_s = 0.71$, $p < 0.01$).

Fig. 10.7 shows the course of a single patient over three years of therapy. Initial biopsy showed severe chronic active hepatitis, and the final biopsy showed mild disease. The improvement in her condition is better shown by the ligandin values, which show a tendency to normality. while the SGOT gives no indication of improvement.

Other liver diseases:-

Twelve patients with metastatic liver disease were seen. Four patients had carcinoma of the stomach, none had elevated ligandin. One of two patients with carcinoma of the lung had a serum ligandin of 30 ng/ml, but the other had normal levels. In six patients the primary site was unknown. Of these 5 had normal ligandin levels, and one had a minimally elevated level of 13 ng/ml. There was no correlation with SGOT levels, which were elevated in all patients.

Six patients with extrahepatic obstructive jaundice were seen. Serum ligandin was not elevated in any of these patients, but SGOT was elevated in all patients.

Twenty patients with cirrhosis were included - 4 with cryptogenic cirrhosis, and 16 with alcoholic cirrhosis. None of these patients had elevated ligandin. None of 8 patients with alcoholic hepatitis had elevated ligandin levels. In these patients SGOT was in the range 20 - 110 U/L.

Ligandin in the serum of 34 patients without liver disease selected at random from a hospital population was in the normal range in all cases. However in three patients who experienced cardiac arrest ligandin was elevated in the serum. The values recorded were 60, 84 and 191 ng/ml.

DISCUSSION

Validation of measurement of serum ligandin:-

Many substances may interfere with RIA's. Since jaundice is likely in the conditions in which ligandin measurement may be useful, i.e. liver disease, it was important to examine the effect of bilirubin on the recovery of ligandin from serum. Since patients with liver disease may have many serum biochemical abnormalities, only some of which are known, it was preferable to test recovery of ligandin from sera from these patients, rather than by adding bilirubin to purified ligandin. This study suggests that acute hepatitis, with and without hepatic encephalopathy, has a negligible effect on the estimation of ligandin, since in these cases ligandin recovery is complete.

Serum ligandin in patients with acute hepatitis dilutes out in parallel to the assay standard curve, indicating that the immunoreactive material released from the liver is similar to native ligandin. This finding provides further evidence against the influence of inhibiting substances.

None of the pharmacological agents tested interfered with the assay. However other agents might well interfere, and further drug inhibition studies may need to be performed. For practical purposes it should be noted that the vast majority of patients in this series, par-

ticularly those with acute hepatitis were not on any therapy at the time.

It is known that rat ligandin adheres to Sephadex. Bass (84) found in the rat that after loading approximately 1400 ng/ml to Sephadex G-100 recovery was only 20%. Therefore failure to recover human ligandin after gel filtration could be due to non-specific adsorption to Sephadex beads.

Ohmi et al (92) found a normal concentration of ligandin to be less than 7 ng/ml. Tsuru et al (93) describe levels of less than 4 ng/ml as normal. Bass (91) in a preliminary study quoted 8.5 ng/ml as the upper level of normal. These figures are compatible with the concentration found in this study in normal volunteers. These concentrations are much lower than those in the rat, (26 ng/ml)(84). The factors responsible for this difference between man and rat have not been investigated.

Acute viral hepatitis:-

These results suggest that the release of ligandin and SGOT differ in some respects in acute hepatitis. Three possible conclusions may be reached; that ligandin is as good an indicator of hepatic necrosis as SGOT, that ligandin is a better indicator, or that SGOT is a better indicator for hepatic necrosis.

Ligandin and SGOT cannot be equally good indicators of necrosis, since the two measurements behave differently during the course of the disease. The correlation between SGOT and ligandin when ligandin concentration is above 96 ng/ml suggests that at these levels ligandin and SGOT are indicators of the same phenomenon. Adachi et al (94) have measured serum GSH-T activity with CDNB in acute hepatitis and have

also shown that correlation of GSH-T activity with SGOT and SGPT is better when these activities are markedly raised than when they are not.

The correlation between ligandin and SGOT below ligandin concentrations of 96 ng/ml is not good, suggesting that at this stage of the disease the release or clearance of SGOT and ligandin are different.

The disappearance curve of ligandin from blood is biphasic, in keeping with the pattern seen with other enzymes (68). This suggests that redistribution of ligandin plays an important role in plasma clearance.

The half-life of ligandin in blood is shorter than the half-life of SGOT (assuming that the curves from which the half-life was calculated are true disappearance curves). This conclusion is supported by finding that the mean, median, and percentage of elevated values falls faster for ligandin than for SGOT. Adachi et al (94) using their assay for GSH-T activity also describe an early peak of GSH-T activity in acute hepatitis, paralleling SGOT activity, but which falls to normal before SGOT. Rats given CCl_4 release GSH-T activity early, before SGOT and SGPT. GSH-T activity returns to normal before transaminase activity (83).

The lack of correlation of necrosis on histology with transaminase levels has been well documented, and discussed previously (57,141). Since SGOT may remain elevated after apparent clinical recovery, the rapid return of ligandin levels to normal may indicate that ligandin is a better index of necrosis. However an alternative hypothesis is that if SGOT elevation indicates necrosis, and SGOT remains elevated longer than ligandin, this suggests that ligandin is undetectable at low

levels of necrosis. Supportive evidence for this contention is provided by the finding that ligandin is not elevated in other liver diseases where necrosis is known to occur, e.g. mild chronic active hepatitis and alcoholic hepatitis.

The bimodal distribution seen in the first week of illness can be interpreted as indicating a group of patients who have very active disease, i.e. those with ligandin)96 ng/ml, and a group with milder disease or who are convalescing. With time the active group merges into the convalsecent group, represented by the tail and those values clustered around normal, while the convalescent group merges with the fully recovered patients. This interpretation is consistent with the known behaviour of the disease, and the fact that by the time the diagnosis is made, i.e. jaundice appears, in some patients maximum necrosis may be past. This interpretation, coupled with the short half-life of ligandin, explains the finding of normal levels of ligandin in some cases in the early stages of the disease. These patients have already entered convalescence and are no longer releasing ligandin from the liver.

SGOT values show a similar frequency distribution. The same interpretation can be applied to the SGOT frequency distribution. In this instance the convalescent group clusters at values higher than corresponding ligandin values, again suggesting that ligandin is cleared earlier than SGOT.

Ohmi and Arias (92) have suggested that necrosis in viral hepatitis is intermittent or progressive. Their conclusion is based on finding a marked difference in maximum ligandin elevation in the serum of patients with fulminating drug hepatitis, and fulminating viral hepatitis. In the former, where they suggest a single massive burst of

necrosis, the ligandin concentration was ranged from 680 - 5350 ng/ml, while in fulminant viral hepatitis, at the same stage of illness, the range was 8 - 780 ng/ml.

Ohmi and Arias (92) have also found higher mean ligandin concentrations in patients dying with fulminant hepatitis than in survivors when studied at the same stage of disease (316 vs 47 ng/ml). They thus suggested that measurement of ligandin could have a prognostic value.

Results of this present study do not permit one to conclude that ligandin is more sensitive than SGOT, but the finding that ligandin returns to normal more rapidly than SGOT in a group of patients who all recovered clinically suggests that ligandin is a better indicator of resolution that SGOT. This hypothesis can only be confirmed by finding that ligandin levels remain elevated in patients progressing from acute to chronic hepatitis. There were no such patients in this series, and so the suggestion that ligandin may provide a better index of recovery remains unproven.

Chronic Active Hepatitis:-

The criteria for initiating steroid therapy in CAH are somewhat arbitrary. These include a transaminase level of more than 5x normal, and/or a globulin level that is more than 2x elevated. Since the criteria used do not correlate perfectly with disease activity as judged histologically, there will be some patients with disease severe enough to warrant treatment, who are not identified, and some patients with high enzymes who might be treated despite mild histological changes. While it is possible that biopsy sampling error could result in inaccu-

racy, it is widely accepted that the histological severity of the disease is more exact than any other criterion of activity.

This study showed that serum ligandin concentration is often elevated in moderate to severe chronic active hepatitis. The degree of elevation correlated significantly with the histological severity of disease. There was no correlation between SGOT and histological severity. In CAH maximum ligandin levels were 6 - 7x normal.

Patients with mild disease have normal or slightly elevated ligandin levels. Although necrosis does occur in mild chronic active hepatitis, ligandin is not sufficiently sensitive to detect this. This lack of sensitivity may be useful. This group do not need therapy, but if conventional indications for therapy, such as the transaminase levels, were used, some would be treated. Ligandin identified this group with certainty.

Patients with moderate disease may have an elevated ligandin Some of these patients may require treatment. In this group ligandin levels do not differentiate between those who do, and those who do not require therapy. However, the SGOT also fails to differentiate between these two groups.

Patients with severe disease all have elevated serum ligandin levels. The magnitude of elevation is more than 2x normal. These patients all require therapy. Ligandin levels identified this group with accuracy. However if only the transaminases were considered, not all would have received treatment.

In this study ligandin concentration correlated better with disease activity than did SGOT. Other series have reported that SGOT correlation with disease activity was better than reported here (97).

This may be because of the small numbers in this series, leading to sampling from a non-representative group.

Neverless these results strongly suggest that a larger study of ligandin in CAH is warranted, to determine whether ligandin indeed correlates better with disease activity than SGOT. If these results are confirmed it would seem that ligandin may be a better test on which to base therapeutic decisions.

Other diseases:-

Serum ligandin was not elevated in liver diseases other than acute and chronic hepatitis, specifically alcoholic hepatitis, obstructive jaundice, and cirrhosis. On the basis of the small series studied here one must be cautious of concluding that ligandin is never elevated in these conditions. Elevated ligandin may be found in hepatocellular carcinoma, but as this forms part of another investigation these results will be reported later.

Ligandin elevation is probably specific for liver disease. Only patients who had had a major episode of hypotension developed ligandinaemia. Although it cannot be proved, it is likely that ischaemic damage to the liver occurred and that this caused the release of ligandin.

CONCLUSIONS

It is possible to conclude that because ligandin is a smaller molecule than SGOT, and because it is present in the hepatocyte in greater concentration than SGOT, that it is released into serum as early as or earlier than SGOT. This has been well shown in experimental animals (83,84). In man release occurs at least simultaneously with SGOT.

Both in man and experimental animals clearance from blood is rapid, with a half life less than that of SGOT. However contrary to expectations, in man ligandin is less sensitive to minor hepatic injury than SGOT, and is not found in serum in our patients with alcoholic hepatitis or cirrhosis in whom the SGOT was mildly elevated. The reason for this insensitivity is not known.

Ligandin estimation appears to be a useful indicator of severe active hepatic disease (with the exception of alcoholic hepatitis), but is probably inadequate for diagnosis of mild disease. Ligandin estimation may thus find its place in the estimation of severity of disease, particularly in CAH, where it may be useful as a guide to therapy.

CHAPTER XI

LIGANDINURIA IN RENAL DISEASE

In this chapter an attempt is made to study the pattern of ligandin excretion in normal individuals and in patients with renal disease, with the aim of ascertaining whether the measurement of urinary ligandin has any diagnostic significance or prognostic relevance.

METHODS

Urine and serum samples were obtained from the following patients;

1. Patients undergoing renal transplant from cadaver donors were studied in the immediate post-transplant period, and throughout their three to four week stay in the intensive care unit. The diagnosis of post-transplant tubular necrosis was made if there was initial non-function in the absence of a demonstrable cause, which recovered spontaneously without treatment, or if renal biopsy showed changes of acute tubular necrosis (ATN). In some patients poor function persisted for more than two weeks, and evidence of rejection was found. In these patients poor function in the first five days was assumed to be due to tubular necrosis (119).

2. Patients admitted with severe infections not involving the urinary tract, who were at risk for tubular necrosis, by virtue of a period of hypotension, or by virtue of severe infection alone. These patients were grouped into those showing no renal impairment, and those showing mild renal impairment which resolved with therapy. Renal impairment

was defined as an abnormality of two of the following; serum urea, serum creatinine, and urine/plasma urea ratio.

3. Patients with severe or prolonged hypotension due to myocardial infarction , cardiac arrest, or trauma.

4. Patients with acute tubular necrosis from other causes. In these patients the diagnosis of ATN was made by finding acute renal failure, with a period of oliguria followed by a period of polyuria associated with recovery, following a recognisable predisposing cause. Patients with non-oliguric acute renal failure, or with suspected ATN but no recognisable cause were excluded.

5. Normal volunteers.

Renal function was assessed by estimation of serum urea and creatinine, creatinine clearance, and urine:plasma urea ratio. Urea and creatinine were measured by standard autoanalyser techniques. Ligandin was measured in urine by RIA.

Validation of measurement of ligandin immunoreactivity in urine:-

1. Immunoreactivity of ligandin in urine. Urine of known concentration of ligandin was assayed in serial dilutions in the RIA. The slopes of the dilution curve were compared to the slope of the standard curve as before.

2. Recovery of ligandin from urine. Known concentrations of ligandin were added to urine from normals and patients with acute and chronic failure. The samples were assayed before and after the addition of ligandin, and the results were expressed as percentage recovery.

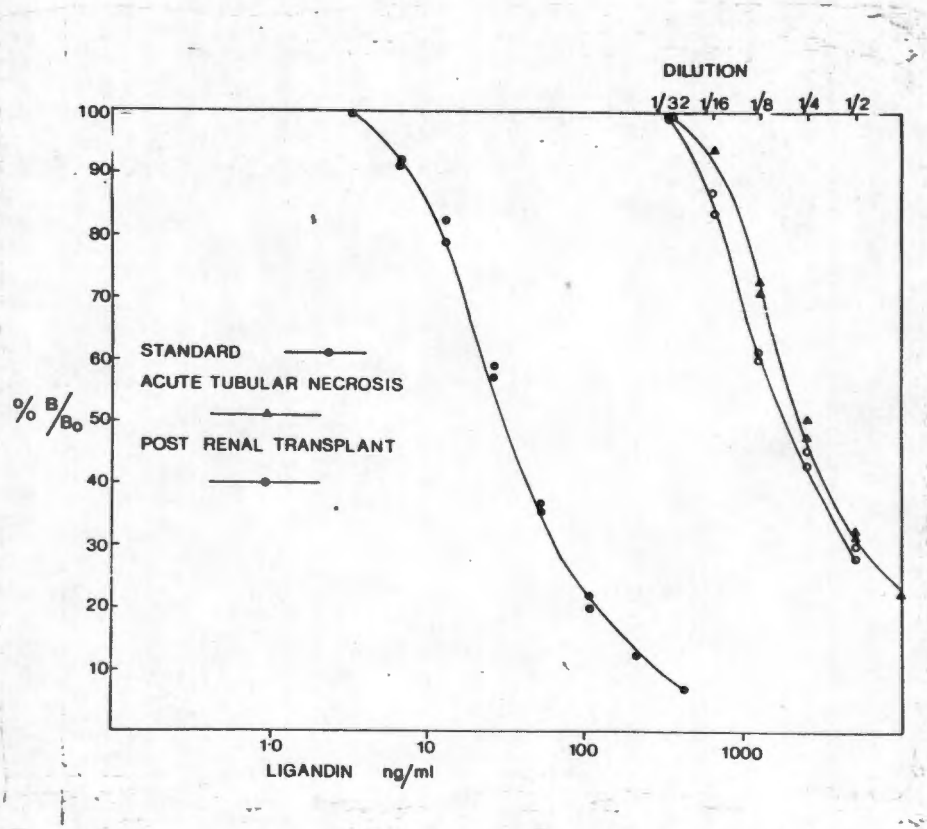


Fig. 11.1. Serial dilutions of urine from a patient with acute tubular necrosis, and following renal transplantation, compared with the standard curve of the ligandin assay. Ordinate and abscissa are as for fig. 10.1.

3. Molecular size of immunoreactive ligandin in urine. Urine from patients with acute tubular necrosis was chromatographed on G-100 Sephadex with molecular weight markers as before.

4. Storage of urine specimens. Ligandin in known concentrations were added to aliquots of normal urine and allowed to stand at room temperaturc, at 4°C, and at -20°C for 4, 8, 12, and 24 hours, and at -20°C for 2 weeks. The samples were then assayed for ligandin immunoreactivity.

RESULTS

Immunoreactivity of urinary ligandin:-

Ligandin in the urine of patients with acute tubular necrosis and post renal transplant diluted out in parallel to the assay standard curve (fig. 11.1). Ligandin in normal urine was diluted below the limits of detection of the assay before sufficient points could be obtained for adequate statistical comparison with the standard curve.

Recovery of exogenous ligandin from urine:-

Recovery of added ligandin from normal urine was complete when either 1 ng or 25 ng ligandin were added. Recovery from patients with ATN in whom endogenous ligandin excretion had ceased was 74% when the dose of added ligandin was 1 ng, and 100% when the dose of ligandin added was 25 ng.

Molecular size of immunoreactive ligandin in urine:-

Following Sephadex G-100 chromatography no immunoreactive ligandin could be detected in the eluted fractions when either normal urine, or

urine from patients with ATN with a known high concentration of ligandin in the urine, was applied to the column.

Ligandin concentration in normal urine:-

Specimens of urine from 50 normal volunteers were examined. Ligandin concentration was 11 ng/ml ± 10.25 ng/ml (mean ± S.D.). The range was 0 - 38 ng/ml. The normal range was therefore assumed to lie between 0 and 40 ng/ml. The upper level thus was slightly more than three standard deviations from the mean.

Storage of urinary ligandin:-

Urine ligandin could be stored at 4°C for 24 hours, or at -20°C for at least two weeks, without loss of immunoreactivity. Storage at room temperature for 8 hours led to a 50% drop in ligandin recovery.

Ligandinuria following renal transplantation:-

Thirty five patients were studied. Five patients were diagnosed as having unequivocal ATN. Of these, one patient had normal ligandin concentration in the urine on the first day. The other 4 patients had elevated levels of ligandin in the initial post transplantation urine. Ligandin concentration in these specimens was 72, 45, 50 and 3696 ng/ml. Normal urinary ligandin concentrations were present in three of these patients on day 2, and in the remaining patient ligandin returned to normal on day 3.

Eleven patients were presumed to have ATN, in that the initial post transplantation function was poor, but did not recover at all, or only recovered with treatment for rejection. In 10 of these patients

ligandin excretion in the first five days was normal. In a single patient on the first day the ligandin concentration was 3236 ng/ml, and within normal limits the next day.

Eighteen patients had no evedence of ATN in the immediate post tranplant period. In seventeen of these patients in the first 5 days the urinary ligandin was in the normal range. In one patient the first post transplant urine specimen showed a ligandin concentration of 4000 ng/ml, on day 2 ligandin concentration was 200 ng/ml, and on day 3 was normal. In this patient the donor kidney suffered a prolonged cold ischaemic time of 48 hours prior to transplantation, but functioned well once grafted.

A single patient experienced hyperacute rejection on day 1. Nephrectomy on day 4 revealed a necrotic kidney. Urine ligandin concentration on day 1 was 1785 ng/ml, on day 2 was 356 ng/ml, and was normal on day 3.

Ligandinuria following acute infection:-

In fifteen patients with severe infections who had no overt renal impairment ligandinuria was assessed for 5 days on treatment. Mean urinary ligandin concentration in these patients was 24.4 ng/ml (range 0 - 147 ng/ml, median 19 ng/ml). Five patients showed elevated ligandin concentrations in the urine. One of these patients had ligandinuria for 4 days, but in the others only a single specimen contained elevated ligandin levels. There were no clinical features distinguishing these patients from the rest of the group.

In 12 patients with acute infection in whom renal function was diminished during the acute illness the mean ligandin excretion was

18.8 ng/ml. This is not significantly different from that seen in the previous group. A single patient in this group developed elevated ligandin in the urine for two days

Ligandinuria associated with hypotension:-

Ten patients suffered prolonged episodes of hypotension followed by oliguria. Th causes were drowning (1), pulmonary embolus (1), ruptured aortic aneurysm (1), cardiac arrest (3), and acute myocardial infarction (4). Six of the ten patients had an elevated ligandin at some stage of their illness. Five of these patients died before the presence of ATN could be established. In this group mean ligandin concentration was 243.3 ng/ml (range 80 - 590 ng/ml). In no instance did urinary ligandin remain elevated longer than 3 days. Neither the sixth patient in this group or any of those without ligandinuria developed ATN.

Ligandinuria associated with acute tubular necrosis:-

Nine patient developed unequivocal ATN. The causes were drug toxicity (3), eclampsia (1), septicaemia (3), sunstroke (1) and intestinal obstruction (1). Two patients were studied early in the course of their disease. In these patients the initial ligandin concentration in the urine was 158 and 174 ng/ml. In the other 7 patients the first available specimen was at least 4 days from the onset of the precipitating cause. In these patients ligandinuria was not detected.

Patients with acute glomerulonephritis (4), and chronic renal failure (9) had normal ligandin concentration in their urine.

DISCUSSION

Bass et al (111) expressed ligandinuria in terms of urinary creatinine. In man the excretion of several urinary enzymes has also been expressed in terms of urinary creatinine (105). Expressing human urinary ligandin per mg urinary creatinine did not result in increased sensitivity and this method has thus not been used.

Only prolonged or severe hypotension or renal ischaenia appear to be associated with an elevated ligandin concentration in the urine. When it occurs ligandinuria is of short duration. This makes the study of ligandinuria in ATN difficult, since patients usually only present when they get symptoms of renal failure, i.e. several days after the onset of the illness. Our data indicate that unless studied early, ligandinuria will not be detected in ATN. For the same reason ligandinuria cannot be used to differentiate between ATN and other causes of acute renal failure. Further the oliguria which is a feature of the disease makes the collection of regular urine specimens difficult.

Marked ligandinuria is associated with conditions causing a drastic reduction in renal blood flow (cardiac arrest, post renal transplant, ruptured aortic aneurysm, drowning). It is likely that ischaemic necrosis of tubular cells is responsible for the release of ligandin into the urine. It is possible that release occurs from damaged cells as well, as not all patients with ligandinuria developed clinically significant ATN following the episode of reduced blood flow.

Post transplantation ATN is thought to occur on the basis of renal ischaemia. In this situation, when ATN could be positively diagnosed, ligandin was elevated in 80% of patients. Of those patients in whom ATN was suspected, but not proven, only one patient had elevated urin-

ary ligandin. Although rejection is said to occur from day 4 onward it is possible that the poor initial function in these patients was due to early rejection.

It is notable that in the patient whose graft underwent prolonged ischaemia the first specimen recorded the highest level of ligandin in the whole series (4000 ng/ml).

Rejection of the transplanted kidney is not associated with ligandinuria. Although tubular damage is seen in this condition presumably the degree of damage is of too low intensity to cause a detectable release of ligandin.

Cho et al (125) have used GSH-T activity in the perfusates of donor kidneys to predict whether ATN would develop post transplant. There was a significantly higher GSH-T excretion in the perfusates of those kidneys which developed post-transplant ATN. The test could be used with 100% predictability. The ultrafiltrates of these kidneys were also studied, and although a difference was found it was not statistically significant. This current study however shows that even high levels of ligandinuria may not be predictive of ATN. The differences between these results could be due to the fact that Cho et al used GSH-T enzyme assay, whereas here ligandin was measured by the more sensitive RIA. Secondly urine produced after transplantation is not equivalent to ultrafiltrate or perfusate.

Patients with systemic infections who develop decreased renal function do not have elevated urinary ligandin levels. It has been said that the decreased function in this situation is due to tubular disease. However on the basis of these results a functional disturbance may be more likely.

In some patients with systemic infection ligandinuria was found without decrease in renal function. These episodes may have been related to drug toxicity, but this cannot be answered with the available data.

PART TWO

GLUTATHIONE S-TRANSFERASE

CHAPTER XII

GLUTATHIONE S-TRANSFERASES

HISTORICAL ASPECTS

Mercapturic acids were first described in 1879 when it was shown that bromobenzene and chlorobenzene given to dogs were excreted as N-acetyl(chlorophenyl)-L-cysteine and N-acetyl(bromophenyl)-L-cysteine, i.e. as conjugates of N-acetyl-L-cysteine (141,142). These N-acetyl-L-cysteine conjugates were termed mercapturic acids (MA).

Most of the experimental work on MA biosynthesis and glutathione conjugation was done in rats but other laboratory animals, e.g. rabbits and guinea pigs were used as well. The findings in different species differed only quantitatively, and were qualitatively similar. Conclusions about the biosynthetic pathway were applicable to all species tested.

Initially interest in the metabolic pathway for the formation of MA's focused on the origin of the cysteine moiety. Possible sources included tissue protein, dietary cysteine, or glutathione (GSH).

Dietary cysteine was eliminated from consideration for several reasons. Varying the timing of administration of food with respect to administration of bromobenzene did not alter the rate of MA formation (143). The amount of MA excreted was proportional to the dose of precursor and not to the amount of dietary cysteine (144). Animals given a MA precursor and kept on a low protein diet continued to excrete MA but lost weight or ceased to grow (145,146). The latter could be reversed by dietary cysteine or methionine. These findings were interpreted as indicating that dietary cysteine was utilized to replenish

tissue thiols, rather than contributing directly to the mercapturic acid biosynthesis. This was confirmed when it was shown that only a small amount of dietary ^{35}S-cysteine was incorporated into MA's when animals were given bromobenzene or naphthalene (147,148).

Having excluded dietary thiols, tissue protein was thought to be the most likely source of the cysteine group. Animals given a fixed dose of bromobenzene excreted MA in proportion to their weight (indirectly therefore, in proportion to tissue protein) and glutathione when given in the diet did not increase MA formation (149).

However from 1934 onwards evidence suggesting that glutathione (GSH) could be the thiol donor began accumulating. (It had been pointed out that the tripeptide GSH, γ-glutamyl-L-cysteinyl-glycine, contains three amino acids which are used in the metabolism of xenobiotics (150). The mercapturic acids contain cysteine; benzoic acid is excreted as hippuric acid (a glycine conjugate), and phenylacetic acid is excreted as the glutamine conjugate. It has not been shown however that GSH is the source of the glycine in hippuric acid).

When in 1934 Nakashima gave naphthalene, which had been shown that year to be a MA precursor, to rabbits, the GSH content of the lens and liver decreased (151,152). In 1940 it was shown that the GSH conjugate of benzyl chloride (S-benzyl GSH) when administered to rats gave rise to the corresponding MA, N-acetyl-S-benzylcysteine, in the urine (153), suggesting that the GSH conjugate was an intermediate in MA formation. In the same year rabbits given bromobenzene were found to show a decrease in liver GSH, which was proportional to the amount of bromophenyl MA formed (154). The finding that p-bromobenzylbromide and its GSH and cysteine conjugate all gave rise to the same MA, N-acetyl-S- (bromo-

benzyl)-L-cysteine, provided further clues to the metabolic pathway (154).

Administration in vivo of a number of MA precursors were shown to decrease liver GSH (156-158). Furthermore addition of dietary cysteine did not alter the rate of MA production (157). Barnes et al placed the final nail in the coffin of the tissue protein theory when they pointed out that the turnover rate of tissue protein generated insufficient cysteine to maintain MA production, while the turnover rate of GSH was capable of sustaining MA synthesis (158).

At about the time that GSH was being shown to be involved at an early stage in MA biosynthesis, progress was also being made in the elucidation of the last step of this pathway. Stekol, who did much of the early work, and others showed that administration of S-cysteinyl derivatives of MA precusors to dogs, rats, rabbits and man, resulted in the formation of the corresponding MA (159-161).

Elucidation of the metabolic pathway of mercapturic acid formation proceeded rapidly. In 1951 bromobenzene was shown to decrease GSH in liver and kidney (162), and liver and kidney homogenates were shown to convert S-benzylhomocysteine to the N-acetyl derivative (163).

Further information on the intermediate steps was provided by the demonstration that a cysteinylglycine derivative of a MA precursor could be cleaved, with the release of glycine, by a peptidase (cysteinylglycinase) which was found in rat liver, kidney, and pancreas (164, 165). In 1960 Booth et al (166) showed the formation of a GSH conjugate of naphthalene by rat liver slices using chromatographic comparison with synthetic compounds. This was the first direct evidence of GSH conjugation as the first step in MA biosynthesis. This group

also showed that kidney homogenates converted the GSH derivative to a cysteine derivative which was then acetylated by liver slices or by kidney homogenate (166). Following administration of MA precursors several other GSH conjugates were demonstrated in rat liver slices, homogenate or bile (165,167-170).

Evidence for participation of an enzyme in the conjugation reaction was first presented by Booth et al in 1960 (165), when they found that the addition of rat liver supernatant increased the rate of conjugation of the epoxide derivative of naphthalene to GSH. Only the GSH conjugate was formed in increased amount. The formation of the cysteine and N-acetyl-cysteine derivatives was unchanged. This effect was destroyed when the supernatant was boiled. Eighty two percent of the enzyme activity resided in the cytosol (171). Activity in the liver was much higher than in other organs (liver > heart > kidney > lung > spleen).

The enzyme was partially purified, using its catalytic activity with 3,4-DCNB and GSH as a marker. No other thiol would substitute for GSH. These workers also described enzyme catalysed GSH conjugation to alkyl halides, epoxides, bromsulphophthalein (BSP), iodobenzene, phenanthrene, naphthalene and chloronaphthalene. The last four substrates required the addition of microsomes and NADPH before conjugation occurred, implying that microsomal oxidation preceded conjugation.

Simultaneously in the United States, Combes and Stakelum were able to show that the reaction between BSP and GSH was catalysed by an enzyme present in liver cytosol (172). No co-factors were required and the reaction was specific for GSH. This specificity for GSH has since been documented repeatedly (173-175). They also suggested that the

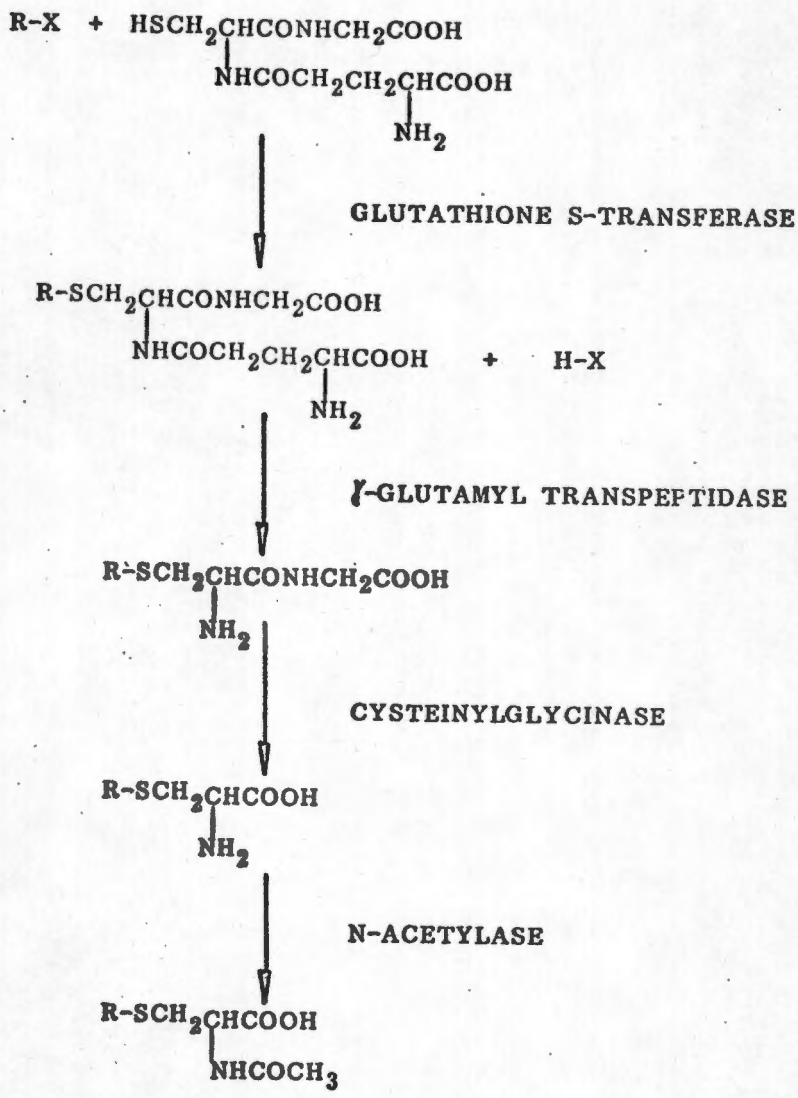


Fig. 12.1. Mercapturic acid biosynthesis. R-X is an electrophilic substrate.

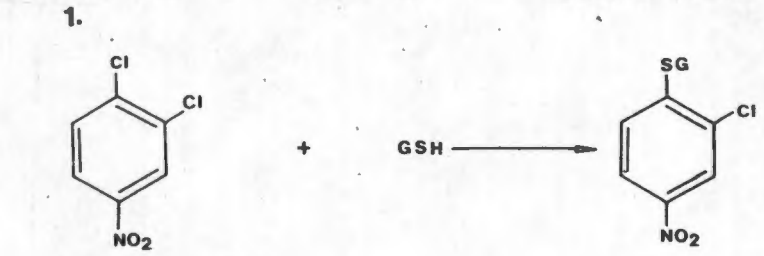
substrate is conjugated to the SH group of GSH in thioether linkage (176,177).

The presence of conjugates of GSH, cysteinylglycine, cysteine and N-acetylcysteine in the bile of rats dosed with naphthalene, and the failure to demonstrate the presence of γ-glutamyl-S-cysteine derivatives led Boyland to propose the scheme shown in fig. 12.1 (178), which has now been accepted as the pathway of MA biosynthesis.

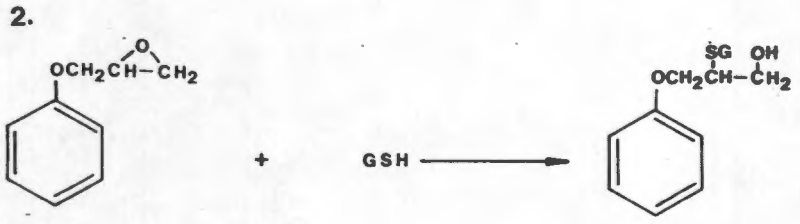
Five forms of glutathione S-transferase (GSH-T) activity were described in the organs of rats and other laboratory animals over the next few years, each ascribed to a separate transferase or group of transferases, and named according to the chemical nature of the substrate used. These were the aryl, alkyl, aralkyl, epoxide and alkene transferases. The enzymes involved were differentiated from each other on the basis of substrate specifity, pH and temperature optima, organ distribution and precipitability in acid, ethanol, and on heating.

