LIGANDIN IN THE STEROIDOGENIC TISSUES OF THE RAT: CHARACTERISATION, DISTRIBUTION AND DEVELOPMENT.

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ABSTRACT

One of the main problems in the field of multifunctional proteins such as ligandin is the possibility that multiple forms and isoproteins may exist. Two forms of liver ligandin [GSH (reduced glutathione) S-transferase B] have been described, a heterodimeric form consisting of equal amounts of Ya (22000 daltons) and Yc (25000 daltons) subunits, and a homodimeric form containing only Ya. Because rat testis ligandin, prepared by the standard technique of anion-exchange and molecular exclusion chromatography, contains more Yc subunit than Ya, it has been claimed that testis and liver ligandin are different entities (Bhargava, Ohmi, Listowsky and Arias (1980) J. Biol. Chem. **255**, 724-727).

This thesis investigated the nature and character of ligandin in the steroid-producing tissues of the rat. A comparative study was undertaken to establish whether testis ligandin differed from liver ligandin. Different methods of purification were used to investigate testis ligandin and its relationship to other GSH S-transferases in steroidogenic tissues.

Testis ligandin purified by immunoaffinity chromatography using anti-liver YaYa ligandin antiserum yielded a product identical with liver preparations (Yc=Ya). This suggests that the differences previously described may be due to contamination of testis ligandin by a closely related species. Testis ligandin prepared by the standard technique was similar to that previously reported, containing more Yc than Ya. Cross-linking studies of standard testis ligandin preparations with dimethylsuberimidate showed more than one band in the 50000 dalton region, further stengthening the

view that these testis ligandin preparations may be contaminated. Since this contaminant was likely to be another GSH S-transferase, sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic analysis was performed on testis GSH S-transferases separated by CM-cellulose chromatography. GSH S-transferase AA which was present in large amounts, was shown to migrate in the same region as Yc subunit. CM-cellulose chromatography of a 'pure' standard testis ligandin preparation revealed significant amounts of GSH S-transferase AA migrating as Yc subunit, in addition to ligandin consisting of equal amounts of Ya and Yc subunits, indicating that testis ligandin is identical with liver ligandin and that previously described differences are due to a contaminant identified as GSH S-transferase AA.

Studies on ligandin in other steroid-synthesising tissues showed that ovary and adrenal ligandin prepared by standard techniques also contained more Yc than Ya. Separation of ovary GSH S-transferases on CM-cellulose showed that GSH S-transferase B, the peak reacting with anti-liver YaYa ligandin antisera contained equal amounts of Ya and Yc subunits, suggesting a situation similar to that in the testis exists. Glutathione peroxidase II activity of testis and ovary GSH S-transferases was investigated. Fractions corresponding to GSH S-transferase AA, A and B exhibited activity with cumene hydroperoxide. The considerable glutathione peroxidase activity of GSH S-transferases in testis and ovary suggest a protective function for the cells of gonadal tissue against oxidative damage to essential intracellular components.

Further attempts to clarify the function of ligandin in the steroid-synthesising tissues were made. The pattern of gonadal

ligandin development during early life, puberty and pregnancy determined by radioimmunoassay was found to parallel serum steroid hormone concentrations. This correlation was not observed in liver or kidney. Ligandin was localised to specific cells of the steroid synthesising tissues using immunocytochemical techniques. These findings suggest that there may be a functional link between steroidogenic cells, or products of their activity and certain GSH S-transferases. Phenobarbital pre-treatment did not have any effect on developing testis, ovary or adrenal ligandin concentrations. Immunocytochemical localisation of ligandin in rat steroid-producing tissues using a peroxidase anti-peroxidase (PAP) technique with anti-liver YaYa ligandin antiserum as the first antibody, showed staining in the testis to be limited to the interstitial (Leydig) cells. Stromal cells of the ovary and the fascicular, glomerular and reticular zones of the adrenal cortex also contained immunoreactive material. PAP staining with anti-testis ligandin antisera (testis ligandin prepared using the standard technique) showed far greater intensity of staining in these tissues, presumably due to reaction with both ligandin and GSH S-transferase AA.

This study has clarified the structural aspects of testis ligandin and demonstrated identity with liver ligandin. Ontogeny of ligandin in the steroidogenic tissues and localisation to specific regions in these tissues suggests a functional link between ligandin, GSH S-transferases, GSH peroxidases and activity of steroidogenic tissue.

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Chapter 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Ligandin, a multifunctional protein found in many organs and species, has been claimed to vary in structure and function, from organ to organ and from species to species. This study was designed to test the hypothesis that ligandin, found in the steroid-producing tissues of the rat, differs in structure and function from that found in liver, kidney and small intestine. In order to provide a background to the hypothesis, as well as a context in which relevance of the results may be judged, the literature review found in this chapter will cover briefly the historical aspects of the subject which include the discovery of Y protein, azocarcinogen-binding protein, cortisol metabolite-binding protein and glutathione S-transferase B (GSH S-transferase B). The distribution of this protein in various species, organs, tissues, cells and organelles will also be described.

The different functions of ligandin and GSH S-transferases have received widespread attention in recent literature. Since many excellent and comprehensive reviews are available, only those functional aspects relevant to the hypothesis tested will be reviewed in detail. In contrast, the recently described function of ligandin and the GSH S-transferases as GSH peroxidase II, which has not been well covered in the literature, will be reviewed in detail here.

The maturation of enzyme systems and the effects of weaning, puberty, pregnancy and senescence on these systems will be examined.

Further, several of the methods of purification of ligandin and the GSH S-transferases and studies concerning its structure and immunological properties will be included.

SECTION A

LIGANDIN AND THE GLUTATHIONE S-TRANSFERASES

HISTORICAL ASPECTS

In 1967, Arias' group at the Albert Einstein College of Medicine, New York, were studying mechanisms responsible for rapid transfer of bilirubin from plasma into liver. In order to study the binding of bilirubin and sulphobromopthalein (BSP) (Fig. 1.1) to proteins present in liver cytosol they subjected a mixture of cytosol and labelled bilirubin to gel filtration on Sephadex G-75, a technique which would not disrupt the anion-protein bond. Their study demonstrated the presence of three protein fractions in liver cytosol which bound to bilirubin and BSP (Levi **et al** 1969a). These fractions were termed X, Y and Z in order of elution from the column. The small amount of bilirubin present in the void volume or X peak was thought to represent non-specific adsorbtion to high molecular weight proteins and as such was not pursued. Subsequent studies thus focused on the isolation and characterisation of the organic anion-binding proteins responsible for the Y and Z peaks. Of the two proteins, Y was considered to be the major organic anion binding protein of hepatic cytosol since it was found to be present in greater abundance and to have a higher affinity for most organic anions than Z.

At about the same time that the Y protein studies were being carried out, other workers in different centres were independently

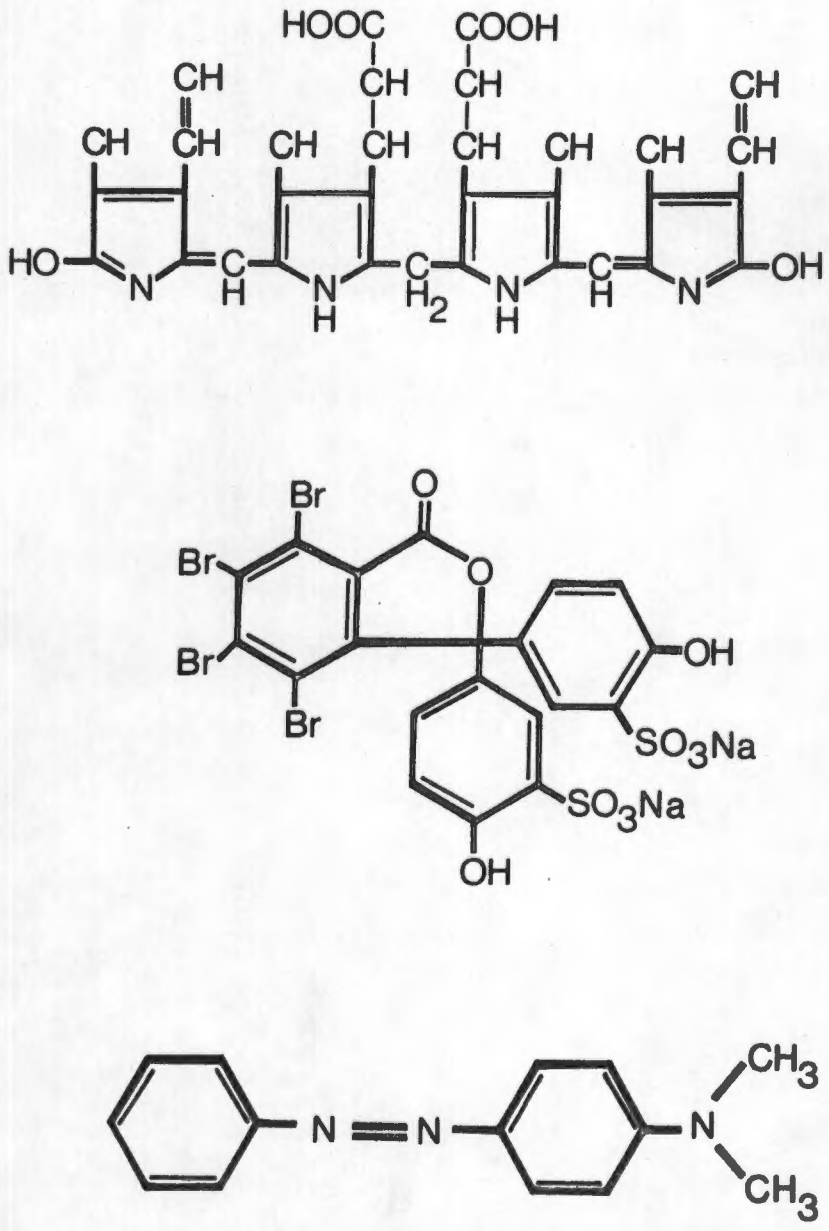


Fig. 1.1 Structural formulae of (top) bilirubin; (centre) sulphobromopthalein (BSP); and (bottom) 4-dimethylaminoazobenzene.