The first GSH-T to be characterized was described because of its ability to catalyse the reaction of the form depicted in fig. 12.2.1 (171), and given the name of GSH S-aryl transferase (179). This same enzyme was found to catalyse the conjugation of other polychloronitrobenzenes to GSH, sometimes with replacement of the nitro group instead of the halogen, e.g. 2,3,4,6 tetrachloronitrobenzene (180). It was thus evident that the enzyme had fairly broad specificity. It soon became apparent however, that this was not the only enzyme catalysing conjugations to GSH, and that several enzymes must exist.

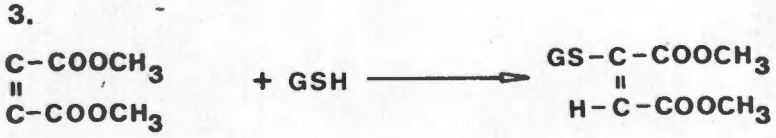
The second GSH-T to be described was GSH S-alkyl transferase and was isolated on the basis of its ability to conjugate iodomethane and GSH (eqn. 12.1)(175,181).



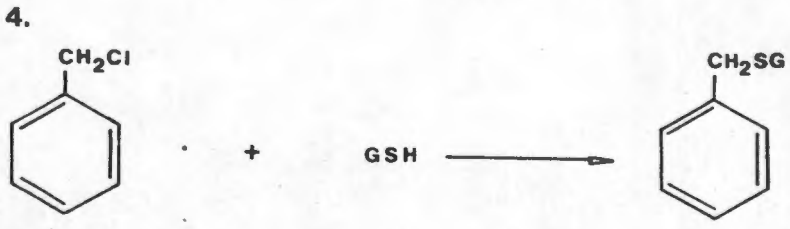
1,2-dichloro-4-nitrobenzene



2,3-epoxypropyl phenyl ether



diethyl maleate



benzyl chloride

Fig. 12.2. Reactions of glutathione catalysed by; (1) GSH-aryl transferase; (2) GSH-epoxide transferase; (3) GSH-alkene transferase; (4) GSH-aralkyl transferase. The reaction with iodomethane which is catalysed by GSH-alkyl transferase is given in the text.

$CH_3I + GSH = CH_3\text{-}SG + H^+ + I^-$ (12.1)

methyl iodide

This enzyme was also shown to be active with several other aliphatic halides (181). It was shown to be different from the previously described aryl transferase.

Since the original description of MA's it has been recognized that MA's derived from certain halogenobenzenes and polycyclic aromatic hydrocarbons are acid labile. These have been termed premercapturic acids, and are of the form of dihydrohydroxy MA's, e.g. N-acetyl-S-(1,2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine or N-acetyl-S-(5,6- dihydro-6-hydroxy-5-benzanthracyl)-L-cysteine. Cold mineral acid treatment would yield N-acetyl-S-(1-naphthyl)-L-cysteine or N-acetyl-S-(5-benzanthracyl)-L-cysteine (fig. 12.3). The term "premercapturic acid" is misleading and unnecessary as these are in fact mercapturic acids (1).

It was suggested that these acid labile MA's were probably formed from intermediate epoxides (182,183). Supporting evidence was obtained when it was shown that conjugation with iodobenzene required the presence of microsomes and NADPH (171), and confirmation came with the demonstration that the conjugation of benzanthracene and phenanthrene occurred via these suggested epoxide intermediates (167,169).

The first attempt at characterization of these GSH-epoxide conjugating enzymes responsible took place in 1965 (174) when, using the substrate 2,3-epoxypropyl phenyl ether, the epoxide GSH-T activity was separated from aryl and alkyl GSH-T activity (fig. 12.2.2).

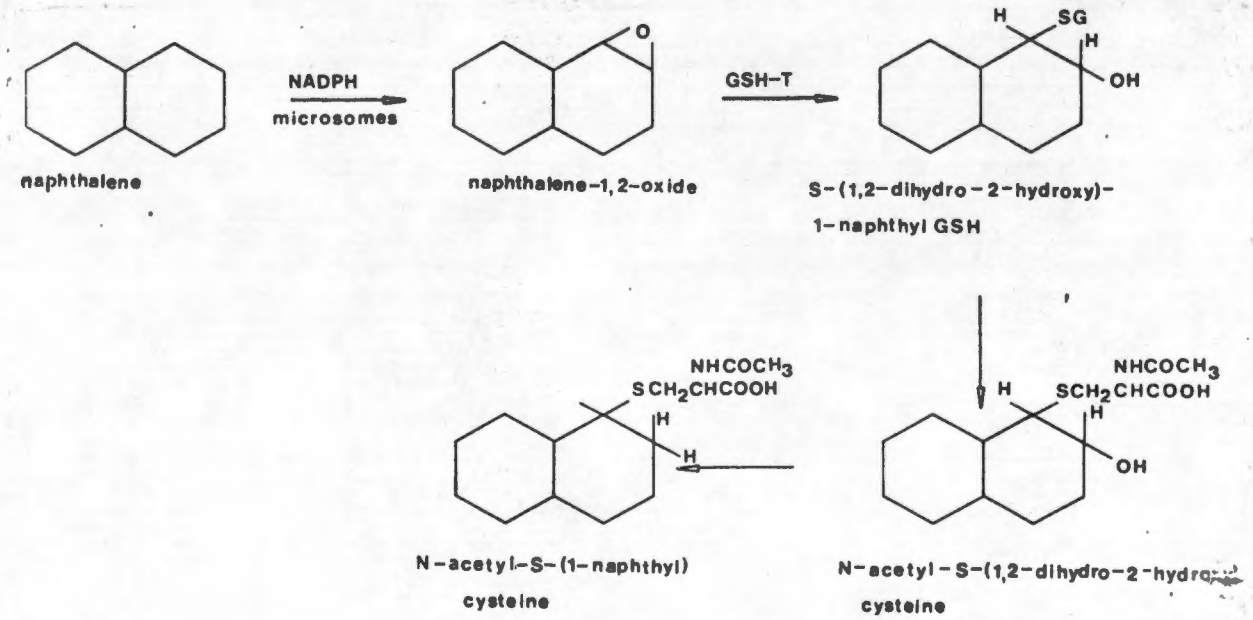


Fig. 12.3. Formation of an hydroxyarylmercapturic acid from naphthalene via an intermediate epoxide. Epoxides are probably the precursors of all hydroxymercapturic acids

Since then a vast number of epoxide substrates have been described including many reactive intermediates of stable hydrocarbons formed as a result of microsomal oxidation.

The first report of GSH conjugation to α-β unsaturated compounds was in 1967 (184). These compounds occur widely in nature and are used in the plastics industry. The enzyme catalysing this conjugation was isolated using its ability to conjugate GSH to diethyl maleate as a marker (fig 12.2.3)(184). It was subsequently shown that at least six enzymes, differentiated from each other by heat inactivation, ammonium sulphate solubility, enzyme inhibitors and by the tissue distribution of activities in liver and kidney, were responsible for reactions with several α-β unsaturated carbonyl compounds (185). Among these were aldehydes, ketones, lactones, nitriles. These six enzymes, the GSH S-alkene transferases were shown by similar techniques to be different from the previously described GSH-T's (184,185).

A fifth form of enzyme which catalysed GSH conjugation to aralkyl halides, e.g. benzylchloride (fig. 12.2.4), was described in 1969 (186). This enzyme, called GSH S-aralkyl transferase, was shown to be different to the other transferases. Yet another enzyme, conjugating GSH to aralkyl sulphate esters, (fig. 12.4.1) was shown to be dissimilar to the GSH S-aralkyl transferase reactive with benzyl chloride (187).

At this stage it was noted that the transferases had not been shown to catalyse conjugation with any endogenous substrates. Although GSH conjugates of several endogenous substances were known to occur in vivo, it had not been shown that the GSH-T's catalysed the conjugation reactions. A glutathione-isovaleric acid conjugate is synthesized in

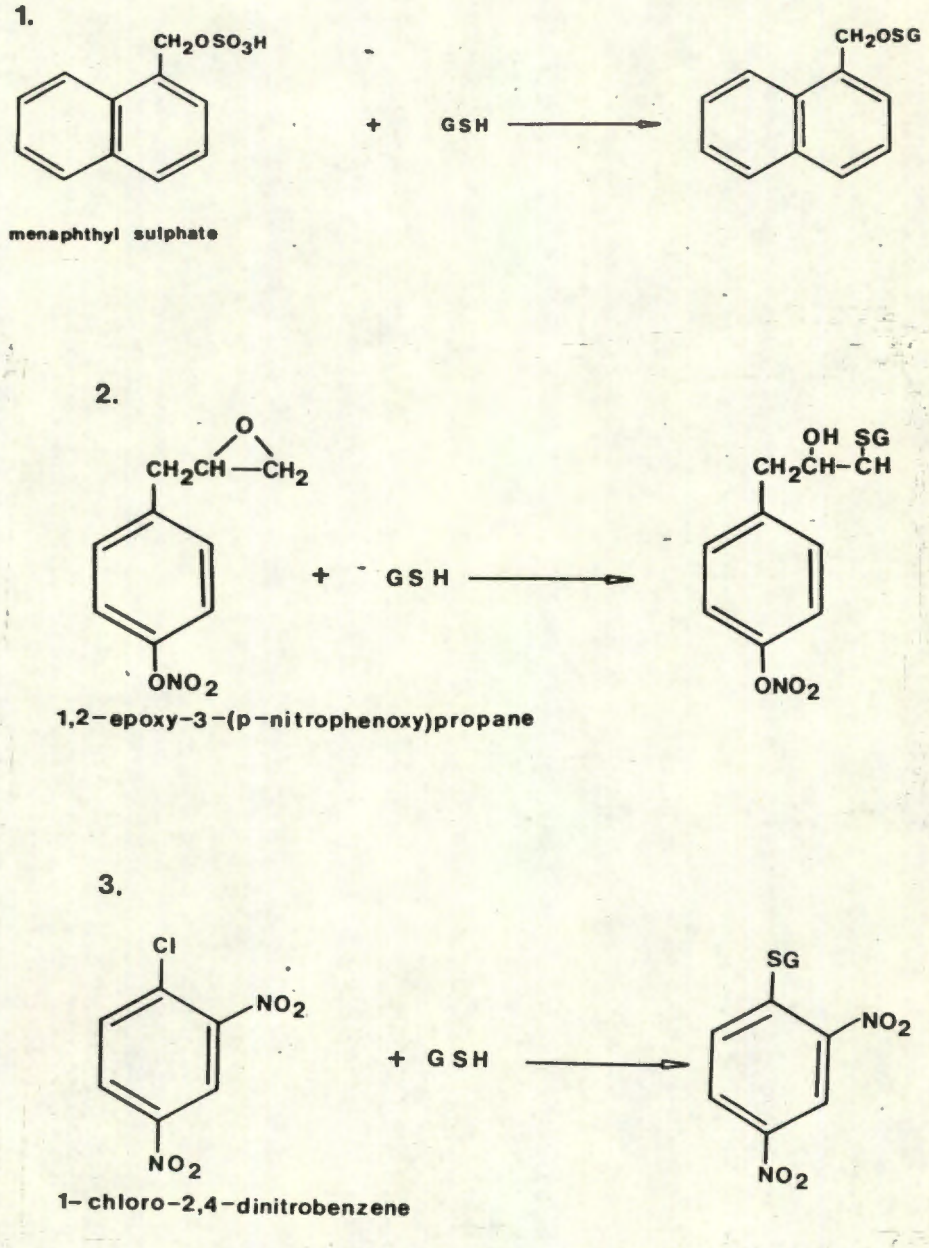


Fig. 12.4. Reactions of glutathione used to purify the GSH S-transferases; (1) reaction with menaphthyl sulphate used to purify GSH-T M; (2) reaction with epoxynitrophenoxy propane used to purify GSH-T E; (3) reaction with chlorodinitrobenzene used to purify GSH-T AA. Iodomethane was used to purify GSH-T B, and dichloronitrobenzene was used to purify GSH-T A and GSH-T C.

the presence of ATP and liver homogenate (188) and it was speculated that this was the first step in the synthesis of isovalthine (S-[1-carboxyisobutyl] cysteine). Other endogenous S-substituted -carboxy cysteines have been found (189-191), but their origin is obscure. Maleic acid conjugates of GSH and cysteine do occur (191-192), (S-[1,2-dicarboxyethyl]-cysteine) but again little is known of their origin. Maleic acid conjugation to GSH occurs spontaneously. Maleic acid is not a good substrate but the presence of liver cytosol does catalyse the reaction to a small extent (184).

Recent work with the rat has suggested that in some species lipid hydroperoxides may be major endogenous substrates for some of the GSH-T's.

Work on the biosynthesis of MA's had at this stage produced an embarrassingly large number of seemingly separate enzymes. At least 10 different enzymes catalysing GSH conjugation had been identified using only a small percentage of the compounds known to be MA precursors, with the possibility that investigation of other substrates would yield further enzymes. Thus far all studies utilized preparations that were probably, by modern criteria, not pure. Each preparation may have contained varying amounts of other GSH-T's. Work with highly purified enzymes began when Fjellstedt et al were able to purify an enzyme capable of conjugating GSH to 1,2-epoxy-3-(p-nitrophenoxy)propane (fig. 12.4. 2)(193). The enzyme was homogenous with respect to gel electrophoresis and analytical ultracentrifugation. However in the early stages of purification there were at least two other proteins with epoxide GSH-T activity. These could be separated on CM-cellulose. The purified enzyme was reactive with a wide range of epoxide substrates, but not with

arene oxides. It was also shown that the three peaks of epoxide GSH-T activity seen on elution from CM-cellulose each, when purified to homogeneity, had overlapping substrate specificities (194).

At about the same time, a GSH-T active with aralkyl substrates had been purified to apparent homogeneity by Gillham (195). Purification was followed using conjugation of GSH to menaphthyl sulphate as a marker (fig 12.4.1).

Clarification came when Jakoby's group demonstrated that there were at least seven GSH-T's in rat liver (127,196,197). The two previously described GSH-T's (193,195) were designated GSH-T M and E respectively (menaphthyl sulphate and epoxide GSH-T). Since it was known that GSH-T E was the first peak eluted from CM-cellulose, the GSH-T's were named in reverse order of elution from CM cellulose, GSH-T A, B, C, D, E (197). GSH-T M was lost in the purification process. Subsequently, when the more sensitive substrate 1-chloro-2,4-dinitrobenzene (CDNB) was used the seventh GSH-T, GSH-T AA was found eluting after GSH-T A (197). With the exception of GSH-T D all the rat GSH-T's been purified to homogeneity. GSH-T B was purified using its ability to conjugate iodomethane to GSH (127), GSH-T A and C by their ability to conjugate 3,4-dichloronitrobenzene (DCNB) to GSH (127,196) and GSH-T AA by its ability to conjugate CDNB to GSH (fig. 12.4.3)(197). Each pure protein had wide overlapping substrate specifity with the other GSH-T's.

Simultaneously Mannervik and colleagues purified two rat liver enzymes with aryl GSH-T activity using DCNB as substrate (198). Their enzymes, designated GSH-T I and II, were later shown to be identical to Jakoby's GSH-T's C and A respectively (199). Also at about the same

time Hayakawa et al using a sensitive radiochemical assay isolated and purified a GSH-T from sheep liver active with several arene oxide derivatives of polycyclic aromatic hydrocarbons. Their purification system was very similar to that of Fjellstedt et al (200). They concluded that their preparation was identical to that of Fjellstedt and the failure of the latter to show arene oxide activity was probably due to the lack of a sufficiently sensitive assay (200).

It was then shown that rat GSH-T B was identical to ligandin (13), a major intracellular binding protein, which had been previously characterised (see chapter I).

In man, using a purification scheme similar to that used in the rat, five GSH-T's were purified to homogeneity and designated α, β, γ, δ and ϵ, on the basis of increasing isoelectric points (16).

At this stage it is necessary to consider a few questions of semantics. Jakoby has pointed out (201) that the terms aryl, alkyl, epoxide, alkene and aralkyl GSH-T, are misleading since they do not refer to single enzymes, but to a combined activity contributed to by several enzymes. He suggested that the enzyme activity be specified by substrate, for example 'GSH-T activity with iodomethane' (201). Therefore having previously dealt with these terms in their historical contest, I will avoid their use except where necessary to refer to earlier papers. Jakoby also pointed out that enzyme kinetics and association constants of binding derived from experiments on cytosol containing mixtures of these enzymes are of little value, being the summation of the activities of the constituent enzymes. This point too has often been ignored.

CHAPTER XIII

PURIFICATION OF GLUTATHIONE S-TRANSFERASES

The earlier methods of purification used a combination of ion exchange chromatography, ammonium sulphate precipitation and adsorption chromatography. The 100,000xg supernatant from rat liver can be applied to DEAE cellulose so that GSH-T M remains on the column (194), but the other GSH-T's pass through unhindered (127). The dialysed redissolved precipitate from an ammonium sulphate fractionation of the pooled eluent is then chromatographed on CM-cellulose. GSH-T D and E appear in the initial wash after which the other GSH-T's are eluted sequentially with a KCl gradient (12). Further purification of each GSH-T is by adsorption chromatography with hydroxyapatite.

A similar scheme is used for the purification of human GSH-T's. The cationic GSH-T's do not bind to the first DEAE-cellulose column and are collected in the initial wash (16). Since the original reports many other methods for purification have been described, mostly using the above techniques in various combinations.

More recently purification techniques have included affinity chromatography with a variety of ligands. Kraus used GSH coupled to aminododecyl-cellulose to purify a GSH-T able to conjugate hexachlorocyclohexane (HCH) to GSH (202). Guthenberg and Mannervik were able to purify a GSH-T from rat lung using GSH coupled to agarose by a hexyl spacer (203). GSH coupled to agarose with 1,6-diaminohexane as a spacer has also been used. GSH-T B did not bind to the affinity gel and appeared in the initial wash. GSH-T AA was eluted with a salt gradient and GSH-T's A and C were partially separated with a GSH dis-

placement (204). BSP coupled to Sepharose 4B has been used to purify a porcine GSH-T, called ligandin (205). A BSP-GSH conjugate coupled to agarose (Sepharose 4B) has been used to purify an insect GSH-T (206) and rat ligandin (207). Human GSH-T has been partially purified using GSH coupled directly to Sepharose (208).

CHAPTER XIV

DISTRIBUTION

The GSH-T's are widely distributed throught the plant and animal kingdoms (209-215). The lowest known organisms found to have GSH-T activity are bacteria. Strains of Salmonella typhimurium used in the Ames mutagenicity test are able to catalyse the conjugation of CDNB and GSH (209-210). Protozoa also have GSH-T's. The GSH-T's of Trypanosoma cruzi have been purified. In this organism the GSH-T's may play a part in resistance to drugs (211-212).

In plants the GSH-T's are similarly highly conserved (213-215). The ability of corn, sorghum and sugar cane to resist the effects of the herbicide atrazine correlates with the levels of the GSH-T catalysing its conjugation to GSH (216). A similar GSH-T responsible for conjugation of fluorodifen (p-nitrophenyl α, α, α- trifluoro-2-nitro-p-tolyl ether) has been isolated from peas (217) and peanuts (218). GSH-T from the corn root has been claimed to conjugate S-ethyldipropyl-thiocarbamate (EPTC) sulfoxide and GSH (215) and to be enhanced by the EPTC antidote dichloracetamide, however the existence of this enzyme has been disputed (219,220). Non-enzymatic conjugation of GSH to several herbicides has been reported (220).

GSH-T's have been found in various species of insect e.g. the wax-moth larvae (206), grass grub, housefly, cockroaches (221,222) and locusts (173,223). The insect GSH-T's have been shown to metabolize organophosphorus insecticides such as methyl and ethyl parathion, diazinon and malathion (224,225). α- Hexachlorocyclohexane is also metabolised by GSH conjugation, as are other polychlorobenzenes (226).

While there is some correlation between insecticide resistance and GSH-T levels (227) the GSH-T's are probably not the main mediators of resistance (228). GSH-T activity can be separated from another GSH-dependent enzyme, DDT dechlorinase (229,230), which is thought to be unrelated.

GSH-T activity has been found in six species of earthworm (Lumbricidae). Worm homogenates subjected to isoelectric focusing have shown species specific profiles of enzyme activity (231).

The gill, hepatopancreas (equivalent of the liver), and excretory gland of crabs and lobsters have GSH-T activity (232,234), as does cytosol prepared from whole molluscs (233).

BSP binding on gel filtration is absent in elasmobranch and teleost fish (234), several of which have GSH-T activity with epoxide substrates (233), suggesting that BSP is poorly bound to the GSH-T's in these species. The rainbow trout takes up BSP avidly, and is able to form BSP-GSH conjugates (235). Tadpoles lack a mechanism for BSP uptake and have no Y protein as determined by gel filtration. In contrast the adult frog and all land animals studied are able to take up BSP and have Y protein (234). GSH-T activity has been demonstrated in the livers of snakes (236), lizards (179), chickens (237), several species of wild birds (179,238,239), laboratory rodents of all kinds (240-242) and apes (243). The hepatic GSH-T's of hamsters (242), guinea pigs (244) and monkeys (245,246) have been purified and characterised and as in the rat and human have been shown to consist of forms with overlapping substrate specificity. A pig liver 'ligandin' which has GSH-T activity has been purified and partially characterised (205).

The GSH-T's are widely distributed within the mammalian body. In the rat liver they comprise about 10% of the soluble proteins (247, 249). Ligandin accounts for about half of the GSH-T concentration, i.e. about 5% of the soluble liver protein. Ligandin is localised to the cytoplasm of the hepatocyte and is not found in the Kupffer cells. Staining by immunofluorescence and immunoperoxidase techniques is more intense in the centrilobular area (99) and nuclear staining is also demonstrable (99,248).

In man the GSH-T's comprise 2-3% of the soluble hepatic protein (249). Using an immunoperoxidase technique the distribution of ligandin in the liver in man has been shown to be similar to the rat (250).

Systematic investigations of the distribution of GSH-T activity using a variety of substrates have shown the liver to have the highest concentrations of the enzymes in all animal species studied (171,243, 251,252). Other sites of relatively high activity are kidney, lung and intestine. GSH-T activity has also been found in heart, spleen, intestine, placenta, lens, erythrocytes, white cells, whole blood, testis, ovary, adrenal, skin, sperm, semen and brain (31,32,251,253-259). In the human kidney ligandin is localised to the proximal tubules and to a lesser extent in the thick descending limb of Henle (250), while in the rat ligandin is found only in the pars convoluta of the proximal tubule (260). GSH-T enzyme activity in the rat is limited to the proximal tubules and the loops of Henle (261). Ligandin measured by radioimmunoassay has been found in significant amounts in rat liver, kidney, intestine, testis, ovary and adrenals, and in smaller amounts in heart, skeletal muscle, brain, stomach and colonic mucosa, lung, pancreas, thyroid and pituitary (262).

Rat hepatoma cells in culture show a line of immunological identity with purified ligandin when tested by immunodiffusion. In addition there was another precipitin line which showed only partial identity with purified ligandin. Cytosol from other rat and hybrid cell lines showed either partial identity or no cross reactivity at all with the purified ligandin (263). In adult rat hepatocytes in culture hepatocytes in culture ligandin is only produced during the early lag phase and the stationary phase of growth, and was markedly decreased during the proliferative phase (264). Other mammalian cell lines have also been shown to have GSH-T activity (265).

Table 15.1. CHARACTERISTICS OF THE TRANSFERASES ISOLATED FROM RAT LIVER AND KIDNEY

SOURCE	GSH-T	MOL. WT. daltons	pI	SUBUNIT SIZE daltons	REFS.
Rat liver	ligandin	37-56,000	8.7-9.1	22,000 & 24,000	4,5,6,8,18 256,295
	T/F AA	45,000	9.6-10	23,000	197,256
	T/F A	45-50,000	8.8-8.9	23-24,000	196,198,156
	T/F B	45,000	9.8	22,000 & 24,000	22,127,256
	T/F C	45-54,000	7.7-8	23-24,000	22,127,198
	T/F E	40-45,000	7.1-7.3	24,700	193
	T/F M		6.9-8.1		187
	mitochondrial	45,000 88,000			271
Rat kidney	ligandin	44,000	9.0	22,000	131,256,470

Table 15.2. CHARACTERISTICS OF THE TRANSFERASES ISOLATED FROM DIFFERENT ORGANS AND SPECIES

SOURCE	MOL. WT. daltons	pI	SUBUNIT SIZE daltons	REFS.
Housefly	50,000		23,000	225
Cockroach	35-37,000			224
Pig liver	51,600	7.3-7.7	27,000	205,244
Sheep liver	80,000	6.5-7.5	23,500	200
Sheep lung	45-47,000		23,500	309
Monkey liver	48,000		24,000	246
Bovine lens	49,000	7.4 & 5.6	23,000	259
Guinea pig liver	45-47,000		25,000	244
Hamster liver		9.0		242
Chicken liver	45,000	8.9 & 7.8	21,000	237

CHAPTER XV

PHYSICOCHEMICAL CHARACTERISTICS

Molecular size:-

GSH-T's purified from different organs of different species have been reported to have molecular sizes ranging from 39,000 to 80,000 daltons (table 15.1). However these differences may be due to methodological artefacts rather than biological variation. A comparative study of the GSH-T's from housefly, rat and mouse showed that on gel filtration the elution volume of the GSH-T's depends critically on the buffers used and the presence of stabilisers such as glycerol and EDTA (both used in the Jakoby method of purification) (266). GSH-T activity from all three species had an identical elution volume in any given system, but the molecular weight varied from 43,000 daltons in a Tris buffer with potassium chloride to 53,600 daltons in Tris buffer without potassium chloride. Crude enzyme and highly purified enzyme had the same elution volume indicating that molecular size did not change during purification (266).

It is generally accepted that rat liver GSH-T's are between 45,000 and 47,000 daltons in size, and that the human GSH-T's are slightly larger at 48,000 daltons (199). The GSH-Ts have all been shown to be dimers, irrespective of tissue of origin (table 15.1 & 15.2).

Rat liver GSH-T's:-

In addition to the seven rat liver GSH-T's (AA, A, B, C, D, E and M) a more acidic GSH-T with a pI of 6.6 has been detected by isoelectric focusing of whole rat liver cytosol (251). This may correspond to

GSH-T M, but has not yet been shown to do so. The physicochemical characteristics of rat liver ligandin have already been discussed. The other rat liver GSH-T's have not been as well studied.

Until recently the GSH-T's were thought to be limited to the cytosol, but reports of GSH-T activity associated with mitochondrial and microsomal fractions (267-270) led to the isolation of three GSH-T's from rat liver mitochondrial matrix (271). Unbroken mitrochondria had little activity (272). Mitochrondrial GSH-T's have been estimated to contribute about 7% of the mitochrondrial protein and are thought to account for approximately 7% of total hepatic glutathione GSH-T activity (268).

Microsomal GSH-T's do not appear to be adsorbed to the membranes, but are thought to be an integral part of the membrane. They resist separation despite several procedures designed to remove loosly bound proteins from membranes (273). Unlike cytosolic GSH-T's the microsomal GSH-T's are activated by agents reacting with sulfhydryl groups. In the absence of the sulphydryl reactive agents, N-ethylmaleimide or iodacetamide, activity of the microsomal GSH-T's was 5-10% of the total GSH-T activity, but in the presence of these agents microsomal activity increases to 30% of total GSH-T activity. Whether the sulfhydryl group was on the GSH-T molecule or on a neighbouring molecule has not investigated (274). Membrane bound GSH-T activity in decreasing order is as follows: rough endoplasmic reticulum, microsomes, smooth endoplasmic reticulum. Other membranes have little activity (272). Membrane GSH-T's were found to resemble the cytosolic GSH-T's A, B and C, but are not inducible with phenobarbitone. Others have found GSH-T activity associated with washed plasma membranes (275).

Rat GSH-T's in other tissues:-

Ligandin has been purified from rat kidney and shown to be immunologically similar to the liver enzyme and to have similar enzyme activity (131). Rat renal Y protein has three bands on SDS-PAGE, Ya, Yb and Yc (18). Rat renal GSH-T activity can be separated into three peaks using isoelectric focusing (136). The most basic peak with a pI of 9.0 is immunologically similar to ligandin. The second peak (pI 8.5) was only poorly recovered on refocusing and could not be assessed further. The third peak focusing at pI 7.0 was different from any of the hepatic GSH-T's separated in the same way. By comparing the elution profile of liver cytosol from CM-cellulose with the elution profile of kidney cytosol under identical conditions, Scully and Mantle (276) showed that the kidney contained a small amount of GSH-T AA, a considerable amount of GSH-T B and a third form, GSH-T X, which eluted from CM-cellulose at a higher ionic strength than GSH-T C did in the corresponding liver cytosol analysis. This peak probably corresponds to the peak at pI 7.0 described above. They were unable to demonstrate a GSH-T corresponding to GSH-T A or C, both of which migrate as YbYb in SDS-PAGE. The Yb subunit detected by Bass et al in kidney cytosol is therefore as yet unexplained.

A testicular protein with GSH-T activity has been purified (277). On SDS-PAGE it consists primarily of a 25,000 daltons subunit running with the mobility of Yc. There is very little of the Ya subunit, in fact in some purifications it does not appear (262,277). This protein has been shown to cross-react with antibodies to rat liver ligandin and

to have similar enzyme activity with CDNB and has therefore been called ligandin, but it lacks a high affinity binding site for bilirubin.

Using elution of cytosol from CM-cellulose, rat testis has been shown to have a considerable amount of GSH-T AA and C, less GSH-T A and only a small amount of GSH-T B (276). The subunits of each GSH-T described in testis correspond to those described in liver. Of interest is the fact that the YcYc dimer corresponds to GSH-T AA and the GSH-T B is a YaYc dimer. To date no YaYa dimer has been shown in the testis (276). In this separation procedure there is no GSH-T with subunits corresponding to those described for testis ligandin, i.e. mostly Yc with about 10% Ya. The protein purified by Bhargava et al may thus not be homogenous and may comprise a mixture of GSH-T B and GSH-T AA.

Purification of the pulmonary transferases showed similar profiles to the liver enzymes (277) using similar purification techniques, but using hexyl-GSH affinity chromatography a peak of activity with CDNB was found which was not present in liver (203).

Human GSH-T's have been discussed previously (see chapter II).

CHAPTER XVI

CATALYTIC ASPECTS

Kinetics:-

Only rat GSH-T A and M have been subject to investigation of their reaction mechanisms. Gillham (195) has presented evidence that for purified GSH-T M, the reaction mechanism with 1-menaphthyl sulphate is of the form termed Ordered Bi Bi, which can be represented thus:

$$E + A = EA$$

$$EA + B = EAB = EQ + P$$

$$EQ = E + Q$$

E = enzyme A = first substrate

B = second substrate P = first product

Q = second product.

GSH-T A has been investigated by two groups with conflicting results. The NIH group (196) proposed a mechanism whereby at low substrate concentration (GSH less than 0.1 mM) the reaction could be described by a Ping Pong mechanism where the first product leaves the enzyme - first substrate complex before addition of the second substrate (right hand wing of fig. 16.1). At higher concentrations of GSH (greater than 0.15 mM) an ordered sequential mechanism is operative (left hand wing of fig. 16.1). Here GSH adds on first, followed by the second substrate. This is the nature of the reaction which is likely to occur in vivo since the physiological concentration of GSH in the liver is about 5 mM.

Mannervik and Askelof however dispute the Ping Pong mechanism and claim that kinetic analysis and statistical comparison of model rate

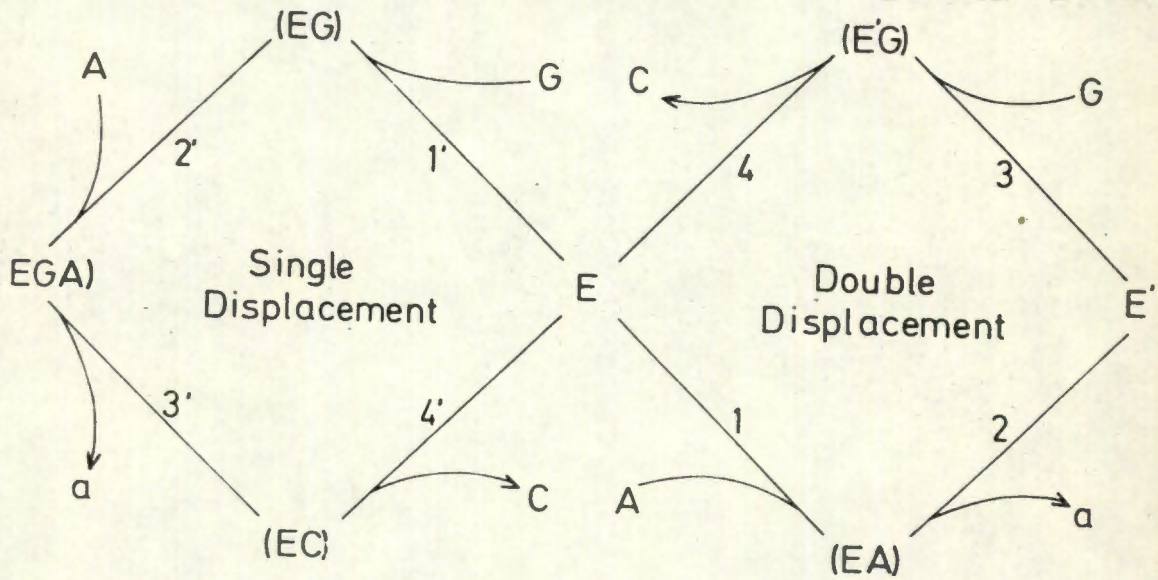


Fig. 16.1. Kinetics of catalysis by glutathione S-transferase A. The reaction proceed counterclockwise from E, the free enzyme, in each case. The left hand wing represents the ordered sequential mechanism which is possibly the physiological mechanism, and the right hand wing represents the 'ping - pong' mechanism. G = glutathione, A = an electrophilic substrate, a = leaving group, C = GSH-conjugate. (From Pabst et al [196]).

equations of the form applicable to a Ping Pong pathway and a random sequential pathway show that using the same substrate as before, the random sequential model fits the experimental data better than the Ping Pong model (278-280). This issue is not resolved. Binding of substrates to GSH-T A is hyperbolic, with a 2:1 stoichiometric ratio (substrate:enzyme). At higher ligand concentrations there is a weaker, probably non-specific binding (281).

Mechanism:-

A wide range of compounds have been described as substrates for the GSH-T's. Criteria used for detection of such substrates include detection of enzyme catalysed formation of GSH adducts in tissue preparations and bile, and the detection of mercapturic acids in the urine. Work with purified enzymes has involved a more restricted range of substrates.