pursuing the same species but in different capacities. Thus Ketterer **et al** (1967) identified a basic azodye carcinogen-binding protein and Litwack's group a corticosteroid binding protein (corticosteroid Binder I) (Morey and Litwack 1969). The realisation that these proteins had similar physical and chemical characteristics led to the exchange of antisera and to the establishment of identity. The term ligandin was suggested since none of the previous terms used described the many ligands bound by this protein (Litwack **et al** 1971).

The possibility that ligandin may have catalytic activity was initially suggested by Kaplowitz **et al** (1973) when it was found that much of the unconjugated BSP added to cytosol as a marker of the Y peak was converted to a GSH adduct. This finding led to the discovery by Jakoby's group that GSH S-transferase B, the major transferase of rat liver, was immunologically indistinguishable from ligandin (Habig **et al** 1974a). The GSH S-transferases first described by Boyland and Chasseaud (1969) catalyze the conjugation of GSH with a large number of compounds bearing an electrophilic carbon (Fig. 1.2) to form the corresponding thioether, which is subsequently acted upon by other enzymes which remove glutamate and glycine, and which acetylate the amino group of the resultant thioether of cysteine. The N-acetyl cysteine thioether is the mercapturic acid of each of the substrates and is usually a non-toxic, soluble product which is readily excreted (Fig. 1.3).

Ligandin has now emerged as a protein able to bind non-covalently to a vast number of ligands, including bilirubin, sulphobromo-pthalein, heme, cortisol metabolites, oestrogens and bile acids

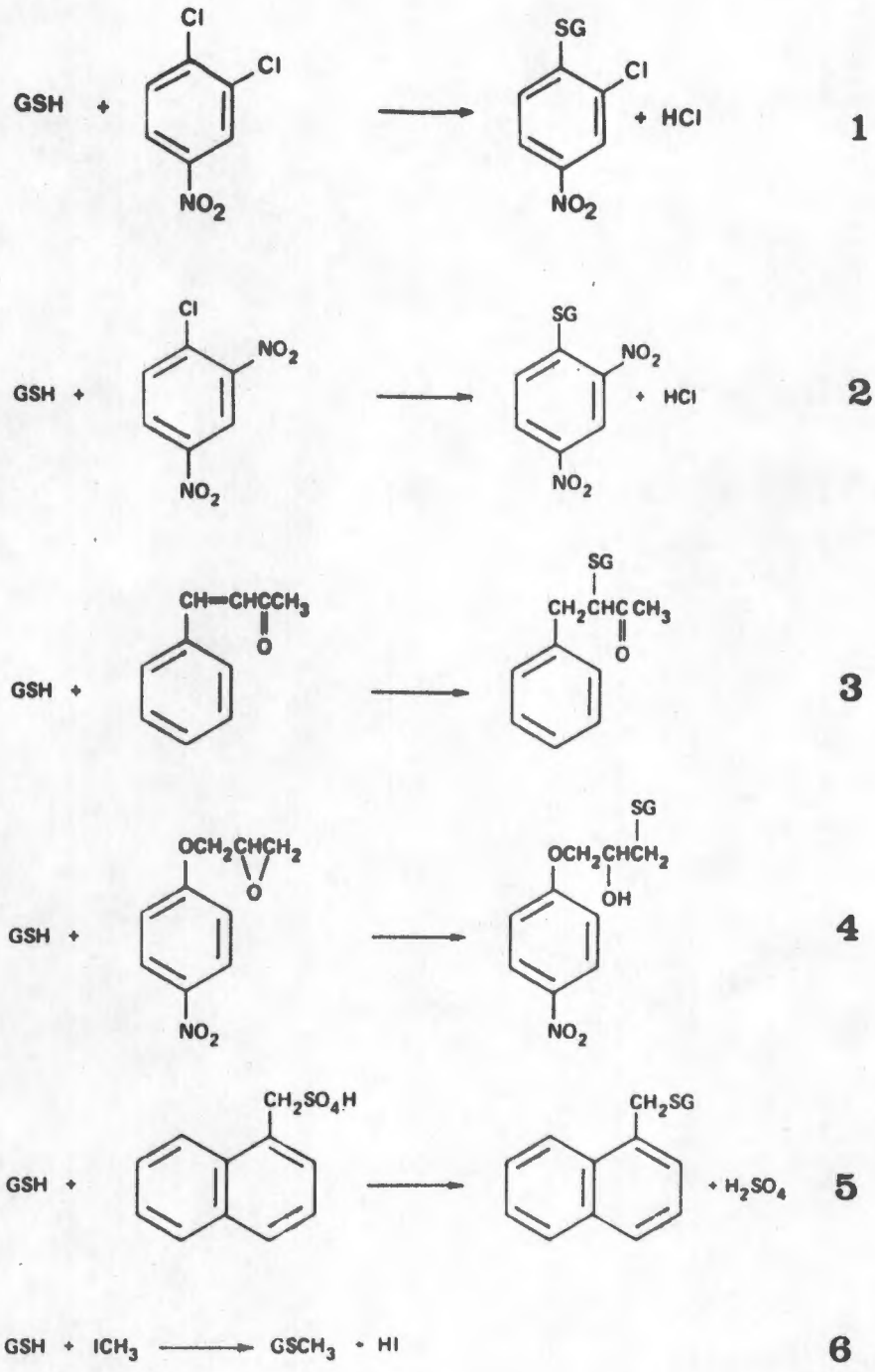


Fig. 1.2 The reactions between GSH and (1) 1.2-dichloro-4-nitrobenzene; (2) 1-chloro-2, 4-dinitrobenzene (both are aryl substrates); (3) trans-4-phenyl-3-buten-2-one (an alpha,beta-unsaturated ketone); (4) 1,2-epoxy-3-(p-nitrophenoxy) propane (an epoxide) (5) menapthyl sulphate (a sulphate ester) and (6) iodomethane (an alkyl substrate). All these reactions are catalysed by GSH S-transferase enzymes.

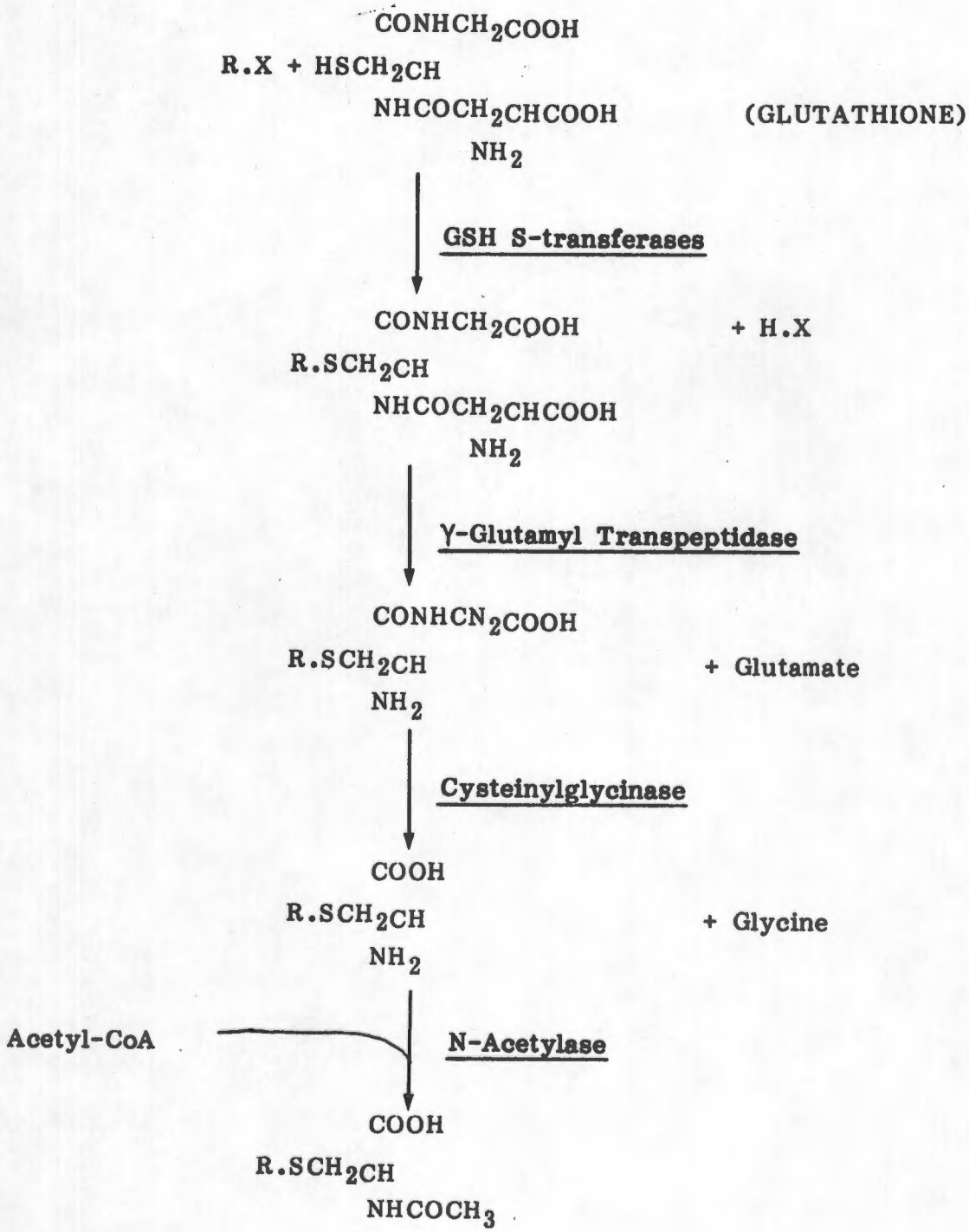


Fig. 1.3 Metabolic pathway for the biosynthesis of the mercapturic acids. R.X represents an electrophilic compound. The initial step is catalysed by the GSH S-transferases.