An essential requirement for a compound to be a substrate for the GSH-T's is that it must have a lipophilic centre. This is a prerequisite for binding to the enzyme (282,283). The leaving group of the substrate may be a halide, sulphate, nitro, or methyl group. Glutathione may add on across the oxirane ring of an arene oxide, or arylhalide epoxide, or it may add on across the ethylenic bond of an α-β unsaturated compound. The electrophilic domain may be a carbon, nitrogen or sulphur atom. The compound can be aliphatic, aromatic or heterocyclic. The wide variety of substrates utilised by the GSH-T's are the subject of several reviews (1,2,3,284-286). The dealkylation and dearylation of phosphoric acid triesters, many of them organophosphate insecticides, are also catalysed by the GSH-T's (for review see refs

222,226,287). More recently some of the GSH-T's have been shown to have glutathione peroxidase (GSH-Px) activity (288) and to be responsible for the soluble Δ^5 3-ketosteroid isomerase ($\Delta^5$3-KSI) activity in the liver (see later)(289).

Many substrates conjugate to GSH at an appreciable spontaneous rate, making the enzymatic catalysis difficult to detect. Consideration of the reaction with organic nitrates and thiocyanates led to the formation of a hypothesis of enzyme mechanism which is now generally accepted. It was proposed (290) and subsequently demonstrated (283) that the diverse reactions of the GSH-T's can be explained on the basis of nucleophilic attack by the enzyme bound GS^- thiolate ion on an electrophilic centre on the second substrate.

The enzyme may increase the nucleophilicity of bound GS^-, in a manner analagous to the behaviour of cysteine thiols in protein (291), but there is no definite evidence for this. Currently it is thought that the sole effect of the enzyme is to bring GSH into suitable apposition with the hydrophobic substrate (292). If the electrophilic group is sufficiently reactive and suitably apposed to the GSH moiety the reaction will occur. Hydrophobic molecules which lack an electrophilic centre may theoretically bind to the enzyme at the same site and act as a competitive inhibitor, and indeed this does occur. Many of the nonsubstrate ligands bound by the GSH-T's fall into this category. Conversely a polar molecule with an electrophilic domain would not be expected to be a substrate. For example, disulphide interchange (eqn 16.1) is a special example of nucleophilic attack on sulphur (282).

$$GSH + RSSR' = GSSR' + RSH \qquad (16.1)$$

This reaction is not catalysed by the GSH-T's if R and R' are polar groups, but if these polar groups are blocked, as with hydrophobic derivatives of L-cystine and GSSG, the GSH-T's do catalyse the reaction.

Similarly maleic/fumaric acid isomerization is not catalysed by the GSH-T's, but more hydrophobic analogues e.g. maleylacetone and maleylacetoacetic acid do undergo GSH-T catalysed isomerization (282). Diethyl maleate does not undergo isomerization, but is subject to thioether formation with GSH, which is also catalysed by the GSH-T's.

Therefore the reason that some nucleophilic reactions of GSH are not catalyzed may be due to poor binding of the second substrate. One can thus propose that any compound containing a sufficiently electrophilic centre and a sufficiently hydrophobic domain is a potential GSH-T substrate. This mechanism can explain all the known forms of GSH-T activity:- organic nitrate reductase, GSH peroxidase, $\Delta^5$3-KSI and prostaglandin isomerase.

The GSH-T's are not good catalysts by enzyme standards, but what they lack in efficiency is made up in versatility. They comprise a low affinity, high capacity system, ideally suited to handle the vast array of substrates presented to them.

Despite the wide array of known substrates only a small number are required to study the rat GSH-T's. GSH-T AA and B are similar catalytically and may be structurally related, but AA is inhibited to a much greater extent than GSH-T B by indocyanine green. Both GSH-T A and C have aryl GSH-T activity (198), but GSH-T A is inactive or nearly so with alkene substrates. GSH-T B can be distinguished from GSH-T A and C by its lack of reactivity with DCNB. GSH-T E is the only GSH-T that

has high activity with 1,2-epoxy-3-(p-nitrophenoxy)propane, and only GSH-T M has appreciable activity with menaphthyl sulphate.

GSH Peroxidase:-

The presence of enzymes catalysing the reaction

$$H_2O_2 = H_2O + O_2 \qquad (16.2)$$

has been known for some time. One such enzyme is GSH peroxidase (GSH-Px)(293) which has been demonstrated in a variety of tissues. The reaction catalysed is

$$H_2O_2 + 2GSH = GSSG + 2H_2O \qquad (16.3)$$

This enzyme is also able to use organic hydroperoxides as substrates. Cumene hydroperoxide is a commonly used example.
The enzyme has a molecular size of 84,000 daltons, and comprises four identical subunits and four molecules of selenium. However another enzyme active with organic hydroperoxides and not with H_2O_2, which was not a seleno-enzyme, was found in rat brain and liver cytosol (294,295). Suggestions that some of the GSH-T's had similar kinetics and inhibition kinetics led to the demonstration that in the rat partially purified GSH-T's (288), and purified GSH-T B and AA were able to catalyse the reaction (296,297). The reaction is presumed to occur via the formation of an intermediate sulfenic acid of GSH, i.e. GSOH (187).

$$GSH + ROOH = GSOH + ROH \quad \text{(enzymatic)} \qquad (16.3)$$

$$GSOH + GSH = GSSG + H_2O \quad \text{(non-enzymatic)} \qquad (16.4)$$

Similar findings have been obtained in rabbits and giunea pigs (298,299). The reaction occurs by nucleophilic attack by GS^- on the hydroperoxide. GSH-T, or the non-seleno enzyme GSH peroxidase ac-

tivity, accounts for 23-38% of peroxidase activity in rat adrenal, liver, kidney, brain and fat, and 91% in testis. Spleen, heart, lung and intestinal epithelium have no non-seleno enzyme GSH peroxidase activity (300). This implies that the GSH-T of these tissues do not have GSH-peroxidase activity. Apart from GSH-T B and AA none of the other GSH-T's have been reported to have GSH-Px activity. There is considerable species variation, ranging from 45% of total GSH-peroxidase activity accounted for by the GSH-T's in the hamster liver to 100% in the guinea pig liver. Eighty four percent of human hepatic GSH-peroxidase activity is non selenium dependent (300).

Burk et al (296) also showed that purified GSH-T B had GSH peroxidase activity and that this activity could be induced by phenobarbitone and in selenium deficiency. The isolated perfused liver from selenium deficient rats was able to produce GSSG when perfused with t-butyl hydroperoxide (301). These results indicate a possible physiological function for the non selenium dependent GSH-Px activity provided by the GSH-T's (see later).

Organic nitrate reductase (E.C. 1.7.99.4):-

Hepple and Hilmoe in 1950 (302) showed enzyme catalysed formation of inorganic nitrate and reduced GSSG from GSH and nitroglycerine or erythritol tetranitrate.

$$RCH_2ONO + 2GSH = RCH_2OH + HNO_2 + GSSG \quad (16.6)$$

Ohkawa et al (303) showed that the general reaction

$$RCH_2SCN + 2GSH = RCH_2SH + GSSG + HCN \quad (16.7)$$

was catalysed in mice and houseflies by a group of soluble enzymes and speculated that the GSH-T's might be responsible. Jakoby's group show-

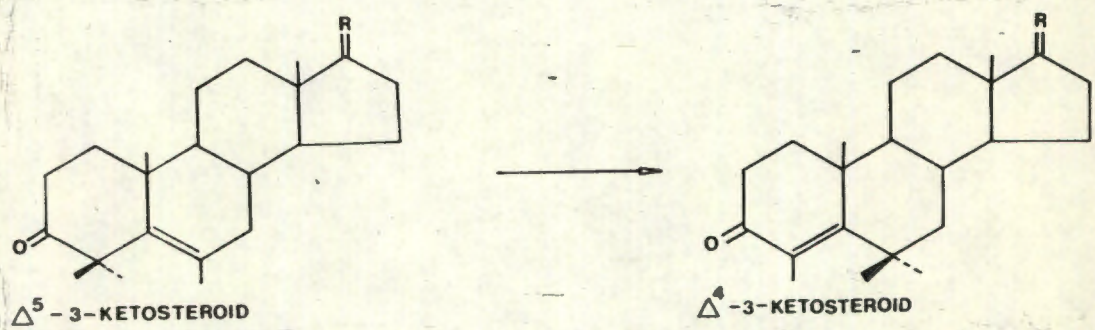


Fig. 16.2. Isomerization of Δ5 3-keto steroids to Δ4 3-keto steroids. In liver cytosol this reaction may be catalysed by GSH-T.

ed these two reactions to be catalysed by purified GSH-T's A, AA, B, C, β, and δ (283,290). Theoretically since the GSH-T's catalyse thioether formation there is a presumed disulphide intermediate (eqn. 16.8) which is unstable and decomposes (eqn. 16.9) to form the product (283,290).

$$RSCN + GSH = RSSG + HCN \qquad (16.8)$$

$$GSH + RSSG = GSSG + RSH \qquad (16.8)$$

Similarly, for organic thiocyanates

$$RCHONO + GSH = GSNO_2 + RCHOH \qquad (16.10)$$

$$GSNO_2 + GSH = GSSG + HNO_2 \qquad (13.11)$$

The presence of the intermediates was confirmed by adding a non-substrate thiol to the incubation mixture, whereupon the concentration of GSSG formed was decreased (283) to an extent that could not be explained by disulphide interchange of the form:-

$$GSSG + RSH = GSSR + GSH \qquad (16.12)$$

<u>Δ^5-3-Keto steroid isomerase (E.C. 5.3.3.1):-</u>

The isomerization of Δ^5 steroids to Δ^4 (fig. 16.2) in steroid producing tissues is catalysed by an enzyme occurring in the microsomes requiring NAD^+ and NADH (304,305). However in rat liver cytosol an enzyme with similar activity, dependent on GSH, was found (306), which was identified as GSH-T B (289). Other rat GSH-T's were less active. All the basic human GSH-T's had activity to varying degree (289). No intermediate conjugate has been found and GSH is not consumed. This subject is discussed further in consideration of the interaction of ligandin and steriod hormone metabolism.

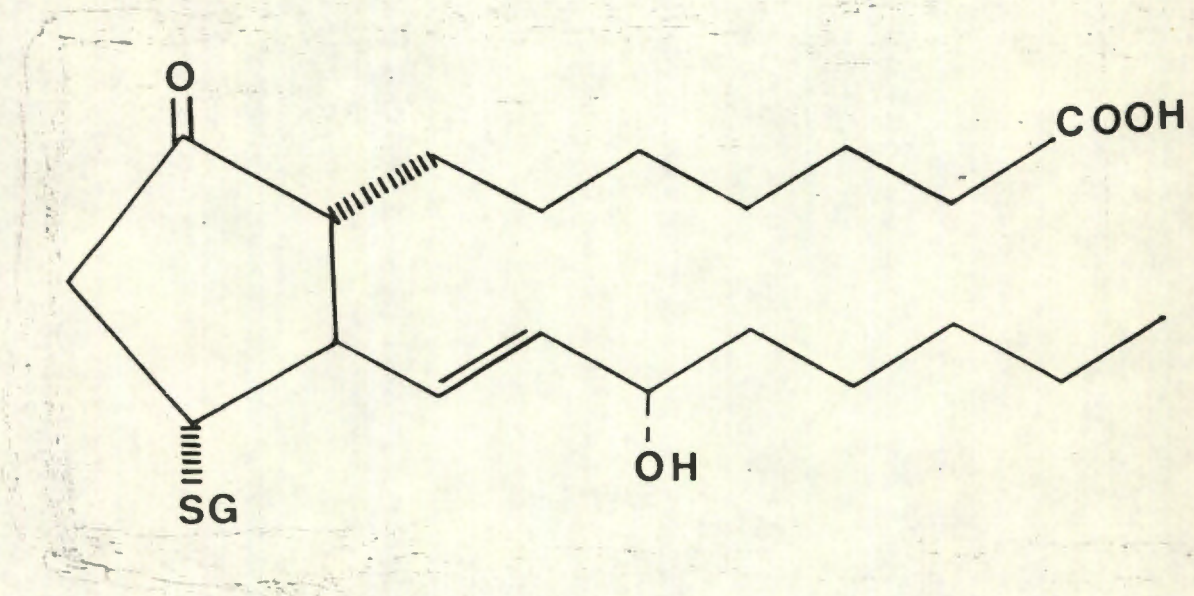


Fig. 16.3. Prostaglandin A_1 - glutathione conjugate. Conjugation occurs at the cyclopentenone ring.

Prostaglandin (PG) Metabolism:-

Several PG derivatives may form GSH conjugates. PGA_1 conjugation with GSH is catalysed by the GSH-T's (307). The formation of a GSH adduct of 15-keto $PGF_{2\alpha}$ is inhibited by the presence of PGA_1 (308). PGA_1 is conjugated to GSH at the cyclopentenone ring, (fig. 16.3), whereas the 15-keto $PGF_{2\alpha}$, is conjugated at the 15-C position. This latter conjugation is also presumed to be catalysed by the GSH-T's.

A preparation from sheep lung with GSH-T activity resembling ligandin in substrate specificity, molecular size, subunit's and pI was shown to catalyse formation of PGD_2, PGE_2 and $PGF_{2\alpha}$ from PGH_2 (fig. 16.4)(309,310). Rat liver GSH-T's also catalysed the reaction to form $PGF_{2\alpha}$, but formation of PGD_2 and PGE_2 was more variable. No No GSH adduct could be demonstrated. This reaction was not catalysed by GSH peroxidase (the seleno-enzyme).

A preparation of bovine vesicular gland microsomes was able to catalyse the conversion of PGH_1 to PGE_1. This enzyme also required GSH but has not been characterised (311).

Slow reactive substance of anaphylaxis (SRS-A) is a GSH conjugate of hydroperoxyeicosatetraenoic acid (HPETE) (fig. 16.5). The parent compound undergoes initial epoxidation at the 6-7 position and is subsequently conjugated to GSH. It is not known whether this reaction is catalysed by the GSH-T's or occurs spontaneously (312).

Despite the above findings the exact role of the GSH-T's in PG metabolism is not known.

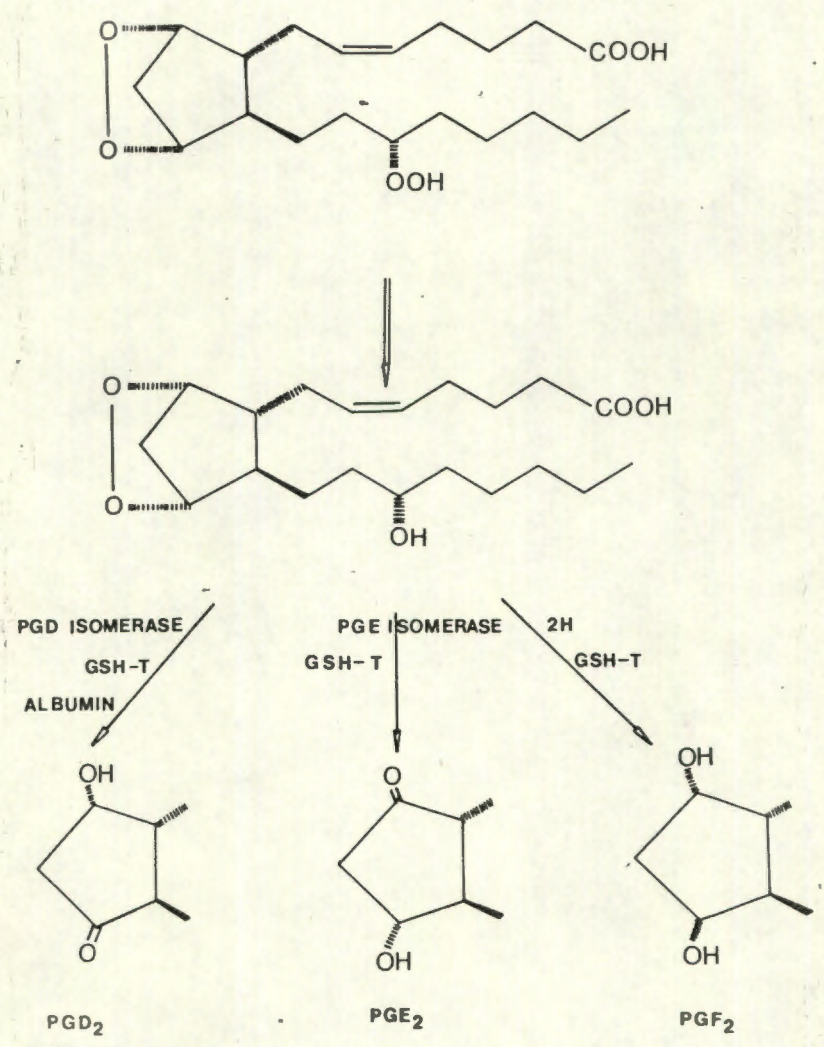


Fig. 16.4. The glutathione S- transferases and prostaglandin metabolism in a sheep lung preparation. (1) 15-hydroperoxy PGH_2. (2) 15-hydroxy PGH_2. The isomerization to form PGD_2 is possibly not a specific enzymic isomerization, but may be a non-specific reaction which occurs in the presence of protein e.g. albumin.

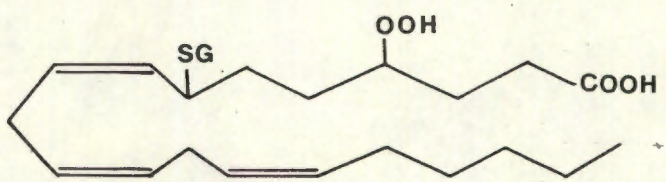


Fig. 16.5. Molecular structure of slow reactive substance substance of anaphylaxis A (SRS-A). This is a GSH conjugate of hydroperoxy-eicosatetraenoic acid.

CHAPTER XVII

NON-SUBSTRATE BINDING

Non-covalent binding:-

In addition to their activity as enzymes (GSH-transferase, prostaglandin isomerase, Δ^5-3-ketosteroid isomerase, organic nitrate reductase, GSH peroxidase) the GSH-T's as a group also bind a wide range of ligands which do not undergo GSH conjugation or other GSH-transferase mediated modifications. All the rat and the cationic human GSH-T's have this activity (table 17.1)(41). Binding affinities for the ligands vary. The binding activity of ligandin is the best studied. Ligandin was originally conceived of as an organic anion binding protein. It soon became apparent that the major requirement for binding is hydrophobicity, and like binding to albumin in the serum, binding to ligandin is non-specific. This binding may serve in part to solubilize hydrophobic molecules in cytosol, much as albumin does in the serum (313). Tables 17.2 to 17.5 list some of the non-substrate ligands which have been shown to bind to ligandin. A variety of methods have been used to study binding, including circular dichroism (20), equilibrium dialysis (42,314), bilirubin difference spectroscopy (315), fluorescence quenching (41) and binding to the Y peak on gel filtration (8). It should be noted that binding to the Y peak includes binding to all the GSH-T's, and not to ligandin alone.

The chemistry of non-substrate binding to ligandin has been discussed in detail in chapter III.

Table 17.1.

DISSOCIATION CONSTANTS FOR SOME RAT & HUMAN GSH-T'S

ligand	K_D for GSH-T- (μM)								
	AA	A	B	C	α	β	γ	δ	ε
Bilirubin	100	15	2	2	65	110	34	18	34
monoglucuronide	4	2	3	6	—	—	—	5	5
diglucuronide	40	23	10	20	—	—	—	33	121
Indocyanine green	100	3	3	1	20	—	—	20	—
Haematin	4	2	0.1	7	10	—	—	10	—
Cephalothin	700	600	2400	800	—	—	—	—	—

Table 17.2.

LIGANDS BINDING TO PURIFIED LIGANDIN

Organic anionic dyes	Ka	REFS
BSP	167×10^5	42,512
	6×10^6	4,514
ICG	3×10^6	20
	3×10^5	196
3,6-DBSP	6×10^4	41
Evans Blue	10^6	131
BSP-GSH		131,20
Antibiotics		
Penicillin	10^3	20
Sulphonamides		521
Tetracycline	10^4	514
Chloramphenicol	10^4	20,
Cephalothin	10^2	519
Polymyxin B	$0-10^2$	131
Bile Salts and Bile Acids		
Deoxycholate	5×10^4	42,514
Taurodeoxycholate	5×10^4	42,514
Chenodeoxycholate	5×10^4	42,514
Cholic acid	2×10^4	42
	10^4	514
Lithocholate		465
Fatty Acids		
Palmitate	10^4	42
Oleate	10^4-10^6	20,38,512

Table 17.3.

LIGANDS BINDING TO LIGANDIN

Radiographic Contrast Media	Ka	Refs.
Iodipamide	10^3	20,512
Iophenoxic acid	10^2	512
Telepaque		512
Hypaque		326
Cholegrafin		512
Diodrast		512
Porphyrins and Derivatives		
Bilirubin	5×10^7	20,41,315
	7×10^6	515
Haematoporphyrin	5.9×10^6	20,472,512
	10^6	
Coproporphyrin I & III	2.6×10^5	472,513
Uroporphyrin I & III	3×10^4	472
Protoporphyrinogen IX		514
Haemin	10^5	20,512
Heamatin	10^8	472
Bilirubin monoglucuronide	3×10^5	461
Bilirubin diglucuronide	10^5	461
Thyroid Hormones		
T_3	10^5–10^6	20,512
T^4	10^4–10^5	20,512

Table 17.4.

LIGANDS BINDING TO LIGANDIN

Steroid Hormones & Metabolites	Ka	refs.
Cortisol	$2x10^5$-10^5	20,512
Oestradiol 17beta	10^5	42,514
Oestrone	10^5	42,514
Oestrone sulphate	4.9-$6x10^5$	42,514
Testosterone	$2x10^5$	42,514
Dihydroepiandrosterone	2.1-$2.5x10^5$	42,514
Pregnenolone sulphate	2.1-$2.5x10^5$	42,514
2-OH oestradiol-GSH conjugate	4.8-$6x10^5$	42,514
Oestrone glucuronide	0.5-$0.9x10^5$	42,514
Diethylstilboestrol	$2x10^5$	42,514
Carcinogens and Metabolites		
N'N' Dimethylaminoazobenzene	$8x10^4$	514
	$5x10^4$	42
N' Methylaminoazobenzene-GSH	3.2-$4x10^5$	9,42,442 514
3-Methylcholanthrene	$4x10^5$	42,514
20-Methylcholanthrene		42
3-Methylcholanthrene metabolite		7,14,15
Benzo(a)pyrene	10^5	20,42

Table 17.5.

LIGANDS BINDING TO Y PROTEIN ON GEL FILTRATION

	Refs		Refs
Gastrin	516	Nitrofurantoin	519
Penicillin	131	Protoporphyrin	514
Phlorizin	131	Coproporphyrin	514
Acetyl salicylic acid	469,470	Folic acid	518
Furosemide	469	Cephalexin	519
Tolbutamide	517	Iopanoic acid	520
Phenolsulphophthalein	131		

N.B. Recently evidence has appeared showing that within the Y peak on gel filtration there are several binding proteins unrelated to ligandin, and therefore these data will have to be interpreted with caution (521).

GSH-T A has been shown to have a single binding site for bilirubin, but the other rat GSH-T's have not been investigated in this respect (41).

Bilirubin binding to the cationic human GSH-T's (ligandin) is complex. There are two binding sites. The GSH-T-bilirubin complex is catalytically active, but at pH 6.5 slowly loses catalytic activity. In the presence of GSH, catalytic activity decreases more slowly and to a lesser extent (316). These studies only considered the cationic GSH-T's.

Covalent binding:-

In addition to non-covalent binding, ligandin and the other GSH-T's are able to bind covalently to certain highly reactive compounds, such as the oxidation products of 3-methyl cholanthrene (3-MC) and benzo(a)pyrene (BP)(26,317). GSH-T A and B in the absence of GSH will bind covalently to CDNB (196,199). Aminoazodye carcinogen binds covalently to ligandin via the thiol groups of cysteine (23). The majority of the cysteines reside on the Yc peptide, and therefore it would be expected that Yc would be the site of covalent binding, and indeed, the activated carcinogens 3-MC and dimethylaminoazobenzene (DAB) bind to Yc in vivo (26). Ethacrynic acid also binds covalently to the GSH-T's. This is the only described instance of a compound binding covalently to the GSH-T's without undergoing prior oxidative metabolism (318).

Inhibition:-

A large number of compounds which inhibit the GSH-T reaction have been identified, some of which are listed in table 17.6. Examples of competitive, non-competitive and uncompetitive inhibition have been described. It is difficult to identify physiologically important inhibitors from the wealth of data available. Two aspects are worth mentioning. Firstly organic solvents in which many of the substrates are dissolved for laboratory studies, are themselves inhibitors (319). This is of importance inasmuch as substrates used by different investigators may be dissolved in different solvents, thereby making comparisons of results difficult.

The second point of interest is the fact that metals, metallic salts and organometallic compounds may inhibit GSH-T activity (320, 321). Further it is known that several metals and their salts may decrease hepatic GSH, and a GSH conjugate of cadmium chloride has been described (322-325). The mechanism of reaction is unknown, nor is it known if this reaction is catalysed or spontaneous.

Other known inhibitors include radiographic contrast media (326), non-substrate ligands, such as bilirubin and indocyanine green (41), and steroid sulphates (327). Product inhibition has also been reported (175,185,195,198).

Table 17.6.

INHIBITION CONSTANTS FOR RAT GSH-T'S WITH CDNB AS SUBSTRATE

ligand	Ki for GSH-T (μM)			
	AA	A	B	C
Bilirubin	7	2	6	2
Indocyanine green	100	0.02	3	0.01
3,6-dibromsulphophthalein	180	22	12	30
Cephalothin	440	200	1500	200

CHAPTER XVIII

REGULATION

Genetic regulation:-

Rat liver GSH-T's are similar in size and have a wide overlap of substrate specifity. The individual GSH-T's have amino acid analyses which are sufficiently similar to suggest that there may be a common origin, but the number of separate gene products are unknown. Based on their belief that GSH-T AA is a YcYc dimer and that GSH-T B consists of both a YaYc and YaYa dimer, Scully and Mantle (276) have proposed that a single gene product gives rise to a YcYc dimer, which is broken down to the Yb and Ya subunits by several peptidases variably present in different organs. Currently available immunological evidence suggests that ligandin is not that closely related to the other GSH-T's. Since conversion of Yc to Ya occurs in the presence of enzyme inhibitors such as EDTA, and since degradation occurs in highly purified preparations, the action of a peptidase is unlikely. However trace amounts of contaminating proteolytic enzymes in an otherwise pure preparation could be responsible for breakdown. An alternative hypothesis is that YaYc is the gene product, and with aging or following phenobarbitone induction, spontaneously loses a $\pm$30 amino acid peptide from the C-terminal end of the Y-chain, to give YaYa. More recently work on the mRNA for ligandin has shown that phenobarbitone induction produces an excess of Ya mRNA as compared to Yc mRNA (328). This suggests that Ya and Yc are separate gene products, that only Ya is inducible, and is in agreement with the findings of Bass et al (262).

GSH-T A and C have similar amino acid analyses, are immunologically identical, and are possibly the products of a single gene, which have undergone post-translational modification. Antibodies to rat liver GSH-T's B and E react only with the eliciting antigen, and do not react with other GSH-T's.

In the human the five cationic GSH-T' are immunologically and structurally similar and are thought to be the products of a single gene. The relationship of the cationic GSH-T's to the anionic GSH-T's is at present not established. Analysis of a large number of human liver homogenates on starch gel electrophoresis showed three groups of GSH-T's, which were asssigned to three genetic loci, named GST 1, 2 and 3 (329). GST-1 was represented by an group of three anodally migrating isoenzymes. This group was not always present. Hardy-Weinberg equilibrium could be achieved if these isomers represented products of a locus with three alleles, two functional and one nul allele. This group would correspond to GSH-T υ. Similarly a cathodally migrating group of GSH-T's could be shown to be consistent with a locus with two alleles, (GST-2). This would correspond to the cationic GSH-T's. A third locus, GST-3, represented by an anodally migrating GSH-T, but faster moving than GST-1, was the product of a single allele, and may represent GSH-T ω. The implication of these studies is that each monomer is coded for by a different allele. This work requires confirmation by running purified human hepatic GSH-T's in the same system for comparison.

Hormonal regulation:-

Of the GSH-T's, only ligandin has been studied to any extent as an individual protein. For the most part changes in enzyme concentration have been detected by following changes in enzyme activity with one or other substrate. Such measurements may be of physiological significance, but they do not reflect changes in individual GSH-T levels.

A sex difference in concentration of the GSH-T's in the liver has been noted several times (179,330-334). Aryl, aralkyl, epoxide and alkyl activities are lower in the female (330), but using the ratio of activity of CDNB and DCNB and immunoprecipitation as a guide to ligandin concentration others have found the male liver to have less ligandin than the female (331).

Hypophysectomy and thyroidectomy both increase ligandin concentration in the rat liver, apparently by stabilising the protein, thereby decreasing the rate of catabolism (335). In these studies ligandin was measured immunologically, but others, using the combination of immunoprecipitation, and the ratio of activity with CDNB and DCNB have shown that hypophysectomy decreases ligandin in the female liver but has no effect in the male (331). These conflicting data can only be resolved when studies measuring the concentration of individual GSH-T's immunologically, or by e.g. isoelectric focusing, are performed.

Hypophysectomy decreases aryl hydrocarbon hydroxylase activity in the testis. This could be reversed with follicle stimulating hormone (FSH) and luteinizing hormone (LH). Similar changes were seen with cytochrome P-450 and epoxide hydrase, but GSH-T levels, using benzpyrene-4,5-oxide as the substrate, were unchanged, either by hypophysectomy or by replacement with FSH and LH (336).

Male gonadectomy results in a decrease in liver aryl hydrocarbon hydroxylase, epoxide hydrase, and GSH-T activity, but in ovariectomised animals there was no change. Only a single epoxide substrate, styrene oxide, was used to determine GSH-T activity (332).

Ovarian epoxide metabolising enzymes change during pregnancy and lactation. GSH-T activity increases during pregnancy and lactation and returns to normal 30 days post lactation (256). GSH-T activity in other organs did not change during this period, and there was no change with oestrus (256).

In the rat kidney variable male:female GSH-T activities have been found, depending on the substrate used (333).

Serum GSH-T activity with epoxide substrates was also found to be higher in the male.

From the above results it is not possible to come to any conclusions about the hormonal control of the GSH-T's, but it seems that thyroid hormone, and the pituitary, may play a part in the regulation of the GSH-T's. The pituitary may exert its efect via TSH. The role of sex hormones or their trophic hormones in the induction of GSH T activity cannot be assessed because studies have not separated the contribution of individual GSH-T's to net enzyme activity. The GSH-T's not subserving epoxide conjugation, e.g. GSH-T B, have not been assessed in this respect.

Ontogeny:-

At birth GSH-T activities in many organs are low relative to the adult. In the neonatal period activity increases to adult levels. In

the liver this maturation parallels the ability of that organ to take up organic anions (334,337-348). In the rat liver GSH-T B, Y protein, BSP conjugation, aryl and epoxide GSH-T activity all reach adult levels at about 30-35 days of life (337,339,344). GSH-T activity is detectable at 18-21 days of foetal life (338,342,349). Karavanova et al (350) have shown that ligandin immunofluorescence is present in more cells and is intense in the late foetal rat liver. In the first post-partum week the number of +ve cells decrease markedly, and persist only around the central vein. From day 7 post-natally the number of positive cells increase to adult numbers, which are reached at 15 - 18 days.

Low levels of ligandin in the newborn primate are thought to contribute to the development of 'physiologic jaundice' (351). Uridine diphosphoglucuronide transferase, the major enzyme responsible for bilirubin conjugation, is also low in neonates and this could also play a role in the physiological jaundice of the newborn (352,353).

A single study has shown that GSH-T levels do not change with senescence, and although epoxide hydrase levels increase, benzpyrene binding to DNA also increased with age (354).

In the steroid producing tissues the picture is somewhat different. In rat testis, activity with epoxide substrates is present at birth, and increases markedly at puberty (344). In rat ovary this activity is first detectable at day 12, increasing to a maximum at day 35 (343). In rat adrenal, activity does not change with maturation (343).

Drug induction (table 18.1 - 18.3):-

Some hepatic GSH-T activity, like the some microsomal mixed function oxidases and other cytosolic drug metabolising enzymes, are subject to induction by xenobiotics. Inducers of the MFO's can be classified into three groups. Phenobarbitone, which causes an increase in liver weight and proliferation of the endoplasmic reticulum induces the cytochrome P-450 group of enzymes. Other members of this group of inducing agents include some polychlorobiphenyls and other insecticides. 3-Methyl cholanthrene (3-MC) and other polycyclic aromatic hydrocarbons induce the so called cytochrome P-448 group of enzymes. This induction is not accompanied by endoplasmic reticulum proliferation or increase of liver weight. 2,3,7,8-Tetrachlorodibenzo-pdioxin (TCDD) and benzo-(a)pyrene (BP) are other examples of this group of inducers. Pregnenolone 16α -carbonitrile (PCN) represents a third class of inducing agents in which the substrate specifity of the induced enzymes is different from the two previous groups.

Induction of the GSH-T's has been shown with members of all three groups. However the response in various organs differs markedly.

In the mouse induction by polycyclic aromatic hydrocarbons is controlled by the Ah (aryl hydrocarbon) locus. This locus also controls induction of uridine diphosphoglucuronyl transferase and at least two other cytosolic enzymes, all concerned in the metabolism of polycyclic aromatic hydrocarbons. Discussion of this system is beyond the scope of this review (see ref 355). However it is known that in the mouse induction of the GSH-T's is not controlled at this locus (356).

Measurement of induction has been mainly by measurement of GSH-T activity with one or other class of substrate, but since any single en-

Glutathione S-transferase activity/ concentration	Inducing Agent
1-chloro-2,4-dinitrobenzene	Pb, 3-MC, TCDD, DAB, PCB's, BHA Rifampicin, Ethoxyquin, DDT,PCN
3,4-dichloronitrobenzene	Pb, 3-MC, Ethoxyquin, BHA
Styrene oxide	Pb, TCDD, PCB's, 3-MC
2,3-Epoxy-(p-nitrophenoxy)propane	BHA, Ethoxyquin, Pb, 3-MC, BPO Rifampicin
Benzo(a)pyrene-4,5-oxide	PCB, Pb, PCN
p-Nitrobenzyl chloride	Pb, 3-MC, BHA, Ethoxyquin
Ligandin	Pb, PCN, 3-MC, DDT, TCDD, TSO
Transferase A	TSO
Transferase C	TSO

Table 18.1. Induction of glutathione S-transferase in rat liver.