(eg. cholic acid and lithocholic acid, Fig. 1.4). Putative functions include that of a storage and transport protein within the cell analogous to that of albumin extracellularly. There is no direct evidence that these proteins are responsible for recognition and uptake of organic anions from the vascular space. However, they do influence net uptake by reducing the efflux into plasma by binding these substances within the cell (Wolkoff 1980). Ligandin is also able to bind covalently to reactive electrophiles such as azodye carcinogens (eg. 4-dimethylaminoazobenzene, Fig. 1.1) and their metabolites, and has been claimed to be responsible for eliminating these harmful substances. Finally, its catalytic activity as a GSH S-transferase, as a delta^5,3-keto steroid isomerase (Fig. 1.5) (Benson **et al** 1977) and the more recent discovery as a GSH peroxidase (Fig. 1.11) (Prohaska 1980), suggests that it may serve as a detoxifying enzyme as well as a barrier to lipid peroxidation.

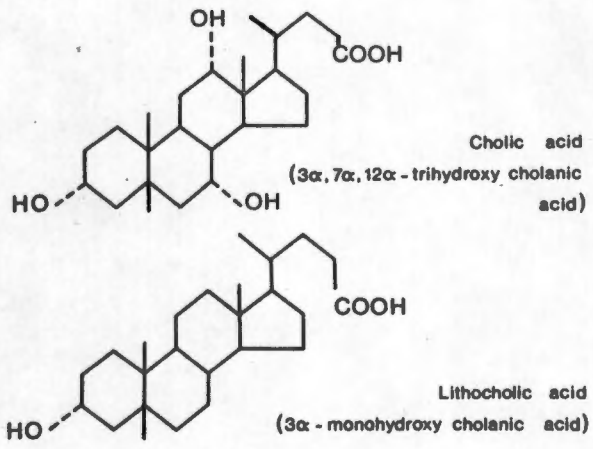


Fig. 1.4 Structural formulae of the bile acids; cholic acid and lithocholic acid.

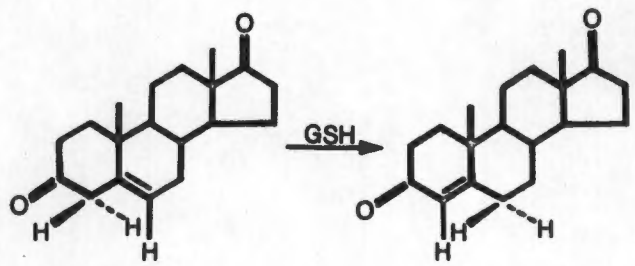


Fig. 1.5 Isomerisation of delta5-3-ketosteroid isomer to delta4-3-ketosteroid isomer Rat liver GSH S-transferases are able to catalyse this reaction (Benson et al 1977).

DEFINITION OF LIGANDIN vs GSH S-TRANSFERASE B

The multiple functions of ligandin have led to the use of a large number of synonyms and definitions which are often confusing. Ligandin, as purified from rat liver (Arias **et al** 1976, Listowsky **et al** 1976), is a basic, cytoplasmic protein existing in dimeric form, with a capacity for binding various electrophiles non-covalently and certain metabolites of carcinogens covalently. It is immunologically identical to GSH S-transferase B, but distinct from the other transferases.

Y protein was the original name of the BSP-binding fraction eluting from a molecular sieving column (Levi **et al** 1969a). This term, including the terms corticosteroid Binder I (Morey and Litwack 1969) and azodye carcinogen-binding protein (Ketterer **et al** 1967) have, as mentioned before, been replaced by ligandin (Litwack **et al**, 1971).

The subunit composition of rat liver ligandin has led to further confusion and will be defined here. Discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the Y peak showed three major bands in the 25000 dalton molecular weight region. Bass **et al** (1977a) named these Ya (22000 daltons), Yb (23500 daltons) and Yc (25000 daltons). Purified rat liver ligandin consists of Ya and Yc in two different dimeric forms, YaYa and YaYc (Yb is removed during purification) (Bass **et al**, 1977a). The dimers have been separated by Hayes **et al** (1979) who have named the YaYa species, Ligandin, and the YaYc species, GSH S-transferase B (see later).

Different groups of workers have their own preferred terminology. Those studying the enzymatic aspects of this protein appear to prefer GSH S-transferase B, while those dealing with its other functions, such as binding, use ligandin.

Unless specifically stated, the terms ligandin or GSH S-transferase B will be used in this dissertation to denote either or both the YaYa and YaYc dimeric species.

SPECIES AND TISSUE DISTRIBUTION

The distribution of ligandin and the GSH S-transferases in different species, in various tissues and in their cellular and subcellular components has been widely studied. This chapter reviews the recent literature with particular emphasis on studies in man and in the rat.

(a) Phylogeny

The appearance of ligandin in the liver coincides with the transition from aquatic to terrestial life. Teleosts, elasmobranchs and several species of amphibia have no ligandin detectable by BSP-binding to the Y peak. In contrast there is an abundance of ligandin (or Y protein) in post-metamorphosis amphibia, reptiles, birds and in mammals (Levine **et al** 1971). A similar phylogenetic pattern has been observed when GSH S-transferase activity was measured in these vertebrate species (Grover and Sims, 1964). Insects, molluscs, **Planaria** (Clark and Smith, 1975), plants (Frear and Swanson, 1970), and peas (Frear and Swanson, 1973) have GSH conjugating activity. Yeast does not (Jakoby 1978). Recently, GSH S-transferase activity has been detected in bacteria. Several strains of **Salmonella typhimurium** contain GSH S-transferase activity in addition to high levels of glutathione (Summer **et al** 1980).

(b) Invertebrate GSH S-Transferases

Tables 1.3 - 1.7 illustrate the different forms, substrate specificities, enzyme kinetics and immunologic properties of GSH

S-transferases found in various species and tissues, some of which have been purified to homogeneity. Purification methods such as ion-exchange chromatography, isoelectric focusing and polyacrylamide-gel-electrophoresis (see Table 1.1 and Chapter 1, Purification Methods) have all been used to isolate these proteins, while different substrates such as CDNB, DCNB, pNBC, SO, EA, IM and cumene.00H (see Table 1.2 for abbreviations) have been used to characterise the wide spectrum of catalytic activities.

In the early 1960s GSH S-transferases were implicated in the metabolism of insecticides (Hodgson and Casida, 1962) and these enzymes were accordingly investigated in insects (Table 1.3). Motoyama and Dauterman (1978) used CM-cellulose to isolate multiple forms of GSH S-transferase activity in an insecticide-resistant strain of the house-fly, **Musca domestica**, while Usui **et al** (1977) were able to separate and purify several of the transferases from fat bodies of the cockroach, **Periplaneta.** The catalytic properties of these enzymes differed from mammalian liver GSH S-transferases but were similar in the regard that they also displayed a wide range of overlapping specificities with various substrates. GSH S-transferase activity has been demonstrated in abdominal homogenates as well as in fat body and gut preparations of several species of insects (Yang 1976). Insecticidal compounds that are substrates for transferase reactions include organophosphosphorus triesters, organothiocyanates and chlorinated hydrocarbons. The GSH S-transferases catalyse two main types of reaction with these insecticides, ie the formation of O-alkyl or O-aryl conjugates, both of which result in detoxication. The former appears to be the major metabolic pathway **in vivo** (Motoyama and Dauterman 1980).

TABLE 1.1. KEY TO TABLES 1.3 - 1.7

Method Used:

(1) Gel filtration (G-100 or G-75 Sephadex)

(2) Ion exchange chromatography
- (a) CM-Cellulose
- (b) CM-Sephadex
- (c) DEAE-Cellulose
- (d) DEAE-Sephadex

(3) Affinity chromatography
- (a) GSH-BSP Sepharose 4B
- (b) hexyl-GSH Sepharose 6B
- (c) GSH-Sepharose

(4) SDS-polyacrylamide gel electrophoresis

(5) Isoelectric focusing

(6) Hydroxyapatite chromatography

ABBREVIATIONS USED IN TABLES 1.3 - 1.7

sa = specific activity in umol/min/mg
s/u = subunit
pk = peak
T/F = GSH S-transferase
m. weights = molecular weights in daltons

TABLE 1.2. SUBSTRATES AND THEIR ABBREVIATIONS

GSH	- reduced glutathione
CDNB	- 1-chloro-2.4-dinitrobenzene
DCNB	- 2.4-dichloronitrobenzene
pNBC	- p-nitrobenzyl chloride
SO	- styrene oxide
EA	- ethacrynic acid
IM	- iodomethane
BPO	- benzo(a)pyrene 4.5-oxide
MS	- 1-menapthyl sulphate
tPBO	- trans-4-phenylbut-3-en-2-one
BSP	- sulphobromopthalein or disodium phenoltetrabromophthalein sulphonate
Cumene.OOH	- cumene hydroperoxide
LAHPO	- linoleic acid hydroperoxide

The insect GSH S-transferases exhibit multiplicity with insecticides as substrates (Usui **et al** 1977), ie several enzymes may be active with a specific compound. Each enzyme may display also activity with several different types of compounds, as do the transferase enzymes of rat liver (Lu 1979). Insect drug-metabolising enzyme activities may be more readily understood since the characteristics of the enzyme in rodents are well documented, allowing for comparisons of the biotransformation mechanisms between the different species. Investigations carried out by Baars **et al** (1979) with the fruit-fly, **Drosophila melanogaster**, an organism often used in genetic toxicology testing (Vogel and Sobels 1976) demonstrated comparable GSH S-transferase activity in this species, which indicated a xenobiotic metabolism function similar to that encountered in mammals.