Abbreviations:-

Pb- phenobarbitone
CPN- 16 -cyanopregnenolone
DAB- dimethylaminoazobenzene
TCDD- 2,3,4,6-tetrachlorodibenzo-dioxin
BPO- benzo(a)pyrene-4,5-oxide
PCB- polychlorobiphenyl
3-methylcholanthrene
TSO- trans stilbene oxide
DDT- dichlorodiphenyltrichloroethane
BHA- ditert butylhydroxyanisole

Glutathione S-transferase activity/ concentration	Inducing Agent
1-chloro-2,4-dinitrobenzene	3-Methylcholanthrene
3,4-dichloronotrobenzene	3-Methylcholanthrene
Ligandin	Phenobarbitone Tetrachlorodibenzodioxin
p-Nitrobenzyl chloride	3-Methylcholanthrene phenobarbitone

Table 18.2. Induction of glutathione S-trasnsferases in rat kidney.

Glutathione S-transferase activity/ concentration	Inducing Agent
1-chloro-2,4-dinitrobenzene	phenobarbitone 3-methylcholanthrene
3,4-dichloronitrobenzene	phenobarbitone
Ligandin	phenobarbitone
p-Nitrobenzyl chloride	phenobarbitone

Table 18.3. Induction of Glutathione S-transferase in rat intestine.

zyme activity can be subserved by two or more enzymes, the changes in activity measured after induction may reflect changes in several GSH-T's. Induction has also been studied by plasma clearance and/or biliary excretion of bilirubin in vivo (357,358), and in vitro by dye binding to Y protein on gel filtration (359). While such studies may be useful in the elucidation of the functional role of the GSH-T's they are insufficient to describe the magnitude of changes in individual GSH-T's. Very few studies have measured the individual GSH-T's directly (by immunological methods or semiquantitatively on ion exchange or IEF columns). In addition the variety of dosage schedules used makes comparison between studies difficult. However some points do emerge.

Phenobarbitone induction results in an increase in the YaYa and YbYb dimers in rat liver (22,262). There is some controversy over whether the YaYc dimer can be induced. Bass et al (262) were unable to show an increase in the Yc monomer on PAGE-SDS, whereas Hayes et al (22) showed induction of GSH-T A (YbYb), but not GSH-T C (also YbYb), and induction of both YaYc and YaYa. Pickett et al (328) have shown an increase in translatable mRNA for Ya with phenobarbitone administration. Yc mRNA was only slightly induced.

Phenobarbitone causes induction of ligandin at all ages (339). Using trans-stilbene oxide GSH-T A, B, and C could also be induced in rat liver. Specific activity of GSH-T A and C were unchanged, but specific activity of GSH-T B was doubled (360). This study did not investigate which dimer (YaYa or YaYc) was induced. Ethoxyquin and t-butyl hydroxyanisole have also been shown to induce GSH-T's (361). Other substances causing induction of GSH-T activity include poly-

chlorobiphenyls rifampicin and dimethyl aminoazobenzene, and polycyclic aromatic hydrocarbons such as 3-MC, TCDD and dichlorodiphenyltrichloroethane (DDT), and a long list of insecticides (179,247,330,341,346-348, 357,362-379). Tables 18.1 to 18.3 lists some the agents which have been used to induce GSH-T activity and concentration in the rat.

In extrahepatic tissue the findings are less dramatic. Parental phenobarbitone induces renal GSH-T's but the response is less than in liver. This has been shown measuring GSH-T activity (333,362,366), and ligandin by RIA (18). TCDD induces ligandin in the kidney (131) but 3-MC treatment only results in induction of aryl and aralkyl activity (333). Epoxide GSH-T activity does not appear to be induced in the kidney (333). It is not possible to say which GSH-T's are induced in the kidney. DDT and PCN could not be shown to induce renal ligandin measured by radial immunodiffusion (131) but this has not been tested by the more sensitive radioimmunoassay. Dietary enzyme inducers e.g. Phenobarbitone and 3-MC however, fail to stimulate renal GSH-T activity (366).

Rat intestinal GSH-T's have been the source of several interesting observations. Immunoreactive ligandin is increased by phenobarbitone from 6.7 ug/mg protein to 10.1 ug/mg, a magnitude of rise too small to have been detected by immunodiffusion (18). Parenteral enzyme inducers, 3-MC, benzo(a)pyrene (BP) and phenobarbitone, increase enzyme activity only in the middle and distal intestine, the proximal intestine being unaffected (370). Others have shown activity more proximally (255). Activity in the jejunal surface and crypt cells could be induced with phenobarbitone (255). However if these drugs are administered in the diet the sites where activity is induced vary according to

the substrate used to test activity and depend also on the inducing agent used (366). These results imply that the route of administration is very important in induction of drug metabolising enzymes. Ingested drugs, which must pass through the liver before reaching the systemic circulation, are sufficiently metabolized to alter the induction pattern, and drugs gaining access to the circulation through other portals of entry e.g. skin or lungs or parenterally, may have an entirely different effect.

Rat lung GSH-T activity with benzpyrene-4,5-oxide is not inducible by phenobarbitone, pregnenolone carbonitrile, 3-MC or cigarette smoke (243). However a more sensitive radioassay using styrene oxide has shown induction by cigarette smoke but not by 3-MC up to 10 days after exposure (380). Mouse lung epoxide GSH-T activity does not respond to 3-MC or phenobarbitone (381). Again, only activity in whole cytosol was measured, and not the individual proteins.

3-MC does not cause induction of GSH-T activity in mouse skin (382).

The testes have been little studied but in the hypophysectomized rat the GSH-T activity with epoxide substrates does not change nor does it change with the administration of FSH and LH to these animals (336).

Membrane bound and organelle associated GSH-T's are not induced by with any substances studied so far (271,273), but their activity is increased several fold in the presence of sulphydryl reagents (274).

Experiments on vitamin A deficient rats have yielded conflicting results. Chhabra et al (383) failed to show any induction of GSH-T activity in the liver, lung and intestine following prolonged vitamin A deficiency. Siddik et al on the other hand, have shown induction of

GSH-T activity in the liver and kidney in vitamin A deficiency. Only enzyme activity and parameters of BSP metabolism were measured. The contribution of the individual GSH-T's was not assessed (384,385).

Low doses of vitamin C have been shown to reduce GSH-T activity (386). The significance of this finding is unknown.

Combined vitamin E and selenium deficiency results in an increase in GSH-T activity in liver, testis and brain. Since Vit A and Vit C are important antioxidants and play a part in the prevention of lipid peroxidation, it is suggested that the ultimate inducer is a lipid hydroperoxide or some other product of lipid peroxidation (387).

CHAPTER XIX

PHYSIOLOGICAL ASPECTS:- DETOXICATION

The GSH-T's are a ubiquitous, highly conserved system. The physiological function of these enzymes is not well defined. Although many chemicals can be shown to undergo GSH conjugation most are purely of research interest. Among the known substrates are many environmental pollutants and therapeutic drugs. It is not known to what extent the GSH-T's are physiologically important in the detoxication of most of these agents.

Toxic xenobiotics may cause cell necrosis (e.g. paracetamol and bromobenzene). Other xenobiotics may be carcinogenic, such as the polycyclic aromatic hydrocarbons (PAH) e.g. benzo(a)pyrene, benz(a)anthracene (BA) and 3-methylcholanthrene (3-MC). Carcinogens also have cytotoxic activity. Study of the mechanism of cell damage in either case has revealed to some extent the role of the GSH-T's in detoxification.

The organs rich in GSH-T's are strategically situated at the portals of entry to the body and thus uniquely placed to deal with toxins, the lung for inhaled toxins, the intestine for ingested toxins, and the liver sitting astride the portal system, through which must pass all toxins which elude metabolism by the intestinal mucosa. The lung, of course, also filters the entire systemic circulation, and is the first capillary bed encountered by metabolites leaving the liver. Furthermore the testis and ovary have high levels of GSH-T activity (256,336). Even the vas deferens and the epididymus have unexpectedly high GSH-T activity (388) as has the placenta (256,336). So not only are the por-

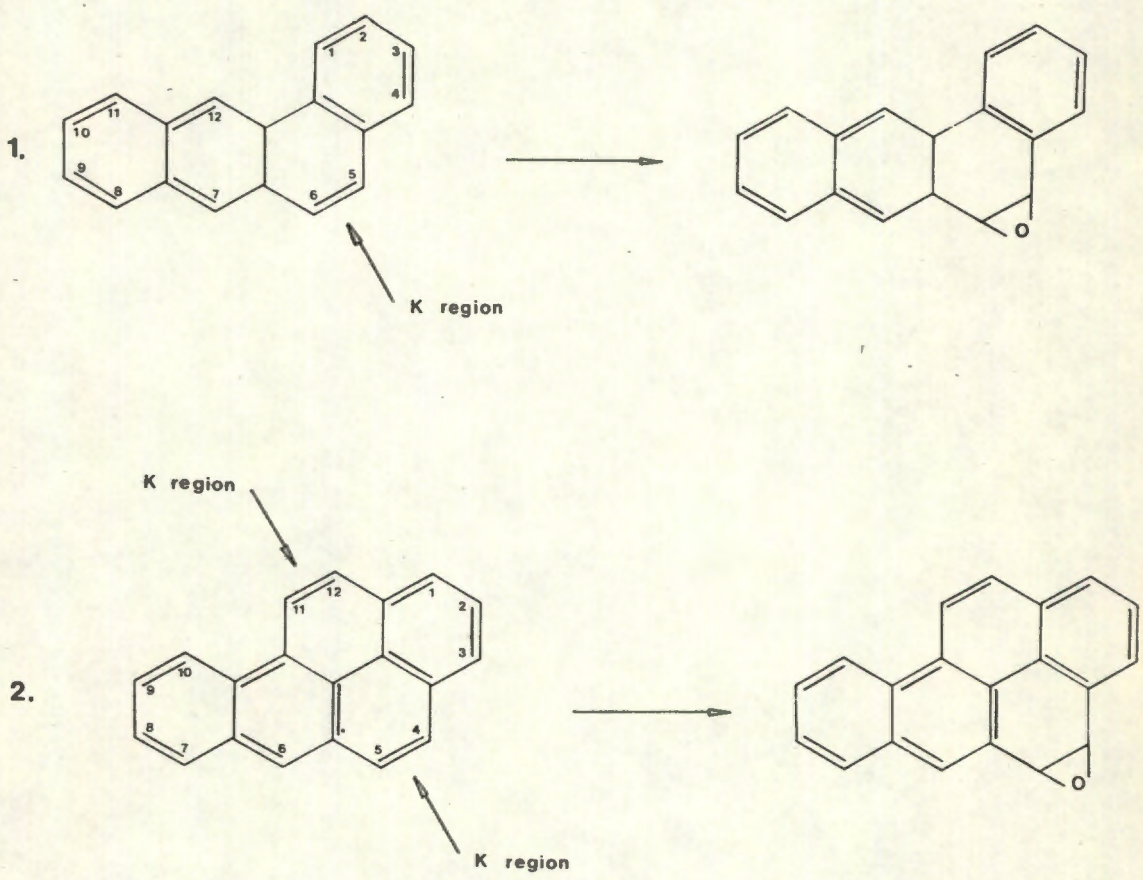


Fig. 19.1. Oxidation of benz(a)anthracene (1) and benzo(a)pyrene (2) to K-region epoxides.

tals of entry to the body guarded, but other sites particurlarly vulnerable to deleterious injury, the gametes and the developing foetus, may be protected.

Detoxification of carcinogens:-

Carcinogens are ingested, inhaled or otherwise gain entry to the organism in an inert form. However their metabolism by the cytochrome P-450 group of enzymes results in the formation of highly reactive intermediates, which in the case of polycyclic aromatic hydrocarbons are epoxides (arene oxides)(389-391).

The polycyclic aromatic hydrocarbons may be converted to either K region or non-K region arene oxides (fig. 19.1) (169,392-399). The arene oxides may then undergo one of the following metabolic transformations; enzymatic hydration to the dihydrodiol by microsomal epoxide hydrase; enzymatic reduction to the parent compound by epoxide reductase; spontaneous isomerization to the phenol; reaction with cell protein; GSH conjugation; spontaneous or enzymatic (fig 19.2)(389,400). Binding to microsomal protein is covalent and irreversible and would render the epoxide inactive. The protein is also denatured. Even though K region arene oxides are more stable than non-K arene oxides, they are better alkylating agents and more likely to undergo reaction with protein or GSH (391). The non-K arene oxides, although they do react chemically and enzymatically with GSH are not good substrates for for the GSH-T's (391,395).

The dihydrodiol may follow one of two paths. It may be conjugated to glucuronide and be excreted, or in the case of the non-K region arene oxides, the dihydrodiol may undergo a second epoxidation at the

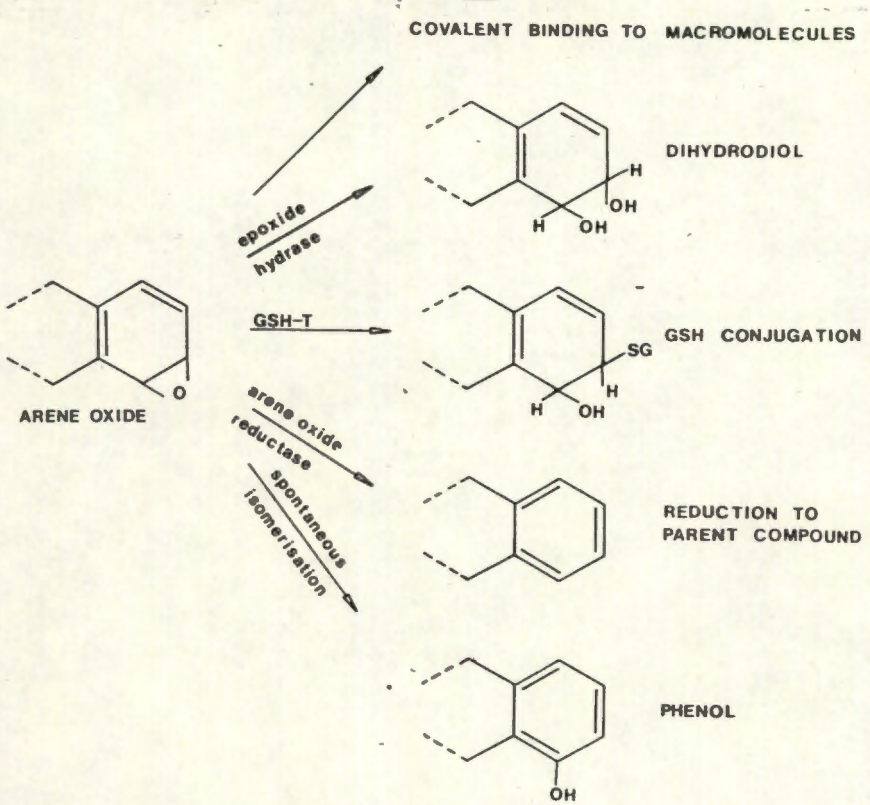


Fig. 19.2. Metabolic fate of an arene oxide (epoxide).

olefinic double bond of the dihydrodiol by the same microsomal mixed function oxidase, to form the diol epoxide (fig. 19.3)(391,401). Although the K-region arene oxides are mutagenic they are not necessarily the ultimate carcinogens. This honour probably belongs to the diol epoxides. These molecules are not good alkylating agents as such, and are therefore less reactive with GSH. They do however, react with DNA and nucleic acids (391).

Benzo(a)pyrene (BP) forms K region epoxides which are conjugated to GSH by purified GSH-T A, C and E. GSH-T B is not very active with epoxides (402). In the isolated perfused rat liver GSH conjugates formed the major biliary metabolites of BP (403,404). In the perfusate the concentration of the dihydrodiol was much higher than the thioether. Within the liver itself formation of the GSH conjugate and the dihydrodiol occurred at approximately the same rate. In the intact animal GSH conjugates predominated in the bile. Although the dihydrodiol was rapidly formed it required glucuronidation, a relatively slow process, for excretion. Both pathways therefore are important in detoxication, but the GSH pathway is the major excretory pathway (405). In rabbit lung GSH-T activity was less than in the liver, and accounted for 15% of the metabolites of BP. The lung has little epoxide hydrase activity. Only water soluble products (conjugates of GSH, sulphate and glucuronic acid) were able to leave the perfused lung. Lipophilic metabolites such as the diol epoxides accumulated in the lung (406). Calculation of kinetic constants of pulmonary benzpyrene metabolism suggested that GSH-T was more active than epoxide hydrase in metabolising benzpyrene. The lung could clear most of the benzpyrene oxide presented to it in a single pass (407). In organs other than the liver

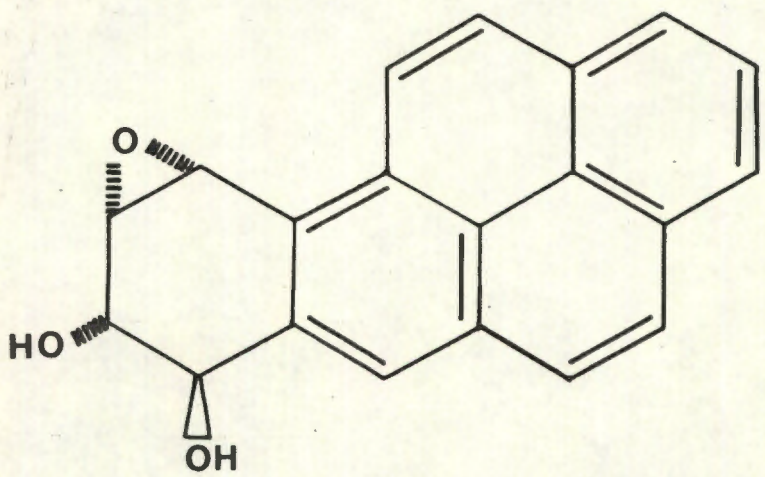


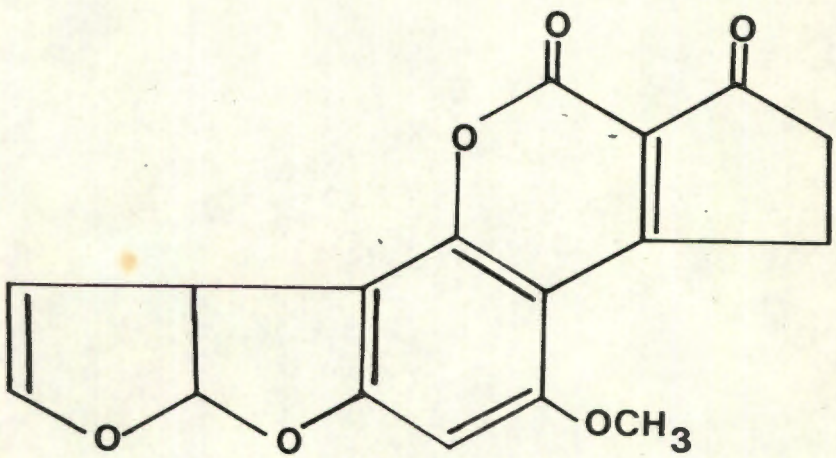
Fig. 19.3. Molecular structure of the diol epoxide of benzo(a)pyrene.

epoxide hydrase activity is low, and in these organs as in the lung the GSH-T's are probably more important in the metabolism of epoxides and arene oxides.

Evidence has recently been presented that as well as conjugation to epoxides, benzpyrene quinones undergo GSH conjugation cataylsed by rat liver GSH-T B (408).

Although some work has suggested that binding of benzpyrene to DNA is influenced more by nuclear epoxide hydrase than GSH-T (409), others have provided more direct evidence that the presence of GSH plus cytosol or purified GSH-T B reduces the binding of benzpyrene metabolites to nuclear DNA. Prior depletion of GSH increased nuclear binding of benzpyrene metabolites. GSH conjugates are formed in roughly inverse proportion to the extent of binding (410). This work was performed in vitro and does not take partitioning into intracellular compartments into account.

Dietary antioxidants used as food preservatives are known to be antimutagenic, although until recently the mechanism was not known. BHA (2(3)-tert-butyl-4-hydroxyanisole) or ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethyl quinoline) added to the diet of rats and mice decreased the mutagenicity of benzo(a)pyrene metabolites in the urine of animals fed benzpyrene (361,411). These dietary antioxidants were shown to induce the levels of GSH-T activity with several substrates (361). Epoxide hydrase activity was also increased (412). This is due to increased synthesis of the enzyme (413). The mechanism whereby antioxidants exert their antimutagenic effect is therefore thought to be by induction of GSH-T and epoxide hydrase which results in more efficient metabolism of mutagens. Mutagenicity of benzpyrene meta-



AFLATOXIN B_1

Fig. 19.4. Molecular structure of the carcinogenic form of aflatoxin, aflatoxin B_1.

bolites in urine was also decreased by addition of purified GSH-T A and B and by cytosol. This protection was not seen if GSH was absent from the assay system (361).

Benzo(a)pyrene also forms K region and non-K region epoxides (395, 414). Both have the potential to form dihydrodiols, but in the presence of GSH and cytosol the K region arene oxide is preferentially converted to the GSH conjugate. The 8,9-diol can be converted to the vicinal diol epoxide (anti-benzpyrene-8,9-diol-10-11-oxide)(fig 19.3) which can then undergo enzyme catalysed GSH conjugation (415).

Aflatoxin B_1 (fig. 19.4), the carcinogenic form of aflatoxin which has been implicated in the causation of hepatocellular carcinoma in Africa and the Far East, is excreted by rats mainly as a GSH conjugate (416). Aflatoxin is metabolised to an epoxide (417) and this is probably the site of GSH conjugation. Binding of aflatoxin to DNA is inversely proportional to hepatic GSH levels (418) and can be decreased by cytosol (419). This inhibition of binding is GSH specific and is enzymatic, i.e. is destroyed by boiling. Although there is no direct evidence for GSH-T involvement in the prevention of DNA binding this work is highly suggestive. In the early studies of GSH-T these criteria, loss of activity on boiling and an absolute requirement for GSH were regarded as good evidence for GSH-T activity. GSH-peroxidase activity would also fulfill these criteria.

A cytosolic protein which resembles ligandin and mouse skin h-protein has been shown to bind to 3-MC. When this protein is incubated with liver cell nuclei 3-MC is translocated into the nucleus (420). The data presented suggest that if the protein is a single homogenous

protein it is probably not ligandin, but perhaps in this work 3-MC is being bound by one or more GSH-T's.

Many other polycyclic aromatic hydrocarbons, some carcinogenic, some necrogenic, are substrates for the GSH-T's, but these have not been studied systematically.

Other relevant carcinogens studied although less completely include vinyl chloride and dimethylaminoazobenzene. Both form GSH conjugates, vinyl chloride via an epoxide intermediate (421), and dimethylaminoazobenzene via N-demethylation followed by sulphation (422). The metabolism of vinyl chloride provides an example of an alternative pathway of metabolism of a GSH conjugate.

The Ames test for mutagens has been used to study the relationship between the GSH-T's and carcinogenesis. The Ames system involves the use of a strain of Salmonella typhimurium which is unable to synthesize histidine. Some strains in addition have increased cell wall permeability. These organisms are grown on a histidine deficient medium in the presence of the mutagen that is being tested. Mutagenic activity manifests as a reversion in the mutated histidine operon, allowing the bacteria to synthesize histidine again. Each revertant will produce a colony. Mutagenic activity is expressed as the number of revertant colonies per plate, after correction for the spontaneous reversion rate (423,424). Mutagenicity correlates with carcinogenicity (425,426). CDNB is mutagenic in this system, but its effects can be reduced by addition of the S9 fraction (9,000 xg centrifugation) containing cytosol and microsomes. The inhibition of mutagenicity is inversely correlated with GSH-T activity of the S9 fraction, and inversely correlat-

ed with with GSH levels (209). As GSH-T levels rose in phenobarbitone induced animals mutagenicity decreased.

The role of the GSH-T's in the Ames test is controversial. To some extent this depends on the mutagen used. CDNB mutagenicity is reduced by the GSH-T's, but epoxide induced mutagenicity is only minimally affected (209,427).

It has also been pointed out that in situations where ligandin concentration is increased within the cell, such as hypophysectomy, thyroidectomy or phenobarbitone administration there is a decrease in aminoazodye induced carcinogenesis (428). However these manoeuvres may have many different effects on different intracellular proteins. To attribute the reduction in carcinogenicity to the effect on ligandin alone is an oversimplification.

Detoxication of cell toxins:-

Paracetamol (acetaminophen) and bromobenzene provide possible examples of the role of the GSH-T's in prevention of cell necrosis. Paracetamol is metabolised by the liver to glucuronides and sulphates primarily, and as a minor pathway undergoes N-hydroxylation and conjugation to GSH. The reactive intermediate has not been identified, but it is able to bind to tissue protein (429,430). There is evidence to suggest that conjugation is catalysed (431), but the GSH-T's have not been definitely implicated. With paracetamol overdosage the sulphation and glucuronidation pathways become saturated and the GSH pathway becomes more important. Toxicity only occurs after liver GSH is depleted, when the arylating metabolites may bind to tissue protein (432).

Bromobenzene undergoes P-450 metabolism to an arene oxide (433, 434), which can also bind to tissue protein. Again depending on dose the amount excreted as mercapturic acid varies. The higher the dose the more GSH conjugation, and therefore mercapturic acid formation. Tissue protein binding only occurs when the intracellular GSH is depleted (435).

With both these agents the extent of necrosis is related to tissue protein binding. Pretreatment which decreases the GSH concentration (eg diethyl maleate) increases necrosis (436), while addition of cysteine, a GSH precursor, decreases necrosis (437,438). Although it is known that bromobenzene oxide is a substrate for the GSH-T's it is not clear to what extent the GSH-T's are responsible for conjugation, and to what extent conjugation is spontaneous.

Detoxication by binding:-

All the GSH-T's, are able to bind non-substrate ligands to different degrees (41). Binding was originally described for ligandin in terms of organic anion binding (BSP, indocyanine green, Rose Bengal). However the list of non-substrate ligands bound by the GSH-T's is very much more extensive.

Experimental data demonstrating detoxication by non-substrate binding are lacking. Most studies have concerned organic anion binding and transport but as these are not strictly detoxication processes they will be considered later.

It has been shown that ligandin has a role in the prevention of bilirubin toxicity. Bilirubin, like many other endogenous end products of metabolism, is toxic. Under normal circumstances bilirubin is de-

toxified by conjugation to glucuronic acid and excreted in the bile. Under situations of bilirubin excess this excretory system may be overloaded, or the excretory system itself may be at fault, and toxicity may occur. This is particularly a problem in the neonate, in whom the blood-brain barrier has not yet developed, and may result in kernicterus. Bilirubin can also be shown to uncouple oxidative phosphorylation in the mitochondria. This effect can be reduced by the presence of ligandin (439). In the liver, even in great excess of bilirubin, mitochondrial respiration is normal, but in the CNS defects in oxidative phosphorylation are observed with bilirubin excess. There is much higher GSH-T activity in liver than in brain.

Dimethylaminoazobenzene is N-hydroxylated by the mixed function oxidases and then undergoes sulphate esterification by the soluble sulphotransferases to form the active metabolite. The active metabolite binds covalently to ligandin (440,441), but also undergoes GSH conjugation (442). The GSH conjugate is non-covalently bound (441). The major hepatic aminoazodye binding protein moves with the electrophoretically slower moving 'h_2' fraction, has a sedimentation coefficient of 5S, and hence was called the slow $h_2$5S azoprotein (443 445). This protein has a molecular size of 80,000 daltons and consists of two apparently identical subunits. However aminoazodyes are also bound, albeit with lesser affinity, by two other proteins in the cytosol, aminoazodye binding protein A (which is probably identical to Z protein or fatty acid binding protein) and ligandin (4,446,447).

On the other hand polycyclic aromatic hydrocarbon binding was first studied in the mouse skin, using benzo(a)pyrene. An electrophoretically slow moving protein, the h-protein was described (448),

which has since been purified from skin and liver (449). This protein has similarities to ligandin; molecular size of 40,000 daltons; two similar subunits of 20,000 daltons; and a sedimentation coefficient of 3.5, but the pI (8.3) is lower than for ligandin (8.9), and the amino acid analyses are different. This h-protein in its currently purest available form has GSH-T activity, but has been shown to be different from ligandin (450,451). Either this protein is not pure and is contaminated with a GSH-T or else it is itself a GSH-T.

3-MC however is known to be bound to ligandin (7). The molecule can be bound non-covalently in its native state, but after activation by microsomes is much more tightly bound. It can also undergo GSH-T catalysed conjugation (392).

The effect of this binding activity is not clear. It maybe a form of detoxication, but equally well non-covalent binding to ligandin may provide a mechanism for intracellular transport of hydrophobic molecules. These hypotheses need to be tested.

Covalent binding to the GSH-T's has been discussed. Jakoby has pointed out that since some carcinogens are non-covalently bound preferentially to the GSH-T's they would therefore preferentially undergo covalent binding to the GSH-T's (199). This has been demonstrated recently. BP-4,5-oxide is bound to four cytosol fractions, all of which have GSH-T activity. Approximately 8-10% of the water soluble metabolites are protein bound, of which binding to ligandin accounted for nearly 50% (317).

In some instances this binding to proteins is thought to protect the cell, in that the carcinogen is trapped and inactivated and excretion may be facilitated. Covalent binding is a form of irreversible

detoxification. Where covalent binding is to a non-essential protein, such as the GSH-T's, especially in view of their high concentration in the cell, this may indeed be so, but essential regulatory proteins present only in small amounts may also be covalently bound. Since covalent binding irreversibly damages protein this may be deleterious.

The covalent binding of activated metabolites to the GSH-T's also inactivates the GSH-T irreversibly. They are no longer able to bind non-substrate ligands or to act as enzymes (317). This form of detoxication has been viewed as a sort of molecular suicide (452).

Despite strong evidence of the importance of the GSH-T's, work in model systems has suggested that the efficiency of the GSH-T's may be severely limited by their cytosolic location, which, it is postulated, would make them inaccessible to lipophilic xenobiotics. The active intermediates of xenobiotics are formed in the endoplasmic reticulum and for reasons of solubility are likely to remain in the hydrophobic membrane environment rather than diffuse into the aqueous cytosol. However the increase in polarity conferred by oxidation may make this argument less relevant. Similarly this arguement does not take into account that protein bound xenobiotics are water soluble, and therefore xenobiotics bound to the GSH-T's may be at home in the cytoplasm.

The experiments on which these arguments are based used the Ames test and showed that if the drug activating system (microsomes) are in direct contact with the target (bacteria) the presence of cytosol does not result in inactivation of the active metabolite, whereas inactivation is greatest when the metabolite has to cross the cytosol to reach the target bacterium (269). Furthermore, others have shown, based on a model of binding to liposomes, ligandin or Z-protein, that 98% of the

hydroxylated metabolites of BP would be expected to be bound to the membranes (453). These models, of course, may not be representative of the situation within the cell. Since the endoplasmic reticulum is in contiguity with the nucleus it is conceivable that activated metabolites could be translocated along the membranes by lateral diffusion and not be conjugated by the cytosolic transferases. In this situation the presence of membrane transferases would be essential. It has been suggested that for epoxides at least the GSH-T's are not involved in the reduction in mutagenicity in the Ames test (454,455) This too may not apply in vivo as other evidence already discussed suggests that the GSH-T's are important in epoxide metabolism (456).

For the most part conjugation to GSH is a detoxifying step as with most phase II reactions, but with bifunctional haloalkanes metabolic activation may result from conjugation. 1,2-Dichloroethane, which is a waste product of the plastics industry, and 1,2-dibromoethane are mildly mutagenic, but their mutagenicity can be greatly increased after activation by liver cytosol. This activation was shown to be GSH dependent and GSH specific and to be catalyzed by GSH-T A and C but not B. It seems that the S-(2-chloroethyl)-L-cysteine moiety was responsible, the hypothesis being that since 1,2-dichloroethane is a bifunctional alkylating agent (2 halide groups) conjugation to one molecule of GSH resulted in a highly reactive compound, a half sulphur-mustard (eqn 19.1)(457,458). Similar findings pertain to dibromomethane and diiodomethane (458).

$$CH_2(Cl)CH_2Cl + GSH = CH_2(Cl)CH_2SG \quad (19.1)$$

A further example of metabolic activation mediated by the GSH-T's is the formation of cyanide (HCN) from organic thiocyanates (eqn 16.8)

(290). This may be the mechanism whereby the organic thiocyanates cause toxicity.

Bilirubin metabolism:-

The role of ligandin in bilirubin metabolism has been partly defined. Unconjugated bilirubin, bilirubin monoglucuronide and bilirubin diglucuronide, all bind to rat and human GSH-T's (16,41,460). Multiple dye dilution studies in isolated perfused livers from normal rats, rats pre-treated with phenobarbitone or rats thyroidectomized to increase ligandin levels have been used to derive rate constants for the influx of bilirubin into the cell and efflux from the cell back into plasma. Mathematical analysis of data derived from a compartmentalized model showed that ligandin is responsible for limiting efflux of bilirubin from the liver into the blood (460).

Bilirubin, like many xenobiotics, is hydrophobic and requires to be rendered more water soluble before excretion can occur. The mechanism whereby this is achieved is by conjugation with glucuronide, catalysed by UDP-glucuronyl transferase, a phase II 'drug metabolising' enzyme. Both mono- and diglucuronides are formed. The controversy regarding the mechanism of of bilirubin diglucuronide formation will not be discussed here. The GSH-T's in man and the rat bind all three bilirubins with varying affinity (41). It has been shown that bilirubin accumulates in the liver, and that 64% of the intrahepatic bilirubin pool in the rat is conjugated, mostly as monoglucuronide. The high concentration of the GSH-T's in the liver cell is thought to be partly responsible for this accumulation (461). The role of Z protein in bilirubin metabolism has not been well defined.

CHAPTER XX

PHYSIOLOGICAL ASPECTS OF TRANSPORT AND STORAGE

Organic anion transport:-

There are several lines of evidence to support the contention that the GSH-T's also play a role in organic anion transport in the cell.

Organic anion transport systems are well described in the liver (BSP, indocyanine green), kidney (penicillin, para amino hippuric acid) and intestine, i.e organs where GSH-T concentrations are high. The GSH-T's are known to bind organic anions. Of course, the simple demonstration that the GSH-T's bind organic anions, and are present in those organs where organic anion transport systems exist is not sufficient evidence for a role in organic anion transport. Such evidence requires that alterations in GSH-T concentration affect, or are associated with, changes in organic anion transport.

It has been shown that drugs which inhibit BSP clearance also inhibit GSH-aryl transferase activity, e.g. radiographic contrast media (462).

Phylogenetic and ontogenetic evidence:-

In the newborn, where the concentration of ligandin and the other GSH-T's has been shown to be very low (338,339) BSP clearance is reduced (8). Phylogenetic studies have shown Y protein to be absent in those species in which the liver is unable to take up BSP (234). Y protein is absent in most aquatic species and in the tadpole. It makes its appearance in the frog (234) and is present in all air breathing vertebrates studied. The presence of Y protein and its binding activity thus correlate with terrestial life. Perhaps animals living in an

aquatic environment do not need a highly developed system to handle hydrophobic molecules. Aquatic species do however have GSH-T's (233). Since in man and the rat all the GSH-T's can bind organic anions they may all be potentially involved in organic anion uptake, but it is possible that in the marine species the GSH-T do not bind organic anions.

Phenobarbitone treatment, which increases cytosolic GSH-T's, also increases cytosolic BSP binding (359) and enhances plasma clearance of BSP, dibromsulphophthalein and indocyanine green (357,463). Fasting reduces ligandin and Z protein and decreases hepatic organic anion uptake (463). There is a high degree of inverse correlation between the plasma BSP half-life and the liver ligandin content in the fasted and phenobarbitone pre-treated fasted states. Kinetic studies with phenobarbitone have shown that the increases in the first order rate constant of transport from plasma to liver (K_1), hepatic BSP and ICG content, and increased relative storage of BSP correlate with increased Y protein (358). Other chemicals, including agents such as 3-MC and BP have similar effects. In most instances changes in Y protein are mirrored by changes in hepatic organic anion uptake, but thyroid hormone deficiency (hypophysectomy or thyroidectomy) increased Y protein but decreased K_1 indicating that other factors must also play a role.

Other kinetic studies on isolated perfused livers suggested that phenobarbitone pre-treatment increased the rate constant for dibromosulphophthalein transport from hepatocytes to bile and decreased the reflux rate from cells back to plasma (464).

In view of the role of ligandin and the GSH-T's in bilirubin metabolism, i.e. regulating the efflux back into plasma, one must consider that the GSH-T's may play the same role for organic anions.

Binding of bile acids is an example of a naturally occurring organic anion which binds to the GSH-T's. Ligandin has been shown to bind lithocholic acid (22), cholic acid (465,466) and chenodeoxycholic acid (466). In rat liver cholic acid is bound in the presence of GSH mainly to a YbYb dimer with enzyme specifity similar to GSH-T C (467), but other GSH-T's also bind cholic acid (465). In the absence of GSH binding to GSH-T C does not occur (465).

There are certain similarities in the binding of bile acids to albumin and to ligandin. Cholic acid binds to a single site on both proteins, whereas chenodeoxycholic acid and lithocholic acid are probably bound at two sites (466). However despite clear evidence of bile acid binding to the GSH-T's the physiological role of the GSH-T's in bile salt and bile acid metabolism is remains unclear.

The question of anion entrance into the cell, by facilitated diffusion or as a result of a membrane carrier will not be discussed here (see ref. 468).

Extrahepatic organic anion transport:-

The possible involvement of ligandin in renal organic anion transport was first suggested by Kirsch et al (131). These workers examined the inhibition of penicillin binding to renal ligandin and showed that BSP and probenecid decreased penicillin binding and delayed plasma clearance. Penicillin is cleared by the kidneys and is known to share an uptake pathway from proximal tubular lumen to cell with probenecid. Others have shown that para amino hippuric acid, acetyl salicylic acid (ASA) and furosemide added to kidney cytosol were eluted from Sephadex G-75 at the elution volume of GSH-T activity (469,470). Para amino hippuric acid, ASA and probenecid, which also share this organic anion

transport pathway in the proximal cells, all inhibited the GSH-T reaction. These data suggest that the renal GSH-T's may play a role in uptake of organic anions. However manoeuvres to alter renal GSH-T levels or organic anion transport, such as 3-MC induction, NH_4 induced acidosis and penicillin treatment, showed that GSH-T activity and organic anion transport varied independently (345). In the rat maturation of the two activities were independent, although in the rabbit the two matured simultaneously (345). In mice enzyme activity was similar in both sexes but organic anion transport was greater in the male (345). It is possible that not all the renal GSH-T's bind organic anions. Each may respond differently to the various inducing agents. To fully evaluate the role of the GSH-T's in renal organic anion transport the GSH-T's will have to be studied individually.

Intestinal organic anion transport has been little studied, but an anatomical dissociation between organic anion transport and GSH-T activity measured with CDNB has been shown (471). Organic anion uptake was maximal in the midportion of the intestine, whereas GSH-T activity was greatest in the duodenum and jejunum.

Thus evidence exists for both a storage function and possibly also a transport function for the GSH-T's. Bilirubin is stored in the liver, and organic anions may also be stored. In the kidney and intestine, where transport is a more important function than storage, the evidence suggests that if the GSH-T's do play a role, they are not the major mediators of this function.

CHAPTER XXI

PHYSIOLOGICAL ASPECTS:- PORPHYRIN METABOLISM

Ligandin in the rat has been shown to bind with relatively high affinity to a number of compounds involved in haem metabolism (472). Haem is one of the most tightly bound of all non-covalent ligands (Ka $\pm 10^7$ l/mol)(473). When labelled δ-amino laevulinic acid is given to rats the label appears in the liver associated with at least five protein peaks on gel filtration (474). The relative amount of radioactivity in each peak changes as the animals are sacrificed at greater intervals after dosing, but at 10 and 20 minutes the ligandin peak located immunologically (and which includes the other GSH-T's) is the major peak. The radioactivity in the other peaks increased at 60 minutes at the expense of ligandin (475). However recovery of haem from the peaks was incomplete at all stages, implying that haem precursors or haem breakdown products were also bound. If haem labelled mitochrondria, haem labelled microsomes or labelled haemin is added to cytosol the binding pattern is different (474). However there is still a major peak of binding eluting at the same elution volume as ligandin. Acceleration of haem synthesis or of the catabolism of haem alters the pattern of binding (475). Ketterer et al (475) speculate that since some hepatic haem must be passed from the mitochondria where it is synthesized to the endoplasmic reticulum, where it is incorporated into haemoproteins, e.g. cytochrome P-450, ligandin could be involved in this process, either as a transport protein or by providing a stable intracellular haem pool, particularly since the Ka is so high.

It is interesting that the differences in binding affinity of porphyrins parallel the differences in their hydrophobicity (474). The more hydrophobic porphyrins are more tightly bound, providing further evidence that the nature of binding to ligandin (and the other GSH-T's) is hydrophobic. Binding of all the porphyrins is inhibited by BSP suggesting that they all bind at the same site on ligandin. At higher concentrations porphyrin binding will inhibit enzyme activity (474).

CHAPTER XXII

PHYSIOLOGICAL ASPECTS:- PEROXIDE METABOLISM

The recent demonstration that the GSH-T's act as a non-seleno GSH-peroxidase suggested a role in detoxication of an endogenously produced toxin. Under aerobic conditions life depends on enzymes which metabolise reactive toxic oxygen intermediates. Superoxide anions, singlet oxygen and the peroxide radical are inevitable products of oxygen metabolism and are all toxic. Cellular defence against these free radicals is mediated by superoxide dismutase, catalase and GSH-Px (see fig. 22.1). Vitamin E also has an anti-oxidant function.

The non-seleno GSH-peroxidase does not utilise H_2O_2 as a substrate, but is able to catalyse reduction of organic hydroperoxides such as cumene hydroperoxide, t-butyl hydroperoxide, and lipid hydroperoxides (294,295,476).

The physiologic importance of this reaction can be judged from several lines of evidence. GSH-T's account for 100% of GSH-Px activity in the guinea pig liver and 84% in the human liver (289,299,276). In selenium deficient rats the proportion of GSH-Px activity subserved by the GSH-T's increases and this is paralleled by an increase in GSH-T activity with CDNB (295,296,310). Vit E deficiency also results in induction of GSH-T activity. It has been postulated that the intermediate endogenous inducer may be a lipid peroxide but there is no evidence to support this hypothesis (387). Indirect evidence for a role for the GSH-T's in protection against lipid peroxidation has been provided by following the production of malondialdehyde as a measure of lipid peroxidation in a NADPH-microsomal system. Dialysed cytosol with

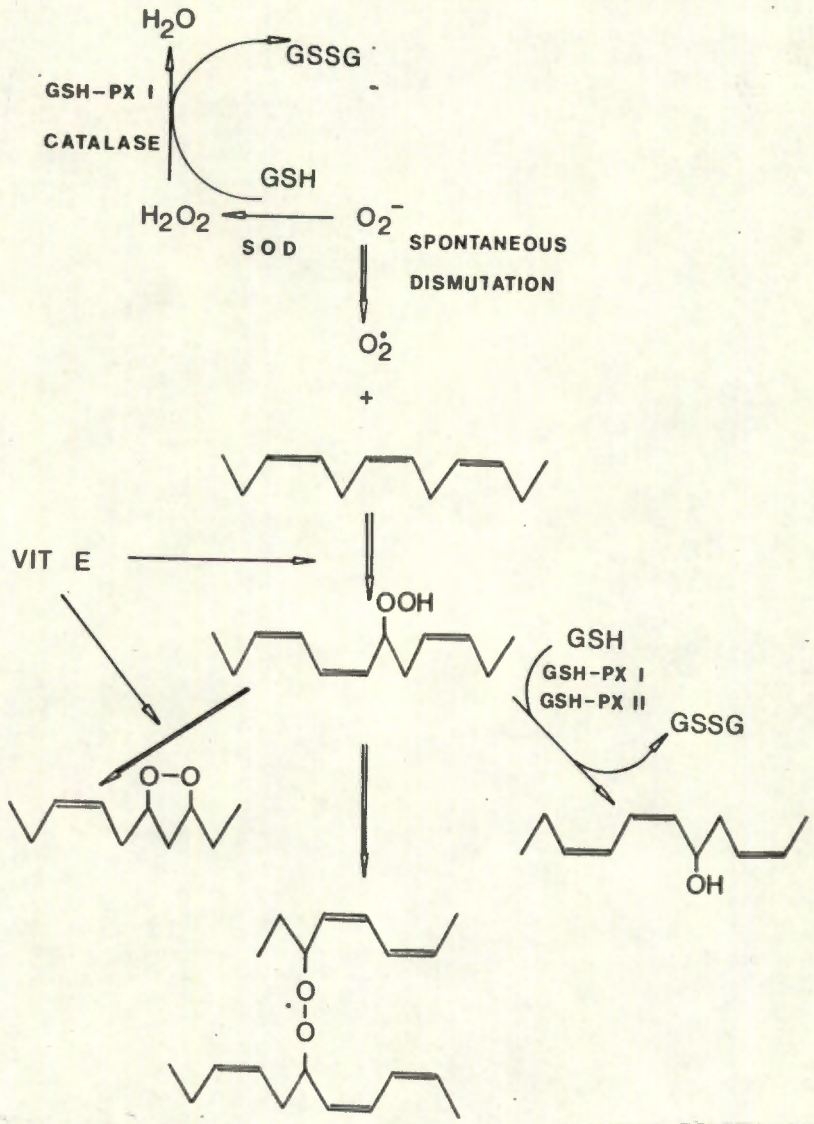


Fig. 22.1. Defence against oxygen toxicity and lipid peroxidation. (S.O.D - superoxide dismutase). Glutathione peroxidase, glutathione S-transferase and vitamin E are said to interfere with lipid peroxidation at the points shown.

GSH added reduced malondialdehyde production. This protective activity copurified with the GSH-T's through several stages of purification. At least two GSH-T's were shown to be protective, but the mechanism has not been investigated (477). The seleno-enzyme did not provide any protection in this system (477).

A soluble GSH dependent factor limiting peroxide formation has been described. It has been shown that this factor reacts with cumene hydroperoxide and fatty acid hydroperoxides, but not with membrane lipids, which are phospholipids (478). This factor was shown not to be GSH-Px (the seleno-enzyme)(478).

A GSH-T partially purified by affinity chromatography did not prevent lipid peroxidation or react with preformed lipid hydroperoxides. However the GSH-T used in this study was not characterised, or shown to have non-Se dependent GSH-Px activity. Since non-Se dependent GSH-Px activity is probably restricted to GSH-T B and AA, and these may not have been included in the test system, the results of this study are of doubtful significance (478,479).

It seems therefore that the GSH-T's may be an important system for detoxifying lipid peroxides, perhaps in some species more important than the seleno-enzyme. Morrisey and O'Brien (480) have postulated that the physiological substrates for the seleno enzyme are H_2O_2 and fatty acid hydroperoxides. Phospholipids may require to be dephosphorylated before the seleno enzyme can act upon them. The GSH-T's may have as their physiological substrates, fatty acid hydroperoxides, and phospholipid hydperoxides. If this is so then the membrane bound GSH-T's assume new importance in maintaining membrane stability.

CHAPTER XXIII

PHYSIOLOGICAL ASPECTS:- THYROID HORMONE METABOLISM

Lichter et al studied the binding of labelled triiodothyronine (T_3) and thyroxine (T_4) to liver cytosol in vivo and in vitro (481). T_3 bound to four peaks on gel filtration including the Y peak containing the GSH-T's. Binding was inhibited by BSP and bilirubin. However in vivo binding to Y was less than in vitro. Maximal binding was to a peak with the mobility of albumin, but its identity was not definitely determined.

T_4 in vitro did not bind to the Y peak, but bound mainly to the albumin-like peak. In vivo however, binding to the Y peak accounted for 33% of protein bound T_4. The difference between the in vivo and in vitro findings could not be ascribed to binding of metabolites. The binding pattern did not change with time. Homozygous Gunn rats which were hyperbilirubinaemic had decreased hepatic T_3 uptake compared to the phenotypically normal heterozygotes. Presumably, increased bilirubin binding by ligandin inhibited T_3 binding. Phenobarbitone pretreatment which induces ligandin also increases thyroxine binding to the liver (482). All this suggests that ligandin binding of thyroid hormones may be physiologically important. T_4 and T_3 can undergo conjugation with glucuronide and sulphate in the liver and kidney (and possibly other tissues). The excretion of T_4-glucuronide is inhibited by BSP (483). It is not known if these conjugates bind to ligandin these conjugates bind to ligandin or whether the inhibition with BSP is due to competition for binding.

Other thyroid binding proteins have been described in liver and kidney cytosol (484-486), but these correlate more closely with the non-ligandin binders in terms of molecular size, and reported binding affinities are different to those of ligandin.

Smith and Litwack do not consider ligandin to be a thyroid hormone receptor (486), but point out that the hormonal effects of the thyroid hormones have a delayed onset and that the existence of long-lived intermediates has been postulated (487). Could ligandin be responsible for storing these intermediates and thus delaying their action? They also discuss the interesting point that in amphibia thyroid hormones are responsible for changes in enzymes which occur at metamorphosis. Thyroxine stimulates tail absorption and limb growth and the appearance of urea cycle enzymes which shift nitrogen metabolism from the production of ammonia in the aquatic stage to urea in the adult stage. Y protein and organic anion uptake are absent in the tadpole but is abundant in the post-metamorphosis liver (234). The interaction between the appearance of ligandin and the shift in nitrogen metabolism and how each is linked to thyroxin is unknown.

CHAPTER XXIV

PHYSIOLOGICAL ASPECTS:- STEROID METABOLISM

The interaction between the GSH-T's and the steroid hormones is poorly understood. Several steroids and/or their metabolites have been shown to bind to ligandin. The question has been asked 'is ligandin a steroid receptor in the liver'? The arguments against this being so have been elegantly summarized by Smith and Litwack (486) and are, in short:

The tissue distribution of ligandin does not correlate with the magnitude of response of those tissues to steroids.

The binding affinities of the steroids for ligandin do not parallel the potency of those steroids.

All the steroids which bind ligandin do so with an affinity far less than the binding affinity of other known receptors.

Ligandin concentrations in the liver and kidney are too great and require too large a concentration of hormone for saturation to allow sensitive modulation of hormone effect.

Since active steroids and their metabolites bind to ligandin with approximately equal affinity they probably compete for binding.

Using immunohistochemical techniques the distribution of ligandin in steroid producing tissues has been shown to be linked to the hormone producing cells, the interstitial cells of the testis, the cortex of the adrenal medulla and the theca granulosa cells of the ovary (250, 488). Others have shown GSH-T epoxide metabolizing activity in both spermatogenic and interstitial cells (256). Ligandin does not have much epoxide GSH-T activity and may be absent from spermatogenic cells.

The demonstration that several rat and human GSH-T's have Δ^5-3KSI activity (289) raises the question of whether there is a role for ligandin in steroid synthesis. Δ^5-3KSI was first shown in the bacterium Pseudomonas testosteroni and has since been purified and crystallized from that source (304). This enzyme has no GSH-T activity. In mammals, in steroid producing tissue, the enzyme exists as a microsomal enzyme, molecular size $\pm$80,000 daltons and is involved in testosterone synthesis as well as synthesis of gluco- and mineralocorticoids. Since these hormones are not produced by the liver it is unlikely that the hepatic Δ^5-3KSI activity has any role in steroid synthesis. However no work defining a physiological role for this activity has yet been reported.

Looked at from another point of view, it is likely that steroid hormones affect the GSH-T levels. It has been shown that there is a male/female difference with respect to GSH-T and ligandin concentration (339). Gonadectomy in the male rat results in decreased levels of GSH-T activity with styrene oxide in the liver, but ovariectomy leaves levels in the liver unchanged (332). There is no explanation for these findings, but it is likely that the sex hormones or related trophic hormones are involved. Glucocorticoids have been shown to induce GSH-epoxide transferase activity in neonatal and young rats up to 12 days of age (489). Activity in the foetus was not inducible when pregnant rats were treated with dexamethasone.

The binding of cortisol and other steroid metabolites to ligandin may have several effects. Binding may be required to facilitate transport of conjugated hormone into the bile canaliculi for excretion. Alternatively, binding to the GSH-T's may prevent the back diffusion of

steroids and their metabolites into plasma (cf. bilirubin). A third alternative is that since the association constants for steroid metabolites and ligandin are higher than the corresponding Ka's for albumin, ligandin may have a role in uptake into the hepatocyte, and may also, in a manner analogous to that of albumin in the plasma, determine the amount of free steroid, and to a greater extent the amount of free metabolite, available for interaction with the receptor or other intracellular proteins.

The GSH-T's may play another role in steroid removal, by catalysing conjugation. A GSH conjugate of oestradiol 17β has been described (490). The reaction is presumed to occur via an epoxide intermediate. Although this particular conjugation is non-enzymatic, oestradiol 17β disulphate has been shown to inhibit several GH-T's both competetively and non-competetively (327). The physiological significance of this is unknown.

CHAPTER XXV

GLUTATHIONE S-TRANSFERASES IN HUMAN TISSUES

INTRODUCTION

GSH-T enzyme activity has been shown to be present in most tissues. Quantitation of GSH-T's in human organs has been limited to studies of enzyme activity only, and has shown considerable interindividual variation. To what extent this variation is due to changes in individual GSH-T's is not known, nor is the nature of the GSH-T's in most organs is unknown.

This chapter considers the quantitation of ligandin in human tissues and also deals with the separation of the different GSH-T's in these organs in an attempt to elucidate some of the qualitative changes that may underly quantitative differences in enzyme activity.

METHODS

Preparation of tissue for assay:-

Human tissue was obtained within 12 hours of death from patients dying by violence. Tissue was either used immediately or was stored at -70°C until used. All procedures were performed at 4°C. 100,000 xg Supernatants were prepared from 25% homogenates in 0.25M sucrose in distilled water if destined for isoelectric focusing, or in 0.01M sodium phosphate buffer pH 7.4, 0.15M NaCl, 0.25M sucrose if destined for gel filtration. Cytosol was used immediately or stored at -70°C until required.

Molecular size of immunoreactive ligandin:-

Cytosol from kidney, liver, testis, adrenal and lung were chromatographed on Sephadex G-100, column size 90 x 1.5 cms. The column was poured and developed in 0.01M sodium phosphate buffer, 0.15M NaCl, pH 7.4. Elution was by pump driven upward flow at a rate of 20 ml/hour. Fractions of 4 ml were collected. Protein content of the eluate was measured spectrophotometrically at 280 nm. Ligandin concentration was measured by RIA and GSH-T enzyme activity with CDNB as before. The column was calibrated with marker proteins to allow the estimation of molecular size of the immunoreactive peaks. These were transferrin (70,000), albumin (63,000), ovalbumin (48,000), cytochrome C (13,000). Blue dextran marked the void volume.

Immunoreactivity of tissue ligandin:-

Serial dilution of cytosol from liver, kidney, adrenal, testis, pancreas, stomach, duodenum, ileum, colon, salivary gland, lung, thyroid, heart, spleen, skeletal muscle, bladder and prostate were assayed for ligandin by RIA. Dose response curves were constructed and linearised by the logit/log transform. The slopes of the transformed curves were compared to the the slope of transformed standard curve of the assay using the RIARUN programme as before.

Concentration of ligandin in tissue:-

Ligandin was measured at a dilution of 1:16 in cytosol from pancreas, colon, ileum, stomach, salivary gland, spleen, prostate, lung, brain, heart, thyroid, bladder and muscle, and in a dilution of 1:100 in cytosol from liver, testis, kidney, adrenal and duodenum. Cytosol pro-

tein was measured by the Lowry method (128). Results were expressed as ug/mg supernatant protein.

Isoelectric focusing:-

This was performed in an LKB 4101 IEF column using a stepwise sucrose density gradient. The water used to cool the column was passed through a coil immersed in water at 4°C. Anolyte was 0.5M H_3PO_4 and catholyte was 0.15M NaOH. The pH range 6.5 - 10.5 was created using a 1:1 ratio of ampholyte pH range 6.5 - 9 and pH range 8 - 10.5. The pH range 4 - 8 was created using a 1:1 ratio of ampholyte pH range 4 - 6.5 and pH range 5 - 8. Final ampholyte concentration in all cases was 3%.

Cytosol was applied to the column as part of the gradient after 50% of the column had been filled. Focusing time was 16 hours. The volume of cytosol used in each instance and the pH range are given in the legend to the figures.

At run completion the column was drained by pump at a flow rate of 2 ml/min. Two ml fractions were collected. pH was measured immediately. The fractions were also assayed for GSH-T activity with CDNB, and for ligandin by RIA.

Assessment of relative contribution of each group of GSH-T to total activity was by estimation of the area under the curve.

Assessment of red cell GSH-T activity:-

The contribution of red cell GSH-T contamination to anionic GSH-T activity in tissue was assessed. Haemoglobin concentration of cytosol

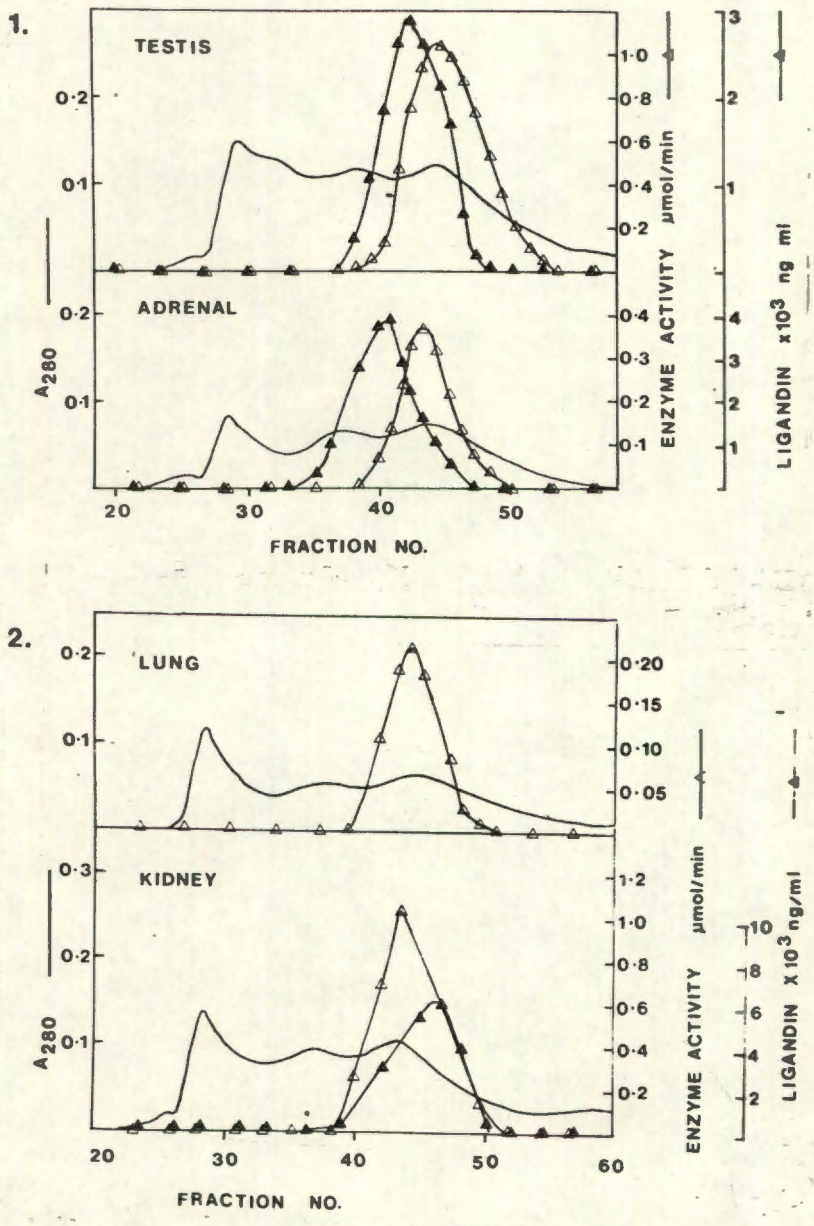


Fig. 25.1. Sephadex G-100 chromatography of cytosol from (1) kidney and lung, (2) adrenal and testis. Ligandin immunoreactivity was measured by RIA, and enzyme activity was measured with CDNB as substrate.

IMMUNOREACTIVE LIGANDIN IN NORMAL TISSUE CYTOSOL

Organ	Concentration μg/ml cytosol	Specific immunoreactivity μg/mg cytosol protein	No. of samples
Liver	375.1	23.2	2
Kidney	119.8	12.8	3
Testis	118.0	19.7	1
Adrenal	86.9	13.4	2
Duodenum	26.7	2.8	1
Ovary	8.8	1.50	1
Colon	2.8	0.38	1
Pancreas	2.4	0.26	1
Lung	1.9	0.17	1
Stomach	1.5	0.16	1
Brain	1.0	0.28	1
Salivary gland	0.8	0.08	1
Heart	0.6	0.07	1
Muscle	0.6	0.04	1
Prostate	0.5	0.05	1
Bladder	0.5	0.08	1
Thyroid	0.4	0.06	1
Ileum	0.2	0.02	1

Table 25.1. Concentration of immunoreactive ligandin in the cytosol from human organs. A single provided specimens of each tissue examined. Additional tissue was available for liver, kidney, and adrenal. In these instances the mean concentration of ligandin in all specimens is given.

was measured at 410 nm. All absorption at this wavelength was assumed to be due to haemoglobin haem. Using the known concentration of GSH-T in red cells (31) the contribution of red cell GSH-T to tissue anionic GSH-T activity was calculated.

RESULTS

Molecular size of immunoreactive ligandin:-

Elution profiles from Sephadex G-100 for 4 tissues are shown in fig. 25.1. Enzyme and immunoreactivity eluted at approximately the same elution volume, namely 1.4 - 1.6x the void volume, but the curves were not superimposable. Purified hepatic ligandin eluted at 1.5x the void volume.

By comparison with molecular weight standards GSH-T activity eluted at a molecular weight of 47,500 daltons, with the entire peak eluting between 39,500 and 54,600 daltons.

Immunochemical displacement curves:-

Dose response curves constructed from serial dilutions of cytosol were parallel to the assay standard curve for each tissue tested. These results are displayed graphically in fig. 25.2 and fig. 25.3.

Concentration in human tissues:-

Table 25.1 shows the concentration of immunoreactive ligandin in cytosol expressed per mg supernatant protein. A specimen of each tissue examined was obtained from a single donor. Additional specimens of liver, kidney, adrenal and lung were also obtained. The highest concentration of ligandin was found in the liver, with considerable

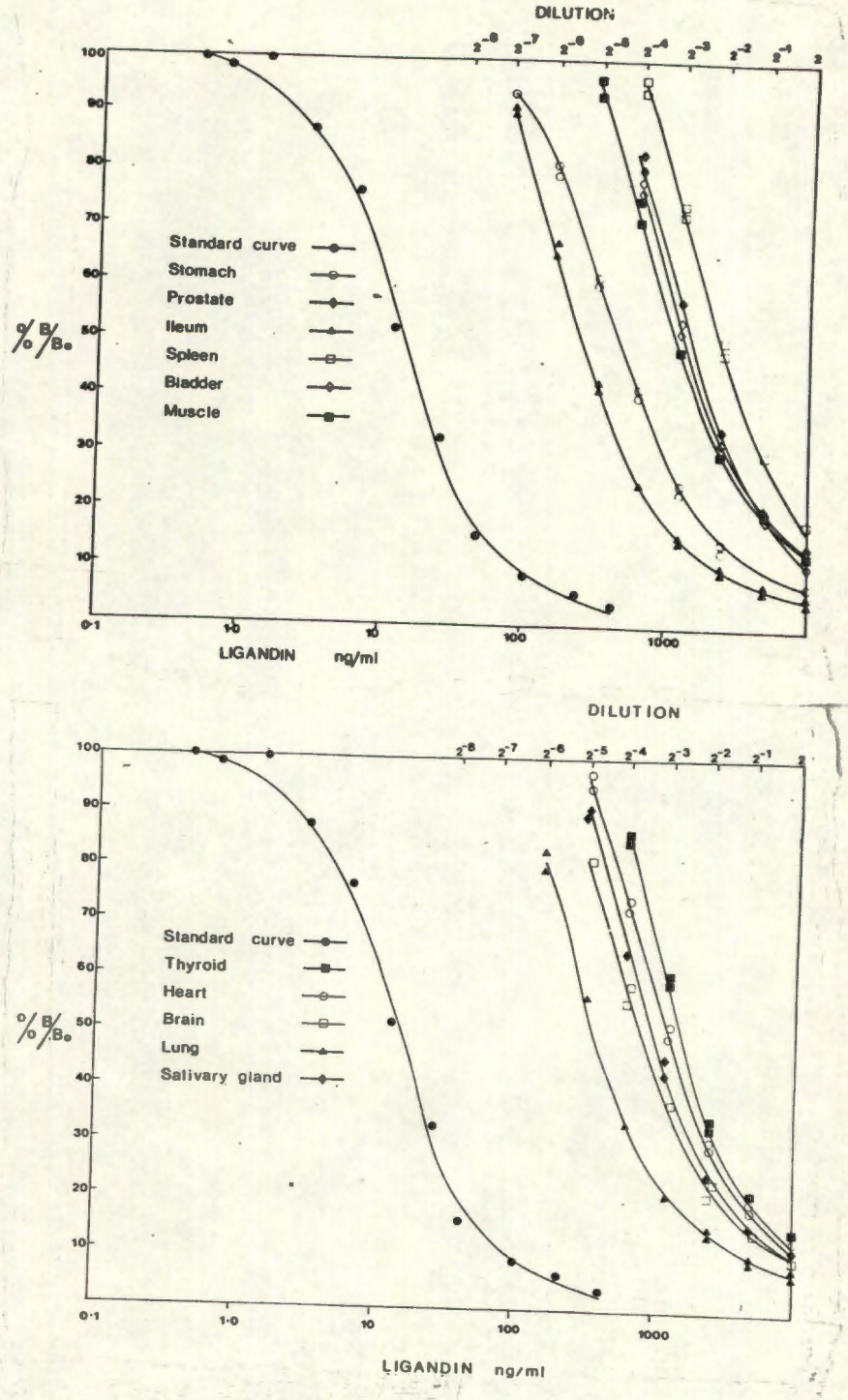


Fig. 25.2. Immunochemical displacement curves of cytosol from human organs compared with the standard curve of the ligandin RIA. Ordinate and abscissa are as for fig. 10.1.

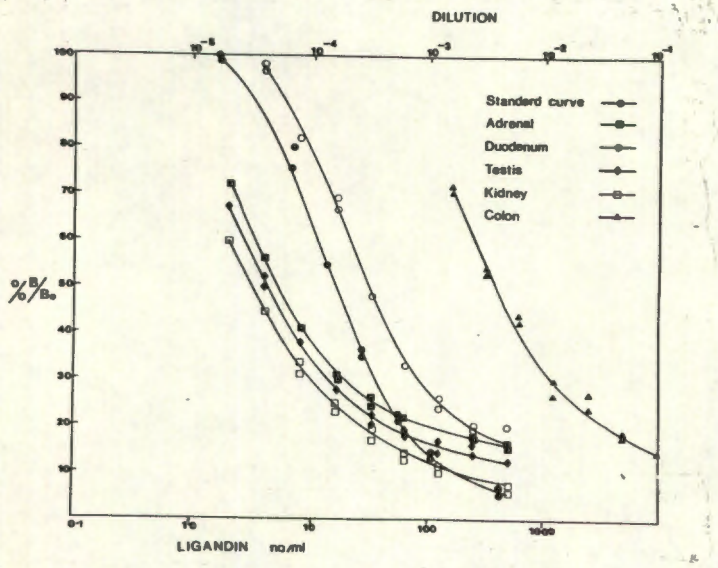


Fig. 25.3. Immunochemical displacement curves of cytosol from human organs compared with the standard curve of the ligandin RIA. Ordinate and abscissa are as for fig. 10.1.

GLUTATHIONE S-TRANSFERASE IN NORMAL HUMAN TISSUES

Organ		GSH-T activity μmol/min/ml	specific activity μmol/mg protein	ligandin immuno-reactivity ng/ml	specific activity μg/mg protein
Liver	II	5.83	0.42	182409	13.2
Kidney	II	5.10	0.44	126471	19.8
Ovary	II	1.66	0.28	8761	1.5
Liver	III	19.97	1.15	567846	33.0
kidney	III	8.34	1.03	211482	26.1
Adrenal	III	3.04	0.48	64512	21.0
Liver	IV	14.02	—	—	—
lung	IV	1.45	—	—	—
Liver	V	16.48	—	—	—
Kidney	V	9.13	—	—	—
Adrenal	V	10.87	—	—	—

Table 25.2. Glutathione S-transferase activity with CDNB and ligandin immunoreactivity in normal human organs. The roman numerals refer to tissue from a single donor.

IMMUNOREACTIVE LIGANDIN IN NORMAL TISSUE CYTOSOL

Organ	Cationic GSH-T % pI above 9.0	Neutral GSH-T % pI 6.0 - 6.9	Anionic GSH-T % pI 3.6 - 5.2
Liver I	99	—	1
Kidney I	66	—	34
Adrenal I	28	3	69
Liver II	97	trace	3
Kidney II	78	—	22
Ovary II	13	6	87
Liver III	100	—	—
Kidney III	76	—	24
Adrenal III	93	7	—
Liver IV	72	28	—
Lung IV	8	—	92
Liver V	63	37	—
Testis V	33	26	41
Adrenal V	24	50	26
Lung V	11	5	84

Table 25.3. Percentage of GSH-T activity which each group of GSH-T's contributes to the total activity. Each roman numeral indicates tissue from a single donor. The pH gradient was unstable above pH 9.0, hindering the accurate asessment of pI in this region.

amounts present in kidney, adrenal, testis and ovary. When more than one specimen of tissue was available for assay up to a 4 fold difference in ligandin concentration (expressed per mg protein) was seen. GSH-T activity and ligandin immunoreactivity in tissues are compared in table 25.2.