Contamination of estuarine and marine environments by organic xenobiotics prompted investigations of the toxicology of aquatic ecosystems. The presence of the GSH S-transferase enzymes were apparent in marine crustaceans such as the Blue Crab (Tate and Herf 1978) and molluscs (Bend **et al** 1977). The hepatopancreas (analogue of vertebrate liver) generally contains the highest enzyme specific activity, although activity can be detected in other tissues as well (See Table 1.3). Earthworms which have been exposed to pollutants and pesticides, display effective GSH S-transferase systems (Stenersen **et al** 1979).

TABLE 1.3. INVERTEBRATE GSH S-TRANSFERASES (See Tables 1.1. and 1.2. for key)

SPECIES	TISSUE	TRANSFERASE FORMS	MOLECULAR WEIGHTS	SUBSTRATE	CATALYTIC PROPERTIES	REFERENCE
Cockroaches (Periplaneta americana)	fat bodies	I, V, II, III, IV, (6)	35-37000 (1)	diazinon methyl parathion	Km=0.13mM (5mM GSH) Km=0.28mM	Usui et al 1977
House fly (Musca domestica)	whole bodies	-	54000-native (1) 37000-dissociated (1) 22700-s/u (4)	DCNB DCNB -	sa = 14.9 sa = 20.0 -	Motoyama and Dauterman 1978
Greater Wax Moth (Galleria mellonella)	larvae	major component pI=7.35 (3a)		IM pNBC	sa = 12.6 sa = 16.4	Clark et al 1977
Grass grubs (Costelytra zealandica)	whole bodies	pI=8.3 (5)		CDNB S-crotonyl-N-acetyl cysteamine methylparathion	800 umol/min 0.2 umol/min 0.02 umol/min	Clark et al 1973
Blue Crab (Callinectes sapidus)	hepatopancreas, excretory gland, gill, testis, muscle (12500 g supernat. fractions)			CDNB - DCNB -	Km = 0.3-0.5mM trace catalytic activity	Tate and Herf 1978
Earthworms (Lumbricus) - (Alldophora) - (Eisenia) -	whole bodies	1 major acidic pk 1 major alkaline pk 1 major neutral pk (5)		considerable CDNB activity, low DCNB	catalytic properties similar to rat liver T/F AA, B	Stenersen et al 1979

(c) Vertebrate GSH S-Transferases

Identification of the GSH S-transferases in rat extracts can be carried out either by the use of specific antibodies or less specifically by measuring enzyme activity with certain substrates and interpreting these patterns. Studies on the GSH S-transferases in animals other than the rat (Table 1.4) are incomplete. Tables 1.5 and 1.6 summarise details on the rat GSH S-transferases.

Liver GSH S-transferases in various vertebrate species have differences in substrate specificity and catalytic properties as shown in Table 1.4. Multiple forms of GSH S-transferase exist in some species, for example rat liver has seven isoenzymes. These vary in amino acid and isoelectric points but are similar in size and overlap in substrate specificity. They are thought to be the product of at least 6 genes. Guinea pig liver has 5 different forms (Irwin **et al** 1980). Chicken liver has 4 GSH S-transferase forms (Yeung and Gidari 1980), while hamster liver has 3 different forms, 2 of which cross-react with both rat liver ligandin and human liver GSH S-transferase epsilon. The five forms of monkey liver GSH S-transferases which can be separated by CM-cellulose are closely related to each other and are similar to the immunologically identical human GSH S-transferases, differing only in isoelectric point and are thought to represent post-translational modifications of a single gene product (Asoaka **et al** 1977). Nimmo **et al** (1980) noted that trout liver contained a transferase similar to rat liver ligandin in molecular weight and isoelectric point, but not in ability to bind BSP or lithocholic acid (Table 1.4).

TABLE 1.4. LIVER GSH S-TRANSFERASES OF DIFFERENT VERTEBRATE SPECIES (See Tables 1.1. and 1.2. for key)