Isoelectric focusing. (IEF):-

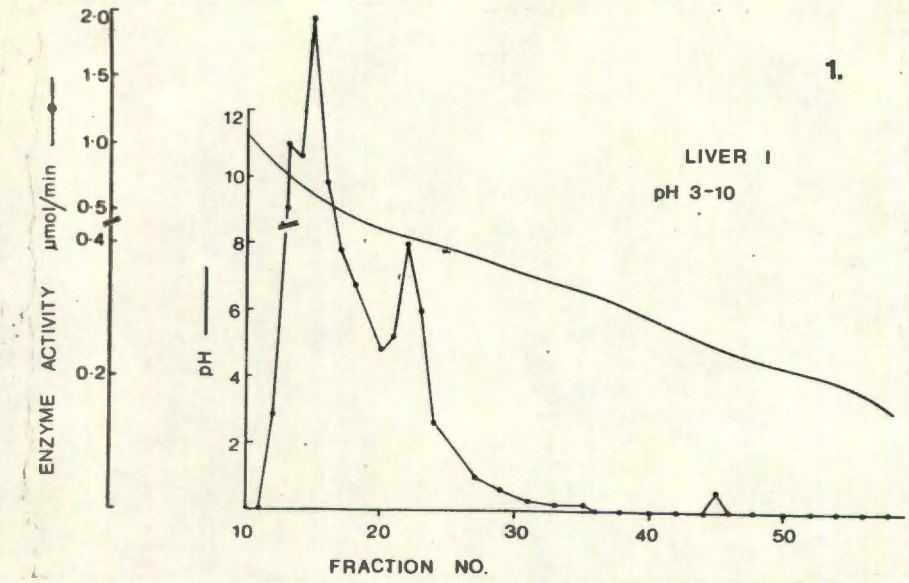
Cytosol from five individuals was subjected to isoelectric focusing. Enzyme activity in the eluted fractions was initially assessed with CDNB, 3,4-dichloronitrobenzene, p-nitrobenzyl chloride and ethacrynic acid. Use of these additional substrates did not increase detection of extra GST peaks, in several column runs, and therefore elution was followed by GST activity with CDNB alone.

Recovery following IEF in all organs was 31-62% (mean 42%). IEF was able to resolve GSH-T activity into cationic (pI > 7.5), neutral (pI 6.0 - 6.5), and anionic (pI < 5.7) groups (table 25.3).

Liver cytosol from 5 individuals was subjected to IEF (fig. 25.4). The cationic GSH-T's were present in all specimens, constituting between 63 - 100% of activity. Up to four peaks of activity were clearly separable, but in two livers only a single peak was detected (fig. 25.4.5 & 25.4.6) .

Neutral GST activity was present in three liver specimens, ranging from a trace (fig. 25.4.2)to 37% of total activity. The anionic GST's were present in 2 specimens (fig. 25.4.1 & 25.4.2), but accounted for no more than 3% of activity.

Kidney cytosol from 3 donors was studied (fig. 25.5). In the kidney cationic GSH-T activity accounted for 66-88% of activity, up to four separate peaks being detected. The pI of the cationic peaks in



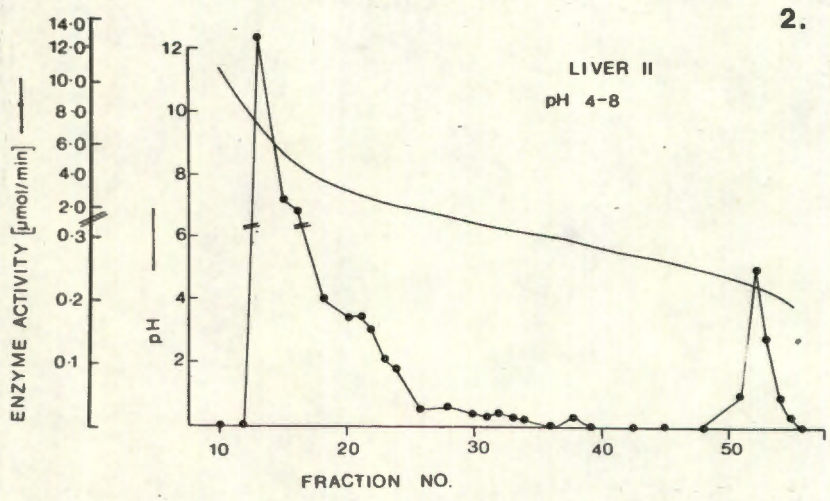


Fig. 25.4. IEF of normal liver cytosol. In the focusing range pH 3 - 10 or pH 6.5 - 10.5 1 ml of cytosol was applied to the column. In the focusing range pH 4 - 8 3.5 ml of cytosol was applied. The elution profile of GSH-T activity with CDNB is shown in all specimens. Immunoreactivity is shown for liver II, III and V. In all cases immunoreactivity was confined to the cationic region.

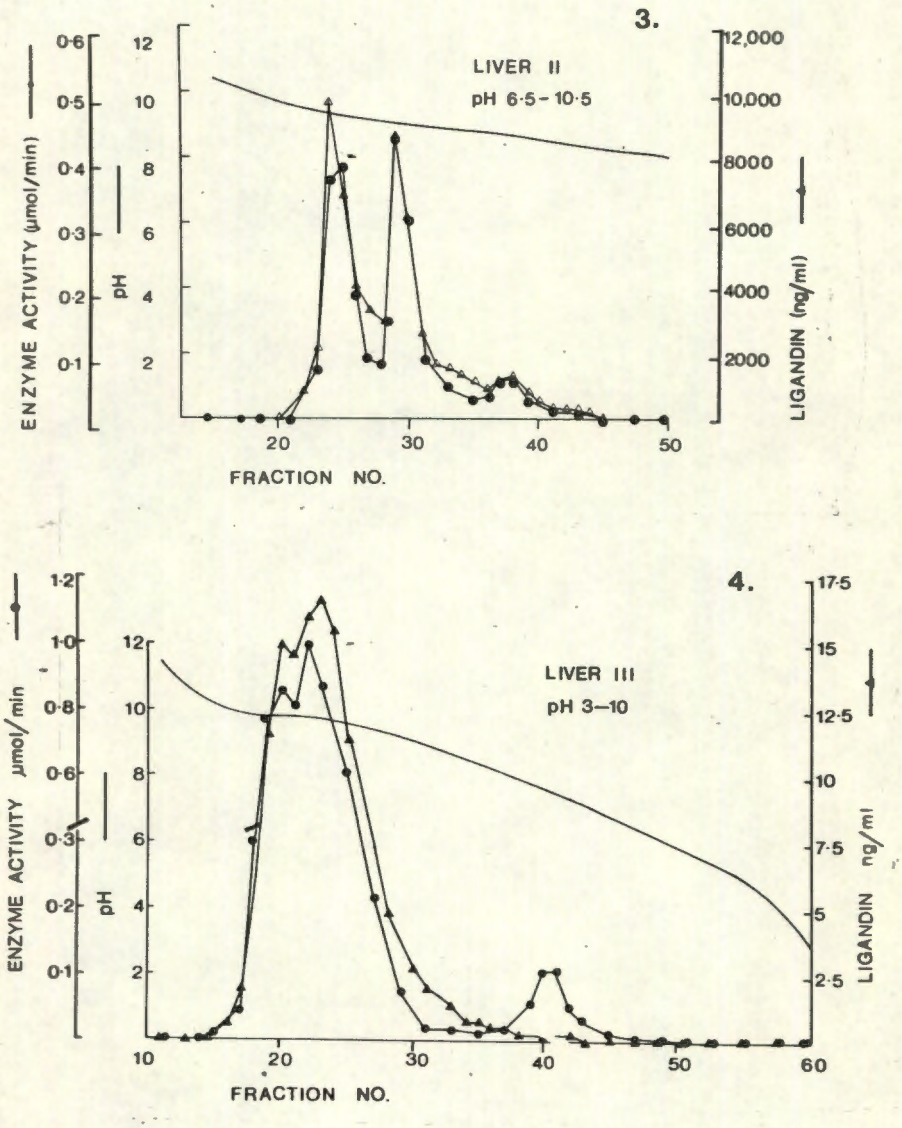
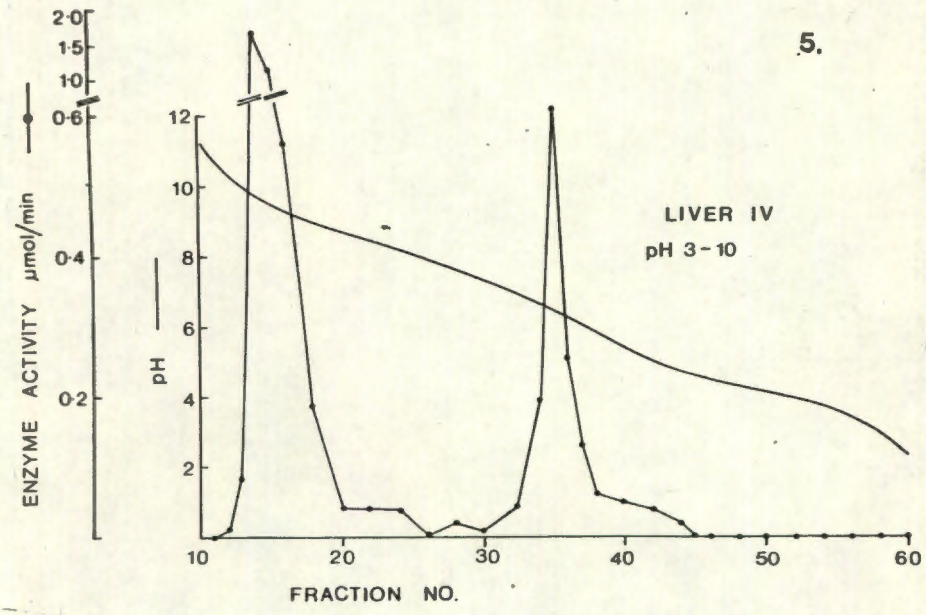


Fig. 25.4. IEF of normal liver cytosol. In the focusing range pH 3 - 10 or pH 6.5 - 10.5 1 ml of cytosol was applied to the column. In the focusing range pH 4 - 8 3.5 ml of cytosol was applied. The elution profile of GSH-T activity with CDNB is shown in all specimens. Immunoreactivity is shown for liver II, III and V. In all cases immunoreactivity was confined to the cationic region.



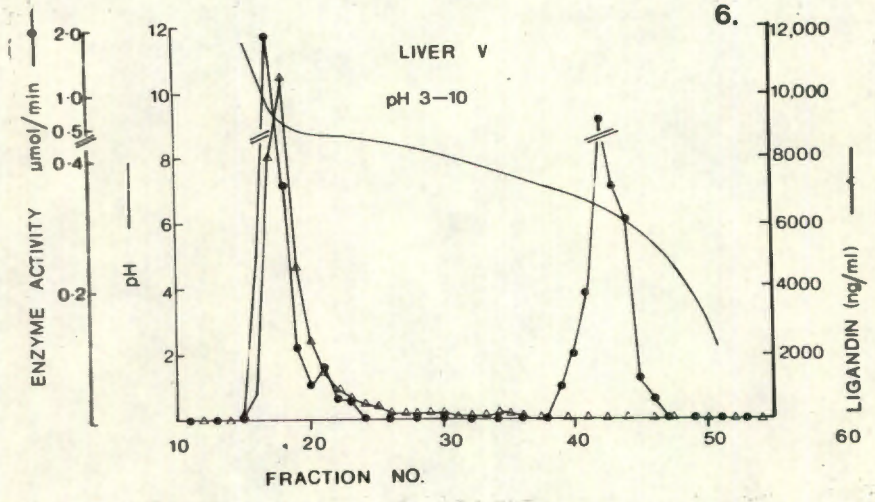


Fig. 25.4. IEF of normal liver cytosol. In the focusing range pH 3 - 10 or pH 6.5 - 10.5 1 ml of cytosol was applied to the column. In the focusing range pH 4 - 8 3.5 ml of cytosol was applied. The elution profile of GSH-T activity with CDNB is shown in all specimens. Immunoreactivity is shown for liver II, III and V. In all cases immunoreactivity was confined to the cationic region.

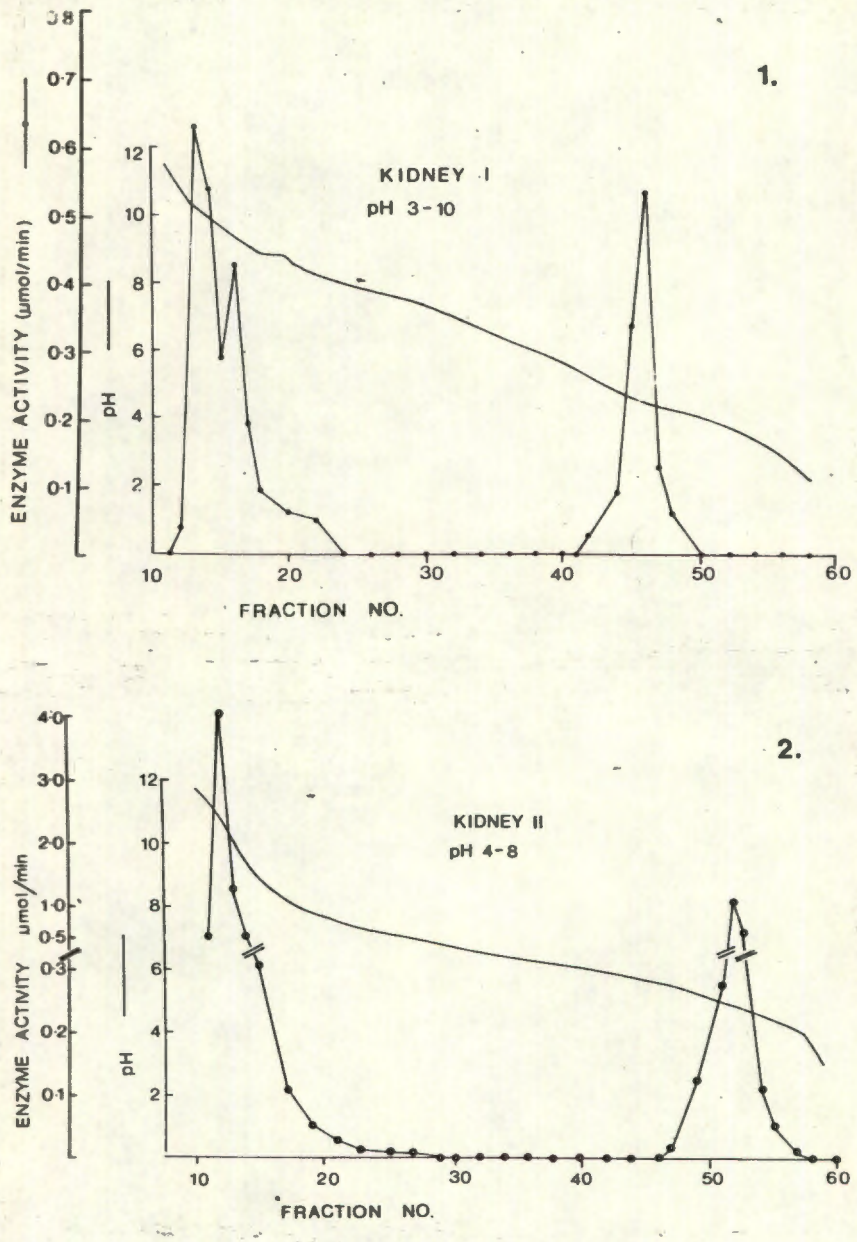


Fig. 25.5. IEF of normal kidney cytosol. In the focusing range - 10 or pH 6.5 - 10.5 1 ml of cytosol was applied to the column. the focusing range pH 4 - 8 3.5 ml of cytosol was applied. The elu profile of GSH-T activity with CDNB is shown in all specimens. Imm reactivity is shown for kidney II and III. In all cases immunoa tivity was confined to the cationic region.

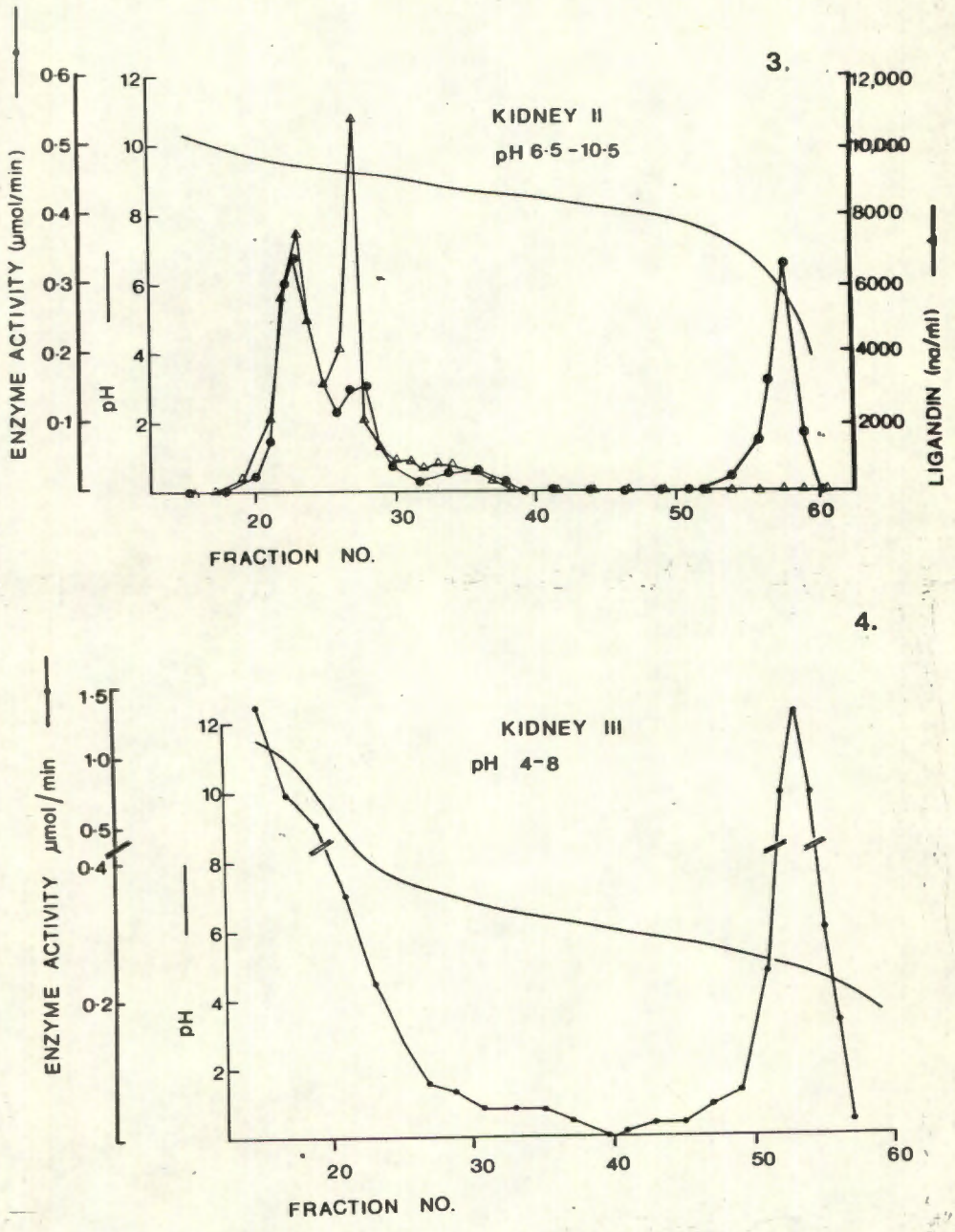


Fig. 25.5. IEF of normal kidney cytosol. In the focusing range pH 3 - 10 or pH 6.5 - 10.5 1 ml of cytosol was applied to the column. In the focusing range pH 4 - 8 3.5 ml of cytosol was applied. The elution profile of GSH-T activity with CDNB is shown in all specimens. Immunoreactivity is shown for kidney II and III. In all cases immunoreactivity was confined to the cationic region.

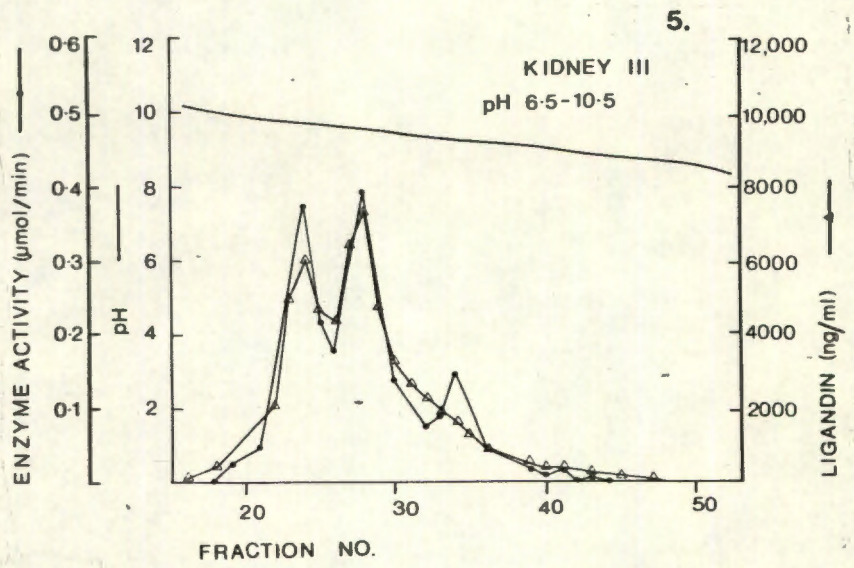


Fig. 25.5. IEF of normal kidney cytosol. In the focusing range pH 3 - 10 or pH 6.5 - 10.5 1 ml of cytosol was applied to the column. In the focusing range pH 4 - 8 3.5 ml of cytosol was applied. The elution profile of GSH-T activity with CDNB is shown in all specimens. Immunoreactivity is shown for kidney II and III. In all cases immunoreactivity was confined to the cationic region.

the kidney did not correspond to the cationic peaks in the liver of the same individual.

Neutral GSH-T activity was present in only one kidney (fig. 25.5.4), but could not be clearly separated fromcationic GSH-T activity. Anionic GSH-T activity was present in all specimens, accounting for 9 - 34% of total activity. A single major peak was present in each case.

Adrenal gland from 3 patients was available for study (fig. 25.6). In the adrenal the relative contribution of the cationic GSH-T's varied from 24 - 28% (fig. 25.6.1 & 25.6.3), to 93% (fig. 25.6.2). Neutral GSH-T activity was present in all specimens, contributing from 3 - 50% of total activity. The anionic species in adrenal was absent in one specimen (fig. 25.6.2), but contributed up to 69% of activity in the others.

Lung from two donors was studied (fig. 25.7). In both samples the major GSH-T activity (84 and 92%) resided in the anionic GSH-T peak, focusing at pH 4.3 and 4.7 (fig. 25.7.1 & 25.7.2 respectively). Only traces of cationic GSH-T activity were present. In lung V a trace of GSH-T pI 6.1 was present, as well as traces of minor anionic species (fig. 25.7.2).

A single specimen of ovary was studied (fig. 25.8). It is not known at what stage of the menstrual cycle the death occurred, or whether menopause had occurred. Focusing of ovarian cytosol in the pH range 6.5 - 10.5 (fig. 25.8.1) yielded a trace of activity at pH 10. Focusing in the range pH 4 - 8 (fig. 25.8.2) yielded a minor rounded peak at pH 6.0-6.1, but the major activity focused at pH 5.0 - 5.2.

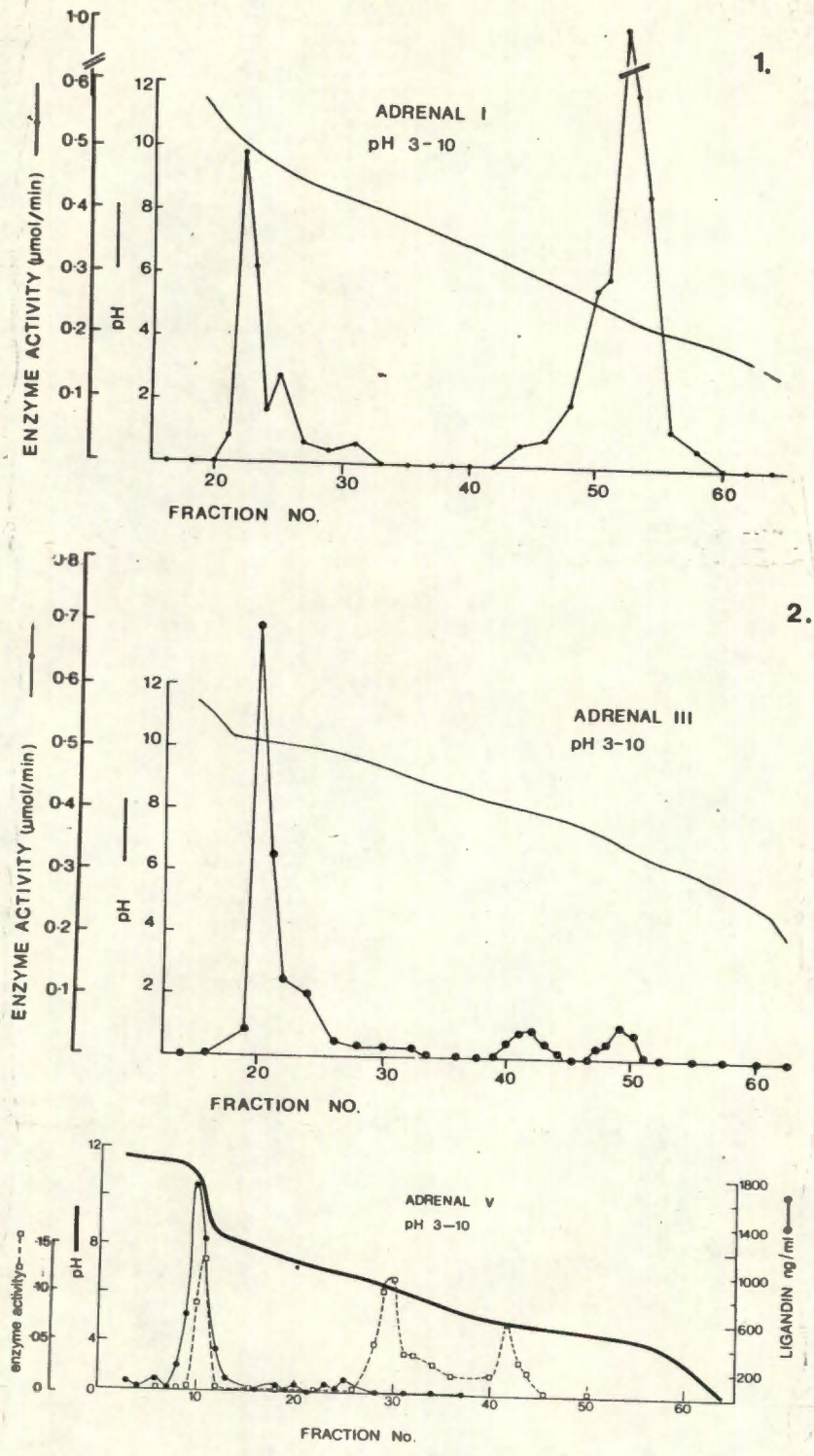


Fig. 25.6. IEF of normal adrenal cytosol. One ml of cytosol was applied in each case. The elution profile of GSH-T activity with CDNB is shown in all specimens. Immunoreactivity is shown for adrenal V.

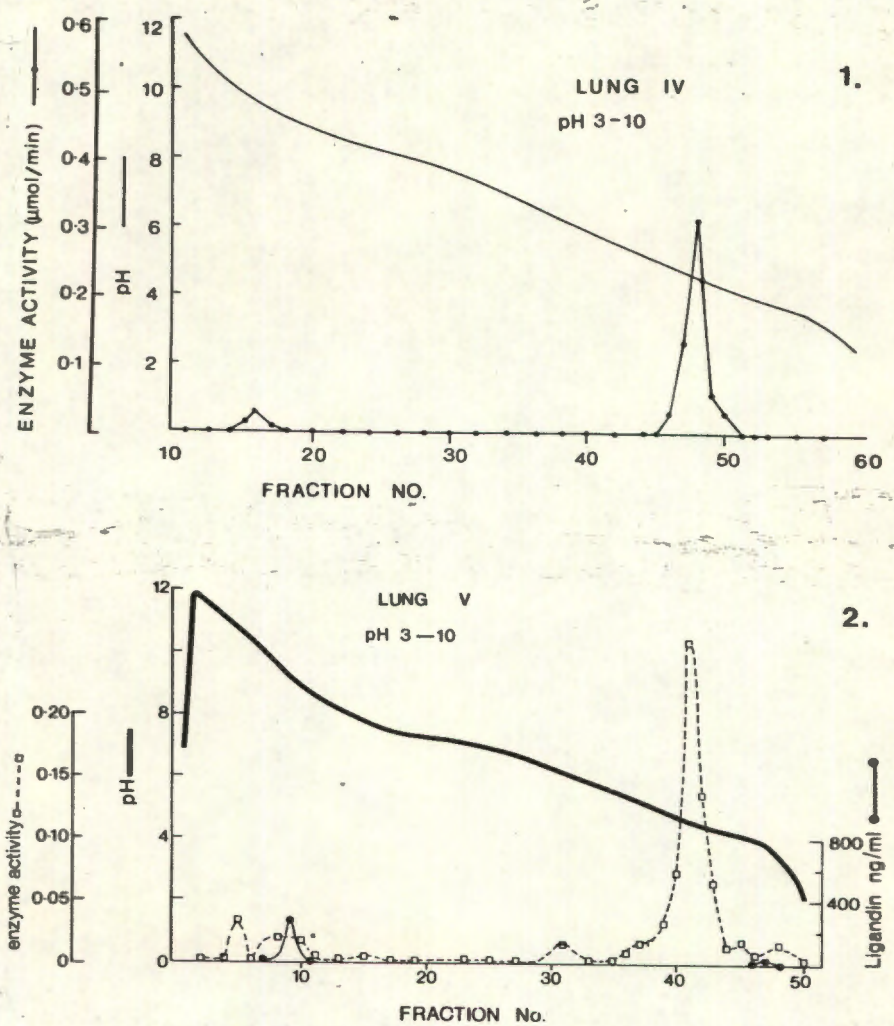


Fig. 25.7. IEF of normal lung cytosol. One ml of cytosol was applied for lung IV, and 3.5 ml for lung V. The elution profile of GSH-T activity with CDNB is shown in both specimens. GSH-T activity is mainly found in the anionic region. Immunoreactivity is shown for lung V. Immunoreactivity was confined to the cationic region.

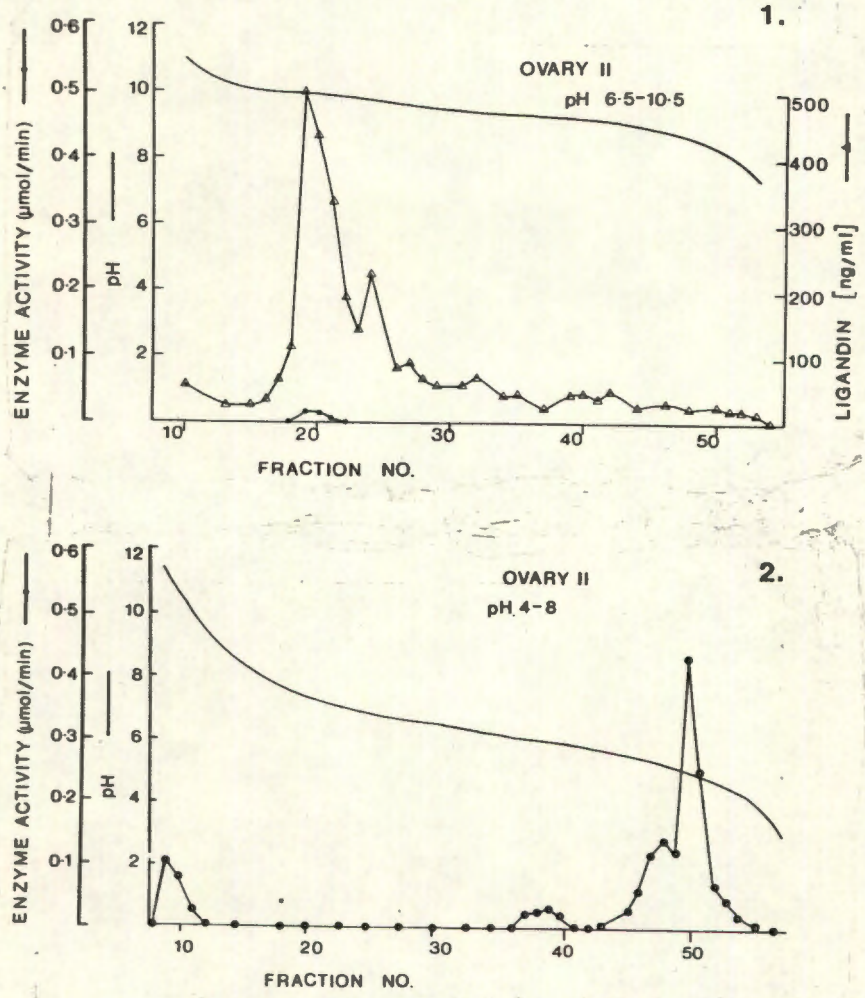


Fig. 25.8. IEF of normal ovarian cytosol. In the pH range 6.5 - 10.5 1 ml of cytosol was applied. In the pH range 4 - 8 3.5 ml of cytosol was applied. Ovarian GSH-T activity is largely confined to the anionic region. Immunoreactivity is mainly confined to the cationic region, but there is a trail of activity in the neutral region.

Cytosol from a single specimen of testis was focused in the pH range 3 - 10 (fig. 25.9). All three groups of GSH;T activity were present, in approximately the same proportions as in the adrenal from the same patient.

Immunoreactivity of the GSH-T's was examined in different tissues. In all tissues studied immunoreactivity with the anti-ligandin antibody was largely limited to the cationic group of GSH-T's. In the specimen of testis (fig. 25.9) there was a significant peak of immunoreactivity corresponding to pH 5.1, i.e not exactly symmetrical with the anionic enzyme activity peak. In lung V (fig. 25.7.2) a trace of immunoreactivity was detectable in the region of the anionic GSH-T's.

In all tissues the cationic enzyme peaks focused identically with peaks of immunoreactivity. The relative heights of the immuno- and enzyme reactive peaks were not identical.

Contamination with red cell GSH-T activity was calculated to be less than 1% in any organ.

In liver III a peak of enzyme activity was detected, focusing at pH 8.5, which was not immunoreactive. This peak was not analysed further.

DISCUSSION

Gel filtration is insufficiently powerful to resolve minor differences in molecular size. The reported size differences between the anionic and cationic GSH-T's are too small to be detected when cytosol from whole liver, adrenal, kidney, testis and lung are chromatographed. However the finding that enzyme active and immunoreactive peaks are not symmetrical indicates that some GSH-T activity is due to proteins not

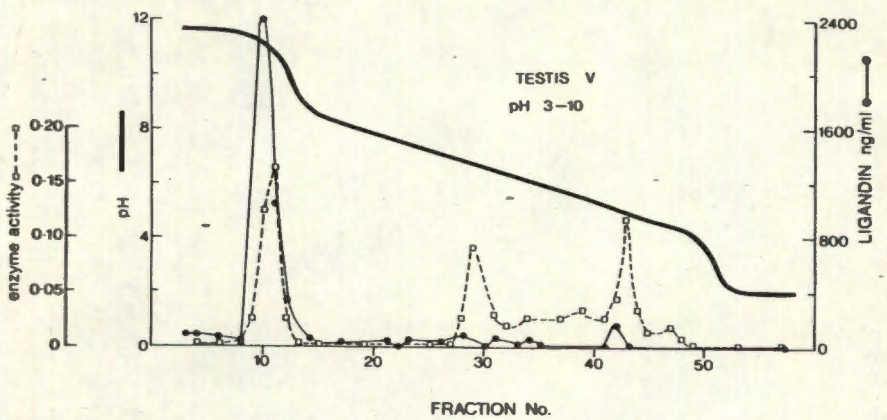


Fig. 25.9. IEF of normal testis cytosol. 3.5 ml of cytosol was applied. Ovarian GSH-T activity is largely confined to the anionic region. Immunoreactivity is confined to the cationic GSH-T's.

detected by RIA. Isoelectric focusing confirms this and demonstrates that contrary to the finding of Awasthi et al (30), and in agreement with Warholm et al (491), anionic GSH-T's do not react with antibodies to cationic GSH-T's. The neutral GSH-T's are also immunologically separate from the cationic GSH-T's. Warholm et al (491) have also reported that the three groups of GSH-T are immunologically distinct. The three groups of GSH-T's are therefore likely to be different molecules, probably derived from three genetic loci.

Human red cells and placenta are known to contain mainly anionic GSH-T's, and to have very little or no cationic GSH-T activity. Human lung and ovary can now be added to this group. In addition the presence of the anionic GSH-T's has been shown to be ubiquitious, and in organs other than liver may contribute a significant percentage of total GSH-T activity.

The anionic GSH-T's may be a heterogenous group. Awasthi et al (30) described one major and two minor anionic GSH-T peaks in human liver. Marcus et al (31) describe a single GSH-T in red cells.

It should be pointed out that the assumption has been made that the neutral and anionic GSH-T's in each organ are the same proteins as in each other organ. Warholm et al (491) have reported that antibodies to the placental anionic GSH-T cross react with foetal hepatic and red cell anionic GSH-T's. This suggests that the anionic GSH-T's in all organs may be similar.

Immunological similarity between the cationic GSH-T's in different organs is shown by the parallel dilution curves and similar elution volumes from G-100, and suggests that these are probably very similar molecules, perhaps differing, as in the liver, by charge only.

Mukhtar et al (523) using enzyme activity with CDNB showed a wide range of GSH-T activity in organs of different individuals, and between organs of a single individual. This marked heterogeneity is reflected in the distribution of the GSH-T's, within and between organs, and in the variable contribution each group makes to overall GSH-T activity.

Column IEF is not sufficiently powerful to separate the five cationic GSH-T's described by Kamisaka et al (16). It is likely that not all individuals have all five GSH-T's, since IEF yielded such different patterns, on occasion only a single peak. A further point of difference is that the pI of the cationic GSH-T's in this study did not correspond to those described by Kamisaka et al, but were more cationic. GSH-T of Kamisaka has a pI of 8.8, but the majority of the cationic GSH-T's in this study focused above 8.8. This may be due to differences in experimental conditions, but may be equally well due to conditions of storage, or depend on vitro degradation.

Enzyme forms of different pI may arise as a result of modifications occurring in vitro processing. Proteolytic digestion may have accounted for the loss of GSH-T activity. In this work all cytosols were handled in like manner, yet yielded different relative concentrations of neutral and anionic GSH-T's. It is unlikely that proteolytic activity had a differential effect on individual GSH-T's.

Sources of interindividual differences in drug metabolising enzymes include genetic factors, age, diet, smoking, drugs, alcohol and environmental pollutants. The genetics of the human GSH-T's have not been well studied, and have been discussed previously (see lit. rev.). This study and immunological evidence from other sources (also previously discussed) suggests that there are at least three gene loci in-

volved, one for each group of GSH-T's. Contrary to the conclusions of Board (329) there is no nul allele for any locus, since all three forms are found in any individual, although not all forms are expressed in all organs.

Inter-organ variation is a manifestation of gene repression/derepression, and is also affected by induction of drug metabolising enzymes. The mechanism for organ variation and protein expression in general (e.g. why do only red cells make haemaglobin) is unknown.

The effect of drug metabolising enzyme inducers is exerted mainly mainly on the liver. work in rats has shown that GSH-T activity in organs other than the liver is difficult to induce. Indeed only the kidney apart from liver, responds to any major degree to inducing agents. Each organ may have it own set of endogenous or exogenous inducers, as yet undefined. Studies of induction of GSH-T activity to date have almost exclusively considered only total enzyme activity and not induction of individual GSH-T's. In the human there are no studies of GSH-T induction and therefore the role that inducing agents may play in human GSH-T concentrations cannot be assessed. It is not known whether any or all of the human GSH-T's are inducible.

In this study tissue was obtained postmortem from victims of death by violence. No medical history was obtainable. No other pathology was found at postmortem. Since e.g. therapy with diphenylhydantoin, or phenobarbitone or drug or alcohol abuse could not be excluded the role of drug metabolising enzyme induction in accounting for the variation found is unknown.

CHAPTER XXVI

GLUTATHIONE S-TRANSFERASE IN HEPATOCELLULAR CARCINOMA

INTRODUCTION

Alterations in protein metabolism and secretion have been described in many malignant tumours, both in man and in experimental animals. In particular hepatocellular carcinoma is well known for its secretion of foetal antigens, α-foetoprotein and carcinoembryonic antigen.

The molecular correlation concept enunciated by Weber (492) relates phenotypic variation in malignant cells to the rate of growth of these cells. This concept was formulated when experimental tumours of different growth rates became available for study, the so-called Morris hepatoma's (493).

Weber and others were able to show that many enzymes of glucose synthesis, DNA catabolism and RNA catabolism are decreased proportionately to the growth rate of the malignant cells, while enzymes of the opposing pathways, i.e. glycolysis, DNA and RNA synthesis, are increased. Specifically, pyruvate kinase and hexokinase activity was increased while glucose-6-phosphatase, fructose-1,6-diphosphatase and phosphoenolpyruvate carboxykinase decreased (494,495). DNA polymerase and thymidine kinase are examples of DNA synthetic enzymes which increase in activity in malignant cells. Thymidine phosphorylase is a DNA catabolic enzyme which decreases in activity (492).

In some instances isozyme shifts were noted, the high Km isoenzymes becoming diminished and low Km isoenzymes becoming increased (492). Hexokinase and glucokinase both convert glucose to glucose-6-

phosphate but hexokinase is a low Km enzyme, while glucokinase has a high Km. With progressive malignancy glucokinase activity decreases and hexokinase activity increases. Similarly high Km liver pyruvate kinase becomes replaced with low Km muscle-type pyruvate kinase (494, 495).

Ligandin concentration and GSH-T enzyme activity have been shown to be decreased in experimental tumours induced in rats (99,496,497). In human hepatocellular carcinoma measurement of ligandin by RIA has shown ligandin concentration to be reduced to a variable extent in tumour specimens. In addition serum ligandin has been shown to be elevated in some patients with hepatocellular carcinoma (92).

In this chapter the distribution of GSH-T in human hepatocellular carcinoma is studied, both biochemically and histochemically. The relationship between serum and tumour ligandin is also explored.

METHODS

Histochemistry:-

Formalin fixed paraffin embedded liver biopsies were studied. The sections were stained routinely with haematoxylin and eosin for morphological assessment. Sections were also stained for ligandin using a peroxidase-antiperoxidase (PAP) sandwich method using an antibody raised against the cationic human GSH-T's (498-500).

The paraffin embedded sections are rinsed in absolute alcohol, then immersed in 0.3% hydrogen peroxide in methanol for 30 minutes. All subsequent reagents are made up in 0.1M sodium phosphate buffer, 0.15M NaCl, pH 7.4, and between steps the sections were washed in the same buffer. The sections were then soaked sequentially in normal

swine serum diluted 1:20 for 10 minutes, rabbit antihuman ligandin antiserum 1:800 for 24 hours at 4°C, swine antirabbit serum 1:20 for 30 minutes, rabbit peroxidase-antiperoxidase complex 1:20 for 30 minutes. The colour reaction is developed by soaking in 5 mg of 3,3'-diaminobenzidine in 10 ml PBS with 0.1 ml hydrogen peroxide. The sections are then counterstained with Mayer's heamatoxylin. Each batch was controlled using non-immune rabbit serum in place of rabbit antiligandin antiserum. Each reagent in turn was left out of the staining procedure to left out of the staining procedure to exclude any staining due to non-specific reagents. Sections were also stained using rabbit anti-ligandin antiserum to which had been added human liver cytosol in the ratio 8:1. The mixture was allowed to stand at 4°C for 48 hours and then centrifuged at 18,000xg for 30 minutes. The supernatant was used in the staining schedule.

Ligandin concentration in the biopsy section was estimated semi-quantitatively using a grading system as follows:-

Distribution of staining:-

No staining	= 0
Few clusters of cells stained	= 1
Many clusters of cell stained	= 2
Diffuse staining	= 3

Intensity of stain:-

Weak	= 1
Strong	= 2

Tumour morphology was graded into well differentiated or undifferentiated tumours. Well differentiated tumours had either glandular elements or a trabecular pattern. Undifferentiated tumours included

those with anaplastic cells, and those which consisted of sheets of cells in which the architectural pattern was lost.

Preparation of cytosol:-

Since young South African Black males have one of the highest indicences of this tumour tissue was limited to that obtained from Black patients dying of hepatocellular carcinoma (courtesy Prof. M.C. Kew, University of the Witwatersrand Johannesburg). The diagnosis was made during life. Tissue was removed within 12 hours after death and frozen at -70°C till used. 100,000x g Supernatants were prepared from 25% homogenates in 0.25M sucrose if destined for isoelectric focusing, or in 0.01M sodium phosphate buffer, 0.15M sucrose, pH 7.4 if destined for gel filtration.

Molecular size of immunoreactive ligandin in hepatocellular carcinoma:-

Cytosol from four tumours were chromatographed on Sephadex G-100, column size 90 X 1.5 cm. The column was poured and developed in 0.01M sodium phosphate buffer, 0.15M NaCl, pH 7.4. Elution was by pump driven upward flow at a rate of 20 ml/hour. Four ml fractions were collected. Protein content of the eluate was measured spectrophotometrically at 280 nm. Ligandin concentration was measured by RIA and GSH-T enzyme activity with CDNB. The column was calibrated with marker proteins to allow the estimation of molecular size of the immunoreactive peaks as before.

Immunoreactivity of ligandin in hepatocellular carcinoma:-

This was assessed by comparing the slope of the dose response curve of serial dilutions of cytosol with the slope of the assay standard curve as before.

Ligandin concentration in cytosol:-

Ligandin in cytosol was measured by RIA. Supernatant protein was measured by the method of Lowry as before. Results were expressed as µg/mg protein. Ligandin was also measured in the serum of patients with hepatocellular carcinoma. Results were expressed as ng/ml.

Isoelectric focusing:-

This was performed in an LKB 4101 IEF column as before. Running conditions were as before.

The volume of cytosol used in each instance and the pH range are given in the legend to the figures.

At run completion the column was drained by pump at a flow rate of 2 ml/min. Two ml fractions were collected. pH was measured immediately. The fractions were also assayed for GSH-T activity with CDNB, and for ligandin by RIA.

Assessment of red cell GSH-T activity:-

The contribution of red cell contamination to anionic GSH-T activity in tissue was assessed by comparison with the haemoglobin concentration of whole blood as before.

RESULTS

Biopsy material from 36 patients with hepatocellular carcinoma were studied. In all patients hepatocellular carcinoma was confirmed on morphology and on clinical grounds. Twenty of these tumours were well differentiated and 16 were poorly differentiated.

The PAP technique stains a brown colour when positive. In normal liver all hepatocytes were uniformly stained a dark brown (fig 26.1). There was no difference in intensity of staining between the three zones of the hepatic acinus. Backgroung staining vas a faint brown colour seen in the portal tracts. The Kupfer cells did not take up stain. Most hepatocyte nuclei also took up stain. Non-immune controls in which antiligandin antibodies were omitted, or in which one of the reagents were omitted stained a faint blue with no brown colour at all (fig. 26.2). Sections stained with antiserum adsorbed with ligandin also showed no brown colour at all.

Staining of tumours varied from absent (fig. 26.3) to intense diffuse staining (fig 26.4 & 26.5). Generally however staining in the tumour was more patchy (fig. 26.6), and was limited to clusters of cells. Generalised patchy staining was the commonest pattern of staining seen. In some sections staining was limited to one or two clusters of cells in the whole section.

Diffuse generalised staining was seen in three tumours, two undifferentiated, and one well differentiated. Completely negative staining was seen in two trabecular carcinomas and 5 poorly differentiated lesions.

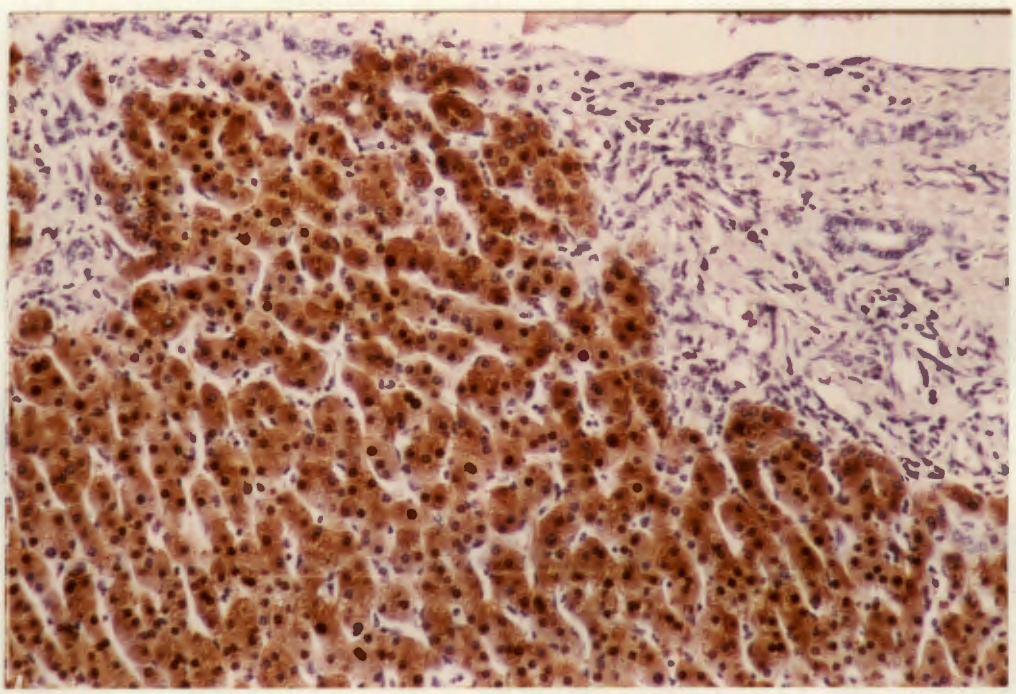


Fig. 26.1. Ligandin immunohistochemistry. Section of normal liver stained with anti-ligandin antiserum with peroxidase - antiperoxidase method (see text for details). Positive staining gives a brown colour. Normal hepatocytes are positive, while the surrounding fibrous tissue is negative.

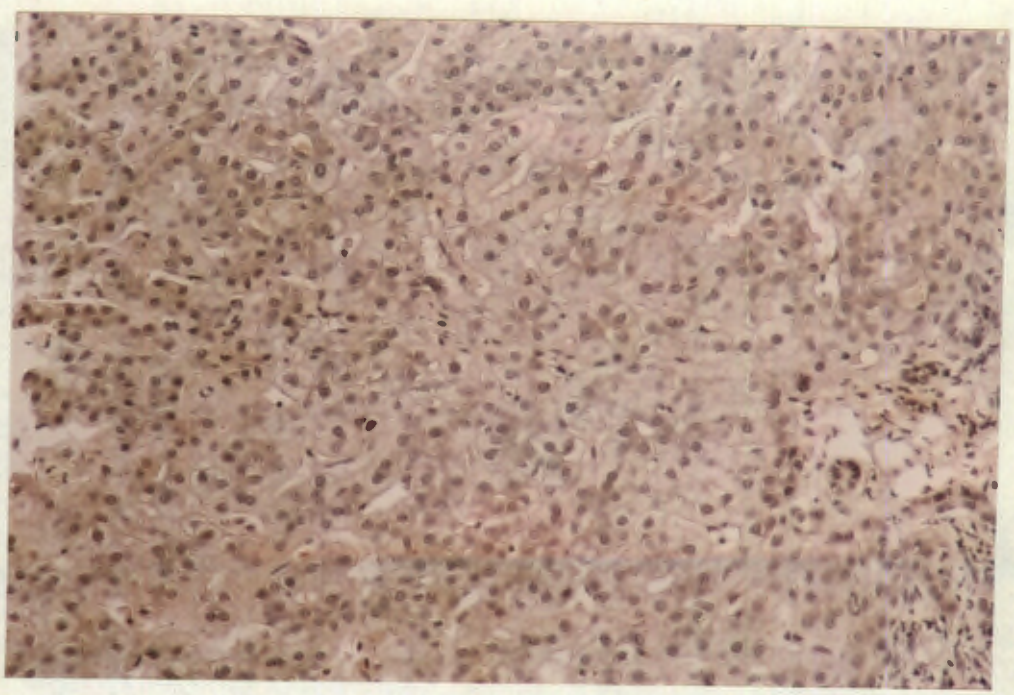


Fig. 26.2. Ligandin immunohistochemistry. Non-immune control of normal liver. Specific anti-ligandin antiserum was ommitted from the staining procedure.

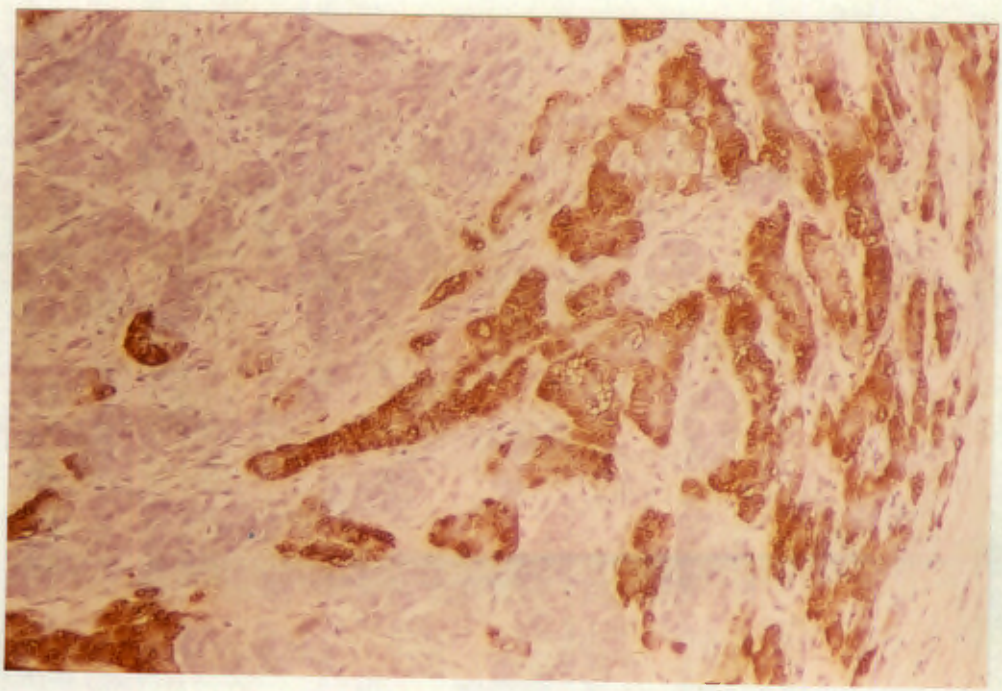


Fig. 26.3. Hepatocellular carcinoma. Section stained as for fig. 26.1. In this specimen normal liver stains positively, but tumour is negative.

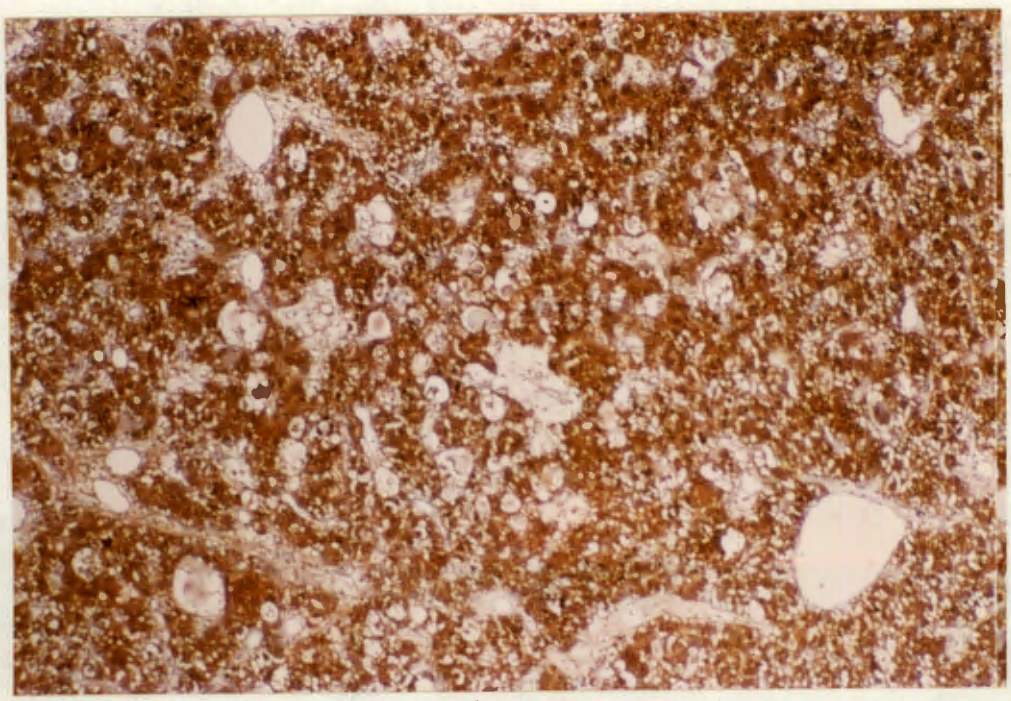


Fig. 26.4. Hepatocellular carcinoma. Section stained as for fig. 26.1. Diffuse intense staining in a poorly differentiated hepatocellular carcinoma.

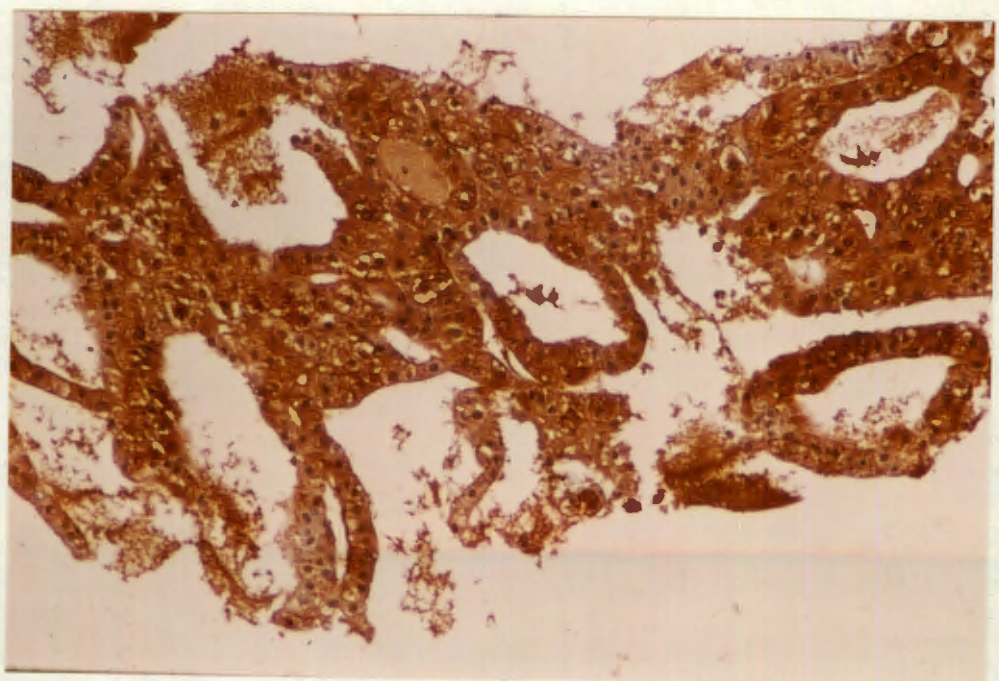


Fig. 26.5. Hepatocellular carcinoma. Section stained as for fig. 26.1. Diffuse staining in a well differentiated hepatocellular carcinoma.

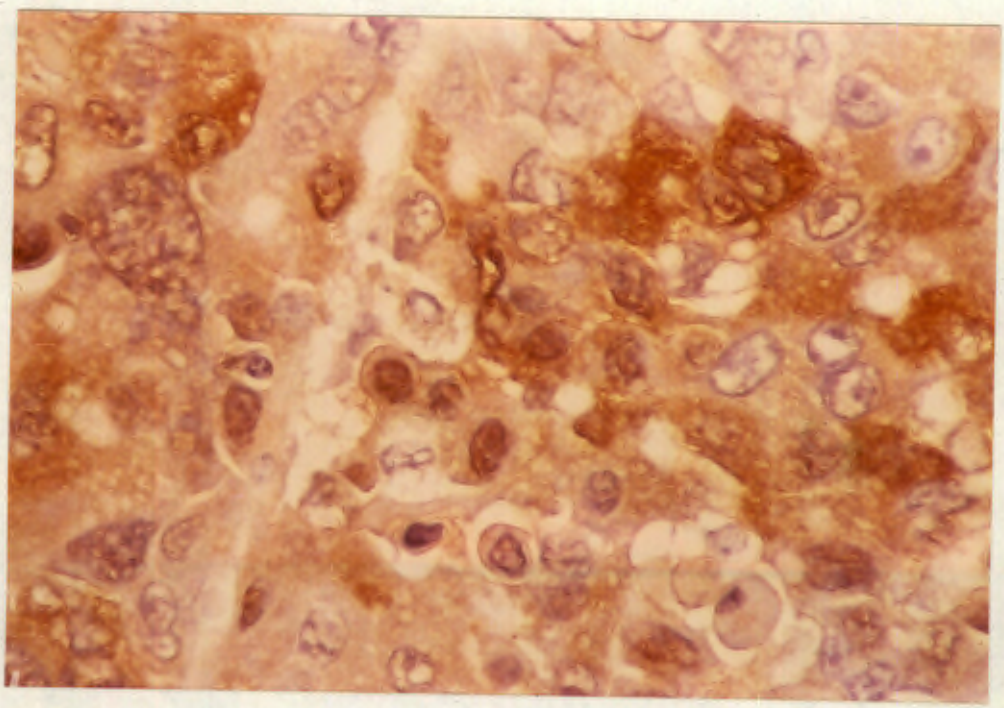


Fig. 26.6. Hepatocellular carcinoma. Section stained as for fig. 26.1. Patchy positive staining for ligandin in hepatocellular carcinoma. Positive staining is limited to small clumps of cells. This was the commonest pattern of staining seen.

	semiquantitative score	% of total
Well differentiated tumours	0	8
	1	35
	2	40
	3	16
	4	0
poorly differentiated tumours	0	38
	1	25
	2	19
	3	13
	4	6

Table 26.1. Semiquantitative estimation of ligandin in hepatocellular carcinoma by immunohistochemistry. A score of 0 indicates no ligandin present, and a score of 4 indicates diffuse intense staining.

Of twenty well differentiated tumours only 2 were completely negative, 7 stained weakly (score 1), 8 moderately (score 2-3), and 3 stained strongly (score 4-5) By contrast 6 of the 16 poorly differentiated tumours stain did not at all, a further 4 stained weakly, three moderately, and three strongly.

Semiquantitative scores for the two groups and the frequency distribution in which the scores occur are shown in table 26.1. The frequency distributions of ligandin in well and poorly differentiated tumours are significantly different using the χ^2 goodness of fit test.

Nuclear staining was present in 8 tumours, 6 well differentiated, and 2 poorly differentiated.

Immunoreactivity of tumour ligandin:-

This was tested in four tumours. In each case the slope of the dose response curve of dilution of cytosol from tumours was parallel to the slope of the assay standard curve (figs. 26.7).

Molecular size of immunoreactive ligandin in tumours:-

Cytosol from 4 tumours were chromatographed on G-100. In each case immunoreactive material and enzyme activity eluted at 1.5 x the void volume, corresponding to a molecular size of 47,500 daltons. The peaks were superimposable (figs. 26.8). There was no immunoreactivity in the void volume, indicating the absence of macromolecular forms, nor was there evidence of smaller immunoreactive forms, which might have been due to protein degradation.

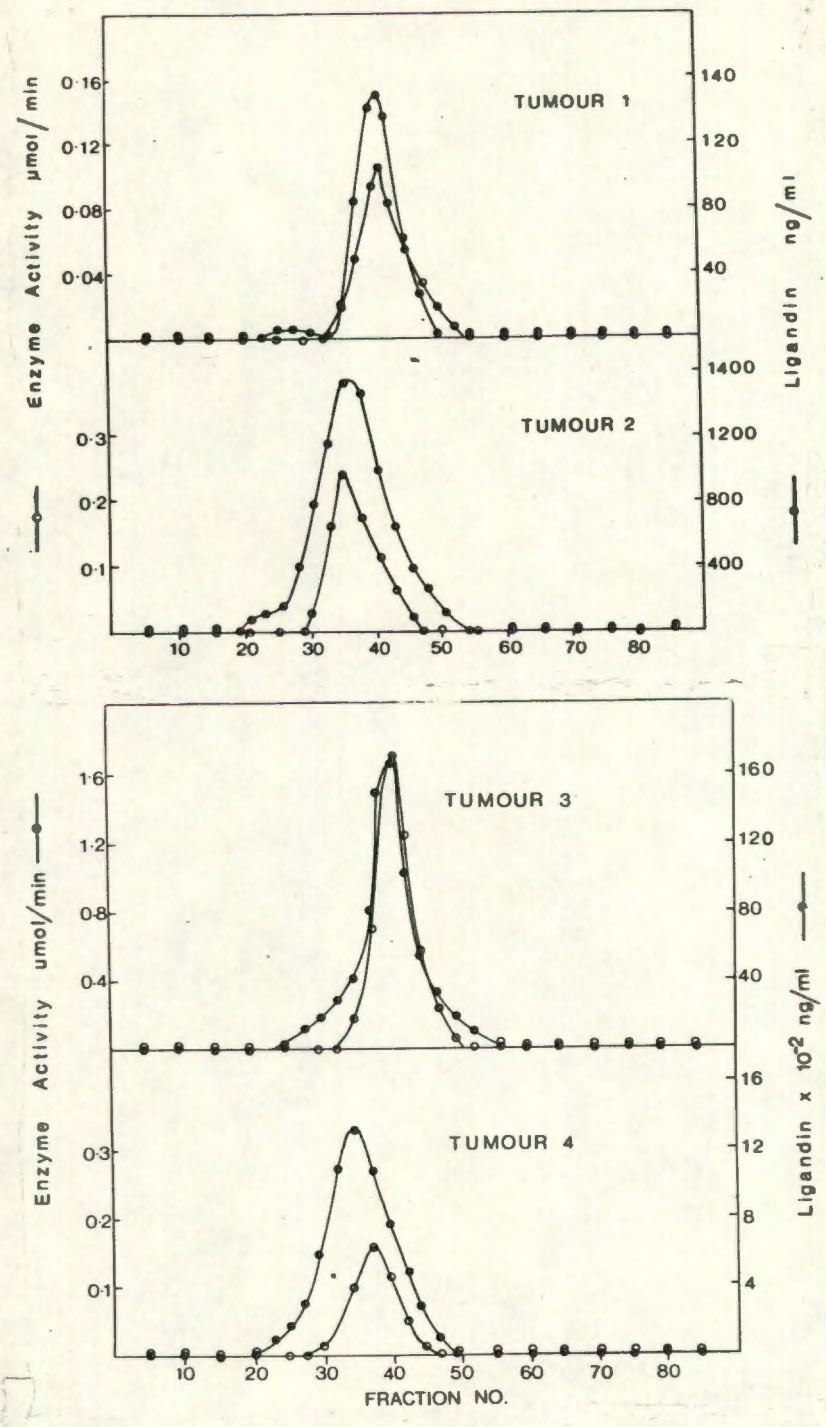


Fig. 26.7. Sephadex G-100 chromatography of cytosol from 4 tumours. Ligandin immunoreactivity was measured by RIA, and enzyme activity was measured with CDNB as substrate.

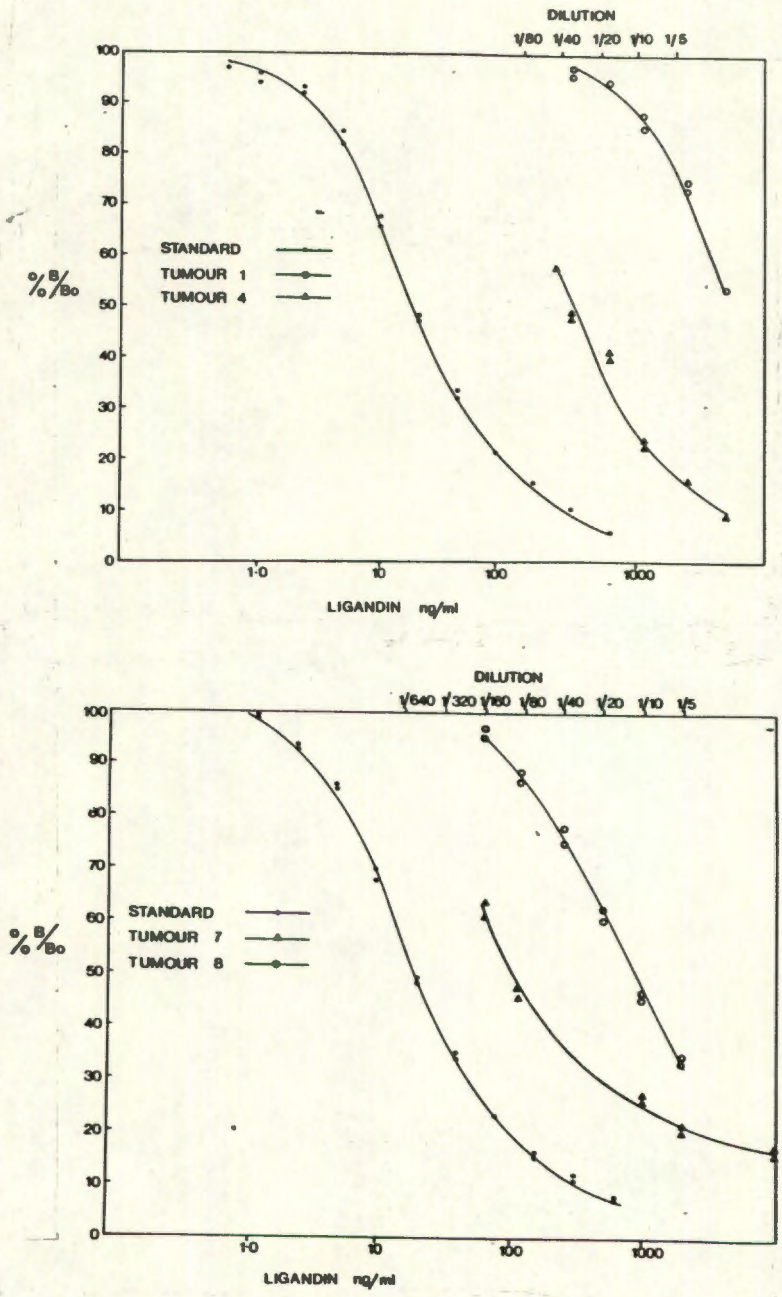


Fig. 26.8. Immunochemical displacement curves of cytosol from 4 specimens of human human hepatocellular carcinoma. Ordinate and abscissa are as for fig. 10.1.

Tumour no.	Immuno-reactivity ng/ml	Ligandin Specific Activity ng/mg protein	Enzyme Activity umol/min/ml	GSH-T Specific Activity umol min/mg
I	146	22	0.11	0.02
II	8815	940	0.31	0.03
III	144000	8150	1.80	0.10
IV	2545	218	0.43	0.04
VII	7979	566	1.25	0.09
VIII	1235	176	3.75	0.53
Liver 7	79512	8737	2.20	0.24
Liver 8	36567	2203	1.50	0.09

Table 26.2. Glutathione S-transferase activity with CDNB, and ligandin immunoreactivity by RIA, in human hepatocellular carcinoma. N.B. Liver 7 and 8 are non-tumorous liver from the patients from whom tumours VII and VIII respectively were obtained.

Quantitative estimation of ligandin in hepatocellular carcinoma:-

Results of assay of tumour ligandin by RIA and GSH-T activity with CDNB are shown in table 26.2.

Serum ligandin in hepatocellular carcinoma:-

Serum from 24 patients whose biopsies were studied was suitable for assay. A further 31 samples from patients in whom biopsy was not suitable for immunohistochemistry, or was not done, were studied. In those patients in whom biopsy was not done the diagnosis of hepatocellular carcinoma was made by finding an elevated α-foetoprotein or by angiography in the appropiate clinical setting. Mean serum ligandin was 11.5 ng/ml, S.D. $\pm$ 16.2ng/ml. The median was 8 ng/ml (range 0-90 ng/ml). Elevated values were seen in 31% of patients. There was no correlation between serum ligandin and HBsAg, α-foetoprotein, age, sex, or SGOT level or tumour morphology or the amount of ligandin in the tumour measured by the semiquantatitive scores.

IEF of tumour GSH-T's:-

Recovery of enzyme activity following IEF was 31-62% (mean 42%). This is similar to that found when normal liver cytosol was subjected to this procedure.

In tumour 1 (fig. 26.9) the cationic GSH-T peak was undetectable with CDNB as substrate. Peak immunoreactivity in this region focused at pH 8.7 and was less than 80 ng/ml. An anionic (pH 4.8) and a neutral (pH 6.3) peak of enzyme activity were present.

Tumour 2 (fig. 26.10) had two major cationic peaks. Immunoreactivity corresponded to the cationic enzyme activity peaks. A neutral

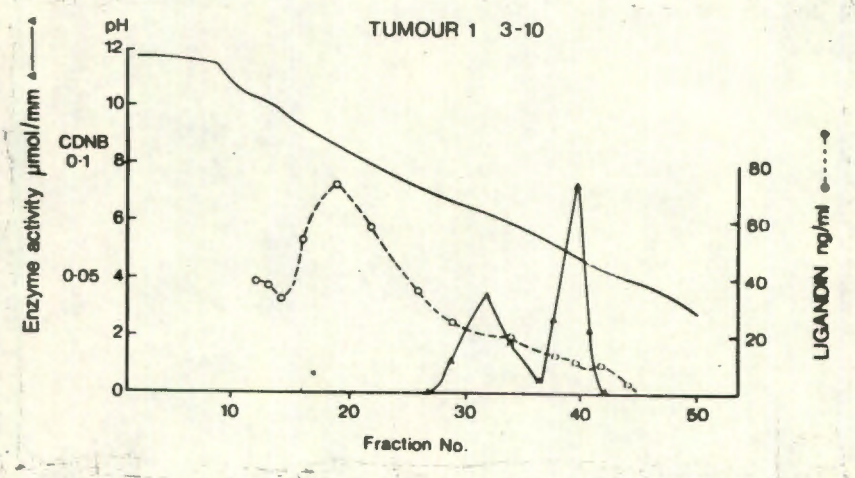


Fig. 26.9. IEF of cytosol from tumour 1. One ml of cytosol was applied. Ligandin immunoreactivity was determined by RIA, and enzyme activity was measured with CDNB as substrate.

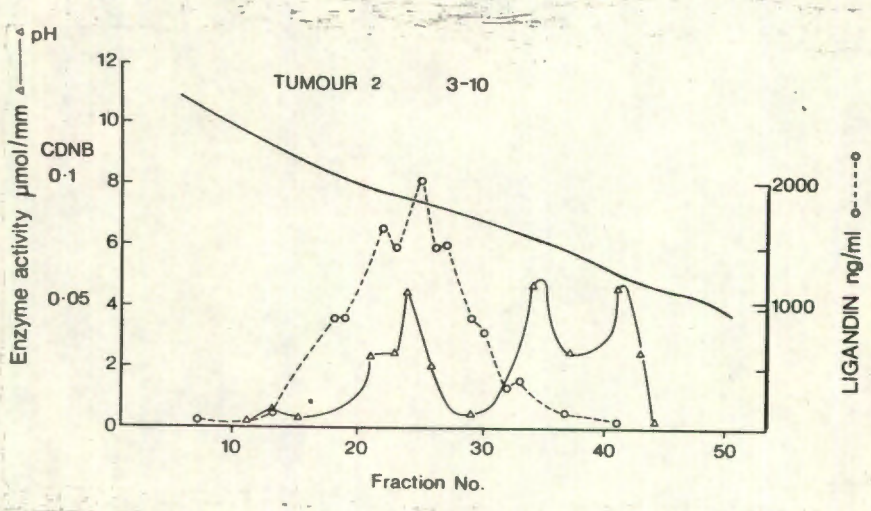


Fig. 26.10. IEF of cytosol from tumour 2. One ml of cytosol was applied. Ligandin immunoreactivity was determined by RIA, and enzyme activity was measured with CDNB as substrate.

peak focused at pH 6 and an anionic peak at pH 4.7. There was a small peak of immunoreactivity corresponding to the neutral peak, but the anionic peak had no immunoreactivity.

Tumour 3 (fig. 26.11) had 3 peaks of enzyme activity. The cationic peak focused at pH 9.2, with a shoulder at pH 8.8 - 9.0. The neutral peak was at pH 6.5. The anionic peak was relatively small, focusing at pH 4.7.

In tumour 4 (fig. 26.12) only a trace of enzyme activity was detectable in the cationic region. Immunoreactivity in this region focused at pH 8.1. Peaks of enzyme activity were seen at pH 6.4 and at pH 5.3. The neutral peak of enzyme activity falls within the curve of immunoreactivity and in this instance it is not possible to be sure whether this enzyme peak is related to GST υ or to the cationic GST's.

Tumour 5 (fig. 26.13) has a large peak of enzyme activity at pH 10 - 10.2, as well as traces of activity at pH 6.5, 5.6 and 4.6. Similarly tumour 6 (fig. 26.14) has a large peak of activity at pH 10 - 10.2, and a tail in the region of pH 8.9. In this tumour there is no enzyme peak in the neutral region. The anionic enzyme peak is minor and focuses at pH 4.8.

Two further tumours and non-tumorous liver from the same patients were available. Tumour 7 (fig. 26.15.1) had two peaks, focusing at pH 6 and pH 10.2. There was no anionic peak. In the liver from the same patient total enzyme activity was reduced (fig. 26.15.2)(see table 26.2). Peaks of enzyme activity were detected in the cationic and anionic range. There was no neutral peak in non-tumorous liver.

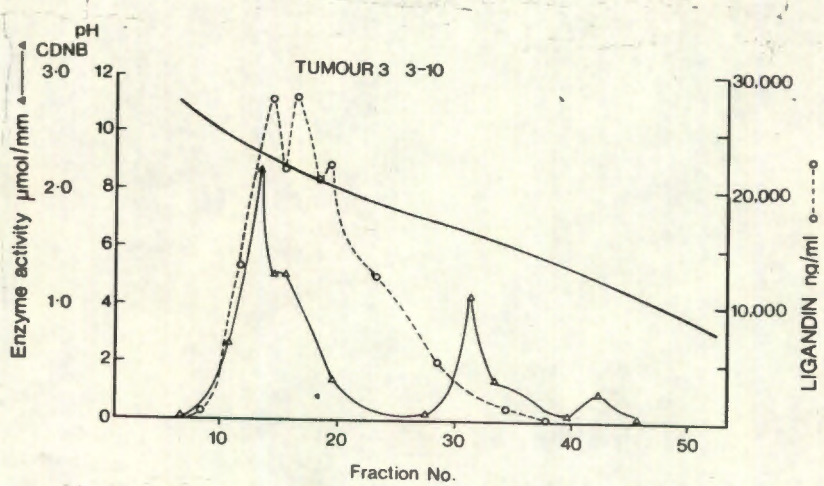


Fig. 26.11. IEF of cytosol from tumour 3. One ml of cytosol was applied. Ligandin immunoreactivity was determined by RIA, and enzyme activity was measured with CDNB as substrate.

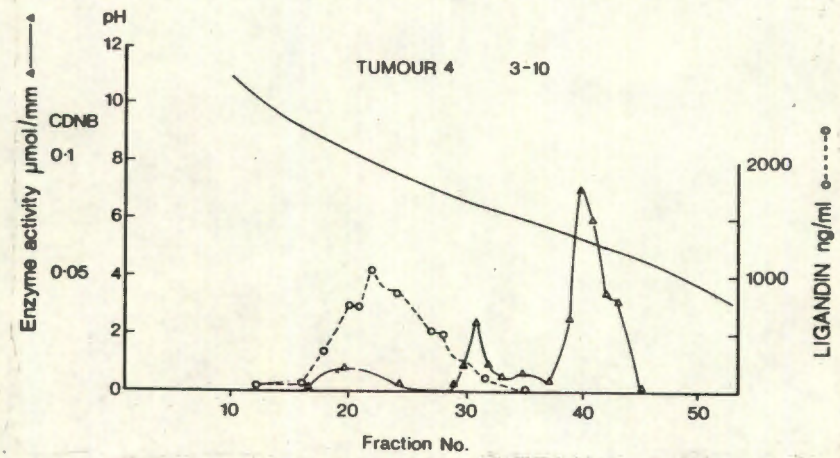


Fig. 26.12. IEF of cytosol from tumour 4. One ml of cytosol was applied. Ligandin immunoreactivity was determined by RIA, and enzyme activity was measured with CDNB as substrate.

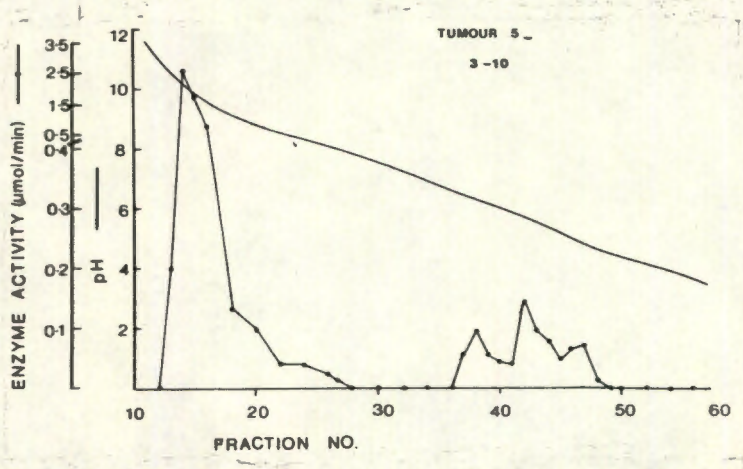


Fig. 26.13. IEF of cytosol from tumour 5. One ml of cytosol was applied. Ligandin immunoreactivity was determined by RIA, and enzyme activity was measured with CDNB as substrate.

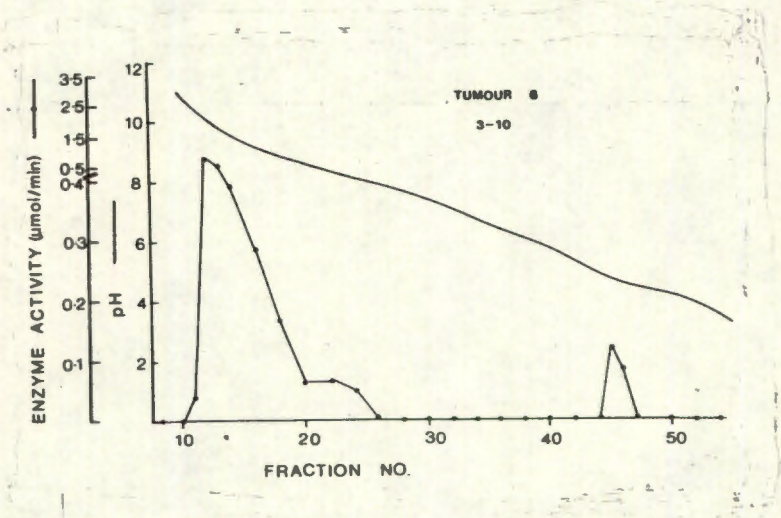


Fig. 26.14. IEF of cytosol from tumour 6. One ml of cytosol was applied. Ligandin immunoreactivity was determined by RIA, and enzyme activity was measured with CDNB as substrate.

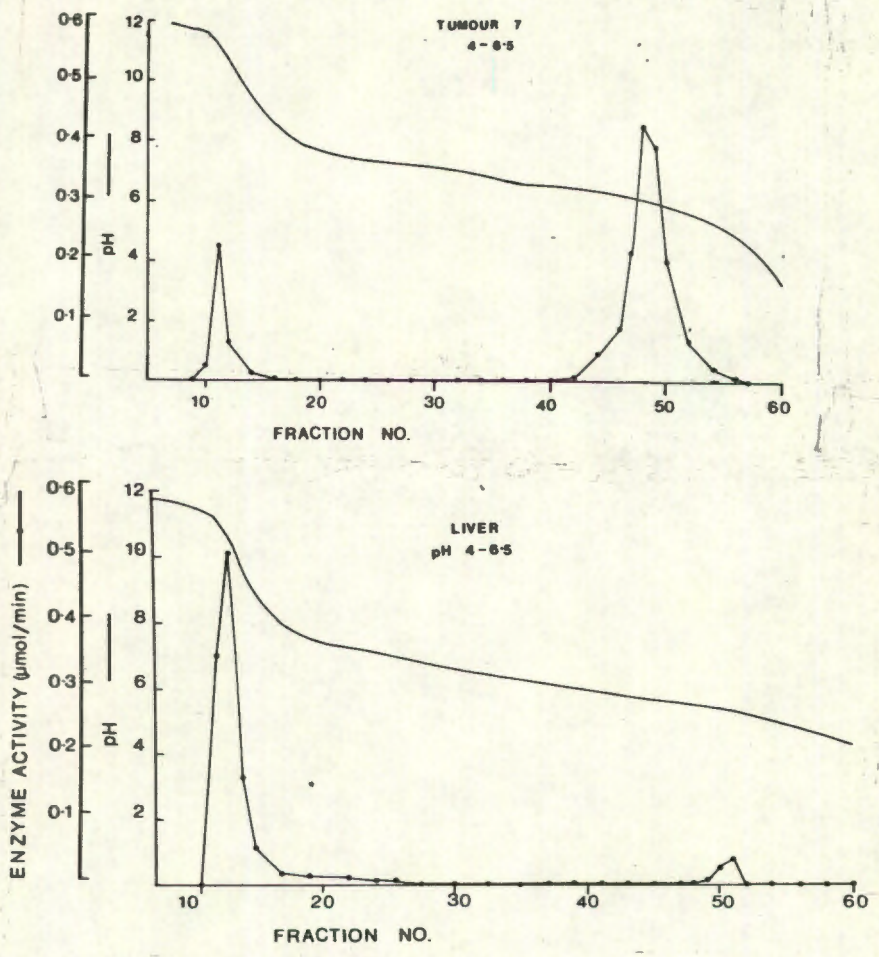


Fig. 26.15. IEF of tumour cytosol and cytosol from the non-tumorous liver from the same patient. Four ml of cytosol was applied in each case. Ligandin immunoreactivity was determined by RIA, and enzyme activity was measured with CDNB as substrate.

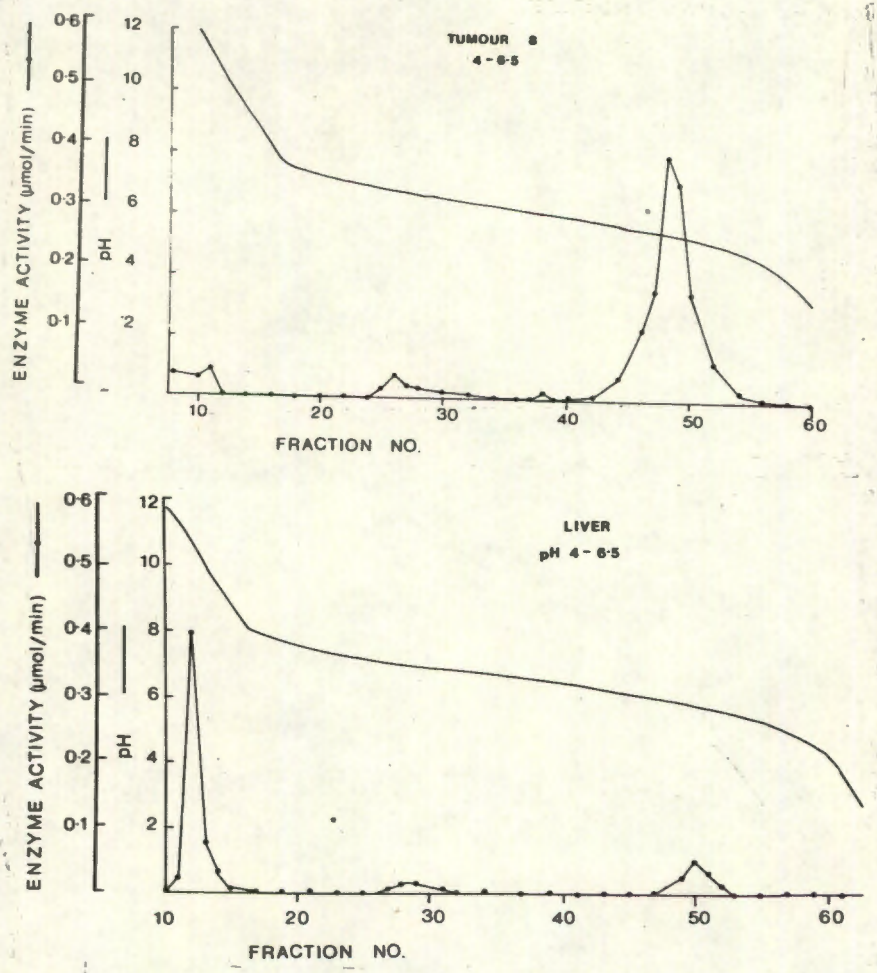


Fig. 26.16. IEF of tumour cytosol and cytosol from the non-tumorous liver from the same patient. Four ml of cytosol was applied in each case. Ligandin immunoreactivity was determined by RIA, and enzyme activity was measured with CDNB as substrate.

Tumour no.	Cationic GSH-T estimated activity umol/min/ml		Neutral GSH-T estimated activity umol/min/ml		Anionic GSH-T estimated activity umol/min/ml	
I	—	—	0.05	(47%)	0.06	(53%)
II	0.11	(34%)	0.12	(39%)	0.08	(27%)
III	1.30	(72%)	0.43	(24%)	0.07	(4%)
IV	0.05	(12%)	0.08	(19%)	0.30	(69%)
V	12.83	(86%)	0.75	(5%)	1.34	(9%)
VI	11.61	(96%)	—	—	0.48	(4%)
VII	0.13	(10%)	1.13	(90%)	—	—
VIII	0.30	(8%)	0.34	(17%)	3.11	(83%)
Liver 7	1.02	(68%)	0.26	(17%)	0.23	(15%)
Liver 8	2.09	(95%)	0.11	(5%)	—	—

Table 26.3. Estimated GSH-T activity in human hepatocellular carcinoma. The estimated activity for each group of GSH-T was derived as a percentage of the activity in cytosol before fractionation. The percentage of total activity due to each group of GSH-T was estimated from the area under the curve of that GSH-T activity on isoelectric focusing, and is given in parenthesis.

In tumour 8 (fig. 26.16.1) the cationic GSH-T'S were virtually undetectable. A trace of enzyme activity was present at pH 6.8, but the major enzyme activity was found in a peak focusing at pH 5.5. In the corresponding liver (fig. 26.16.2) total GSH-T activity was again reduced. Two peaks of activity focused above pH 9.0, with traces of activity at pH 9.3, pH 5.7 and in region of the neutral GST's.

DISCUSSION

It is widely accepted that the term malignancy implies loss of control of growth, and that growth rate of tumours is a measure of their degree of malignancy (501,502). This concept has its pathological equivalent, in that highly malignant tumours, i.e. rapidly growing, often have histological features which distinguish them from less malignant tumours. Features such as anaplastic cells, i.e. cells with no distinguishing features, suggest high grades of malignancy. It is also widely accepted that as a cell deviates from its original phenotype, i.e. as it becomes more malignant, it alters more and more in its phenotypic expression. This has been demonstrated for enzymes of glucose metabolism, (503) RNA metabolism (504), DNA metabolism (492), and purine metabolism (505) amongst others.

Drug metabolising enzymes however are not well studied in neoplasms. This present work demonstrates that in keeping with concepts of enzyme expression in tumours in general, the GSH-T's are present in decreased amounts in human hepatocellular carcinomas. There is a rough correlation between tumour differentiation and the amount of GSH-T in the tumour.

It is known that occasionally morphologically well differentiated tumours behave in a highly malignant fashion and vice versa. If the concept expounded by Weber, the molecular correlation concept (492) is correct, then heavy staining for ligandin in poorly differentiated tumours and the lack of staining in well differentiated tumours may be examples where the biochemical phenotype is a better guide to malignancy than the morphological characteristics. Unfortunately clinical data to prove this assumption are lacking.

Many workers have occupied themselves with the problem of phenotypic variation between tumours derived from a single cell type. However less work has dealt with phenotypic variation within a single tumour. This present study shows that although in some tumours ligandin is diffusely present, in others it occurs in scattered islands, the cells of which might stain quite strongly.

Although malignant tumours are believed to be clonal in origin morphological variation within a single tumour is self evident. Several lines of evidence suggest that within a malignant tumour a significant proportion of cells are not malignant (506,507).

With respect to glutathione transferases in hepatocellular carcinoma it may be that islands of persistent staining in an otherwise anaplastic sample represent normal or relatively normal cells that have retained many of the phenotypic features of the parent cell. An alternative but complementary hypothesis is that since it is known from cell culture work with rat hepatocytes, that ligandin is produced during the early lag phase and stationary phase, and is decreased during the proliferative phase, that these persistent islands of staining rep-

resent post-mitotic, non-dividing resting phase or stable cells. Such cells have been described in other tumours (508).

A final possibility is that these rests of cells represent normal non-neoplastic liver isolated from the rest of the liver by invading tumour.

Ohmi et al (100) have shown by transplanting hepatoma cells into nude mice that serum ligandin rises as the tumour grows, and that the tumour is the source of the serum ligandin. In man however this current work does not allow such a conclusion since although elevated serum ligandin levels are seen in some patients with hepatocellular carcinoma there is no correlation with the intensity of tumour staining. Nor is it possible to exclude ligandin release from non-tumorous liver. Expanding tumour may cause adjacent cells to become necrotic, thereby releasing ligandin.

Immunoreactive material in human hepatocellular carcinoma is probably identical to purified ligandin, since the immunoreactive material elutes at the same volume from Sephadex G-100 as purified ligandin, has similar affinity for the antibody, as shown by dilution studies, and has a similar range of isoelectric points as ligandin in normal liver cytosol.

Both GSH-T activity and ligandin immunoreactivity may be markedly reduced in human hepatocellular carcinoma. Ohmi et al (100) have reported similar data with a ligandin RIA. As with normal tissues immunoreactivity is restricted to the cationic GSH-T's.

Although the relative contribution of the neutral and anionic GSH-T's to total GSH-T activity increases, the absolute amounts of activity under the neutral and anionic peaks after correction for differ-

ent loading volumes is not much different in tumours and normal liver. Therefore the major abnormality in tumours is the reduction in the cationic GSH-T, but this is variable. The activity of the cationic GSH-T's ranges from barely detectable to within normal levels.

Non-tumorous liver from patients with hepatocellular carcinoma has decreased GSH-T activity. In one patient liver and tumour had the same GSH-T's, albeit in different concentrations, but in the other patient the 'normal' liver had no neutral GSH-T, while the tumour arising from that liver had no anionic GSH-T.

It has been pointed out that suitable control tissue for studies of the metabolic abnormalities in tumours is somewhat controversial (492). Tumour bearing liver is probably not a good control tissue since it may be subject to many unknown influences secondary to the tumour itself (509). Regenerating liver is considered the best control tissue since it is growing at approximately the same as that of the most rapidly growing tumours (501). However regenerating human liver is for practical purposes unobtainable.

One may speculate that tumour bearing liver, which has been subject to the same influences as the original neoplastic cell, will show some phenotypic changes which may be considered as preneoplastic. It is known that, in the rat, ligandin immunochemical staining patterns mature with progressive nodule formation during chemical carcinogenesis, resembling the maturation patterns seen during normal postnatal maturation of the liver (511). The earliest nodules of induced carcinogenesis have reduced ligandin staining. It is feasible (if the theory of chemical carcinogenesis applies to human hepatocellular car-

cinoma) that the non-tumorous liver represents this early stage carcinogenesis. Alternatively decreased concentration of ligandin may be a non-specific result of the host of metabolic abnormalities induced by the tumour, and which affects a many other proteins as well as GSH-T.

It is also of interest that in the human foetal liver the anionic GSH-T's constitutes a greater proportion of total activity than is ever seen in the adult (491). In the adult the anionic GSH-T's never contribute more than 5% of total activity, but in the foetus the anionic GSH-T's contribute approximately 30 - 40% of total activity. The increased contribution of anionic activity in tumours may thus be an example of retrodifferentiation which is said to be the mechanism of alteration in phenotypic expression of enzymes in neoplasms.

SUMMARY AND CONCLUSIONS

This thesis describes both clinical and biochemical studies on the glutathione S-transferases. In the first part of the thesis the purification of ligandin is described, together with the development of a RIA for ligandin which has allowed its measurement in human subjects. Although the human cationic GSH-T's have been previously purified and characterised, the purification procedure used in this work has not been described before. Using a modified scheme, similar to that used to purify rat ligandin, a human ligandin has been purified to apparent homogeneity. Only gel isoelectric focusing shows inhomogeneity, which is probably due to minor differences in net charge, since all other criteria of purity are met. The protein purified here resembles that purified by others, in structure, and to a lesser extent in enzyme specific activity and kinetic parameters. The purification scheme makes use of anion exchange and gel filtration, in five relatively simple steps. TEAE-cellulose used in the first chromatographic step is somewhat tedious to prepare, but an anion exchange resin which is easier to use, eg DEAE-cellulose or DEAE-Sephadex may yield similar results. It may well be that the newer technique of chromatofocusing may considerably simplify purification of ligandin.

Using the previously purified ligandin a monospecific antiserum was raised in rabbits. The antiserum gave a single precipitin line when run in immunodiffusion against pure ligandin, further proof of purity of the antigen. Indeed when the pure ligandin was run against a heterogenous antiserum raised against soluble hepatic proteins, a single precipitin line was obtained.

The requirements for a successful radioimmunoassay are:-

1. A pure antigen.
2. A monospecific antibody of suitably high affinity and titre.
3. A pure labelled protein of high specific activity which retains immunoreactivity.

To provide the labelled protein the chloramine-T method of iodination was used. The intact labelled protein was separated from damaged products of iodination and from free labelled iodine by a combination of gel filtration and anion exchange in a single column. This technique achieved good separation as was shown by TCA precipitation and chromatelectrophoresis of the peaks eluted from the purification column.

The RIA developed utilised the double antibody method to separate antibody bound from free labelled antigen. The assay protocol allowed rapid initial processing of a large number of samples. Runs of 300 samples were not uncommon. Although the two incubation times meant that a single assay took 4 days to complete (including counting time) this was not a major obstacle since ligandin in tissues, serum and urine could be stored. The assay compared favourably to other RIA's in that it was sensitive in the low nanogram range; the least detectable concentration was less than 1 ng/tube; it was reproducible with a low coefficient of variation and was free from non-specific interference as far as was tested. Although there was some degree of cross reactivity with rat ligandin, the assay could not be used to measure rat ligandin as the dilution curve of rat ligandin was not parallel to the assay standard curve.

The RIA was used to study the release of ligandin in human hepatic diseases. These studies suggested that serum ligandin levels were not a useful diagnostic aid, since the high levels seen in initial specimens often returned to normal within the first few days. This was probably due to the fact that the half-life of ligandin in blood is very short. However the fact that normalisation of ligandin was associated with clinical recovery suggests that ligandinaemia may be particularly suitable as an index of recovery, which may thus be determined at an early stage. Although ligandinaemia persisted in the few patients whose illness ran a prolonged course no patients in this series developed chronic hepatitis following acute hepatitis. The hypothesis that persistent ligandinaemia preceded chronic hepatitis could therefore not be tested.

In a separate study in patients with established chronic active hepatitis the degree of ligandinaemia was significantly correlated with histological severity of disease activity while there was no correlation between histological severity and SGOT levels. Although the series is small, the results are encouraging, and suggest that a larger study is warranted.

Studies of ligandinuria in situations in which acute tubular necrosis might be expected to develop, i.e. post renal transplantation, following hypotension, and in the setting of acute infection, revealed that ligandin release does indeed occur in these patients, but that the release is not predictive of ensuing renal failure. The highest levels of ligandin in the urine were seen post renal transplantation and following severe prolonged hypotension. When ligandin release did occur it was of short duration. The measurement of ligandin in the urine

has limited clinical application for these reasons.

Part 2 of this thesis deals with the distribution of the glutathione S-transferases in tissues. The occurrence of GSH-T activity in humans is not as simple as first thought. It is now known that there are three groups of GSH-T activity in human liver, and that at least two of these groups may consist of multiple isoenzymes. Prior to this work no information has been available on interindividual variation of the GSH-T's in organs other than the liver, or in various organs of the same individual. This thesis has shown that, like red cells and placenta, the lung and ovary have predominantly anionic GSH-T's. Furthermore in the kidney the anionic GSH-T's comprise a significant proportion of total GSH-T activity. The neutral GSH-T 's , which had previously been described only in the liver, have now been shown in testis and adrenal tissue as well. The small number of individuals surveyed precludes any firm conclusions on the pattern of distribution. However the findings are sufficient to challenge the previously held concept, that the neutral GSH-T is coded for at a locus which has a nul and two functional alleles, since the neutral GSH-T was detected in organs in individuals in whose liver it was absent.

It is obvious that there is wide variation in the distribution and the concentration of the GSH-T's both between organs and between individuals. The factors governing this variation are at present conjectural. To what extent genetic factors and environmental factors are important is unknown. Presumably the human GSH-T's, as in other animals, are subject to induction by ingested chemicals. To what ex-

tent this occurs in man, and what chemicals may be responsible, are also unknown.

In hepatocellular carcinoma there is also a wide variation in the pattern of GSH-T activity. Similar variation is seen in the distribution of ligandin immunoreactivity within these tumours. Immunohistochemical studies reveal a rough correlation between the degree of differention of the tumour, and the amount of stainable ligandin present. These findings may be in accord with the current concept, that the activity of an enzyme varies with the degree of malignancy of the cell, but in a heterogenous tissue such as a tumour such a conclusion is difficult to prove.

This work may have clinical importance, in that a tumour which is known to be deficient in a drug metabolising enzyme should theoretically be more sensitive to the effects of chemotherapeutic agents which are metabolised by that enzyme. If on the other hand, a chemotherapeutic agent requires activation by the enzyme in question, then a tumour deficient in that enzyme will be resistant to the chemotherapeutic agent.

The role of the GSH-T's in drug metabolism is not clear. Although many drugs are conjugated to GSH in vitro, the extent of in vivo reaction is unknown. Until further information is available the activity of drug-metabolising enzymes in tumours will remain a subject of academic interest only.

This thesis is the culmination of four years of pleasant labour in the laboratories of the Liver Research Group Of the University of Cape Town and the Medical Research Council of South Africa. It is my hope that these bodies are suitably rewarded for their support, by the

fruits of my labours, and that this work will stimulate those who come after me to seek answers to the questions that this work has raised.

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