SPECIES	TISSUE	TRANSFERASE FORMS	MOLECULAR WEIGHTS	SUBSTRATE	CATALYTIC PROPERTIES	REFERENCE
Rainbow trout (Salmo gairdneri)	liver	2pks low pI 1pk high pI Corresponds to rat liver ligandin (2b)	45000 (1)	CDNB activity	Does not bind BSP or lithocholic acid	Nimmo et al 1980
Snake (Agkistrodon piscivorus)	liver			methoxy fenitrothion (6a)		Stafford et al 1976
Cattle	liver	3-4 different forms (2a)		CDNB		Awasthi et al 1980a
Monkey	liver	I, II, III, V (2a) IV (6)	all have similar m. weights	CDNB CDNB pNBC	sa=90 1mM GSH sa=1.3	Asoaka et al 1977
Sheep	liver	4-5 different forms (pI 6.5-7.5) (5)	40000 (1)	GSH SO Napthalene-1.2-oxide	Km=10 (pH 6.5) Km=0.13mM (pH 8) Km=0.11mM (5mM GSH	Hayakawa et al 1974
Guinea Pig	liver	2 forms (6.1) (pI=7.3-7.7) (5)	45000 (s/u= 25000)	SO	Km=1.7mM (2mM GSH)	Hayakawa et al 1977
Guinea Pig	liver	(e,c,) (a, aa, aa') aa' purified (pI=8.9) (2a)	45000 (s/u 26000)	all forms CDNB, DCNB activity cumene.OOH activity CDNB	sa=46	Irwin et al 1980
Hamster	liver	I (2a) II(pI=9.0) (5) * III(pI=9.0) * *(II and III cross-react with rat liver ligandin and human liver T/F epsilon antisera)		CDNB, DCNB	similar Km's but higher Vmax's compared to rat and human forms	Smith et al 1980
Chicken	liver	4 forms (2a) 1 major form purified (pI=8.9)	45000 (1) (s/u 23000) (4)	CDNB GSH	Km=1.92mM (1mM GSH) Km=0.35mM	Yeung and Gidari 1980
Rabbit	liver	1 major peak 4 minor peaks (2a)		SO BPO	95% activity 40% activity	Maguire et al 1977
Rabbit	Liver	3 peaks (2a) (only one had GSH peroxidase II activity)	45000	CDNB LAHPO	Km=0.105mM (5mM GSH)	Morrisey and O'Brien 1980

The range in molecular weight of the GSH S-transferases is from 40000 - 46000 daltons (enzyme); 23000 - 26000 daltons (subunits). The isoelectric points of hepatic GSH S-transferases (pI = 6.5 to 10.0) do not vary greatly from species to species. The GSH S-transferases discussed here represent mainly the cationic and in less detail the neutral groups of enzymes (Tables 1.4, 1.5 and 1.6). The GSH S-transferases isolated from rat tissues other than liver resemble the hepatic forms with regard to molecular weight and isoelectric point (as shown in Table 1.5).

Species of snakes with naturally higher hepatic GSH S-transferase activities than others appear to have survived in contaminated ecosystems, this success of species survival perhaps being due to the presence of an efficient detoxifying system (Stafford **et al** 1976).

(i) Immunological Studies

Following isolation of homogenous preparations of rat and human hepatic ligandin, monospecific antibodies were used as a tool to study the antigenic relationship of these proteins with other hepatic GSH S-transferases and with similar proteins in other tissues and species. Development of a radioimmunoassay for rat ligandin facilitated the quantitation of this protein in different tissues (Bass **et al** 1977b). Immunodiffusion showed no cross-reactivity with anti-rat ligandin antiserum and liver preparations from guinea pig, mice, rhesus monkey, Shetland ponies, chickens, rabbits, fish, amphibia or reptiles (Fleishner **et al** 1972). This antibody was used to establish identity between Y protein, cortisol metabolite binder I and azocarcinogen-binding protein.

The liver supernatants from different strains of rats (Long Evans, Gunn and Sprague-Dawley) all contained ligandin which cross-reacted with the antibody as did the ligandin from different rat tissues. Hamster tissue demonstrated partial immunologic identity with the rat antibody (Fleishner **et al** 1977). Recently, Smith **et al** (1980) using a highly purified monospecific antibody to rat liver ligandin, were able to show cross-reactivity with both human and hamster liver preparations.

The five basic human transferases react similarly with monospecific antibody prepared against each of the other four proteins (Fleishner **et al** 1976). Antisera against the human proteins showed cross-reactivity with rhesus monkey liver preparation but not with rat.

(ii) Rat GSH S-Transferases : Liver

Table 1.6 lists the properties of the seven rat liver cytosol GSH S-transferases designated E, D, C, B, A and AA in order of their elution from carboxymethylcellulose (CM-cellulose) cation exchange columns (Habig **et al** 1974b; Habig **et al** 1976). Gillham (1973) has described an additional transferase, GSH S-transferase M, which binds to diethylaminoethylcellulose (DEAE-cellulose) at pH 8.0 and conjugates the reaction of GSH to 1-menapthyl sulphate. All but transferase D have been purified to homogeneity. The GSH S-transferases represent approximately 10% of soluble rat liver proteins. The major transferase of rat liver is GSH S-transferase B which accounts for 5% of total cytosol protein (Fleishner **et al** 1972).

Earlier work with crude preparations led to enzymes being named on the basis of substrate reactive groups or carbon (skeleton) backbone, and resulted in terms such as glutathione aryl, alkyl, alkene and epoxide transferases (Boyland and Chasseaud 1969). These terms are no longer considered applicable since there is considerable overlapping of catalytic capabilities of isoenzymes and all are able to catalyse conjugation of GSH with several substrates (Jakoby 1978 and Chasseaud 1979). Rat GSH S-transferases A and C are structurally and immunologically similar and differ only in isoelectric point. None of the other rat liver GSH S-transferases appear to share immunological identity. The specific activity of GSH S-transferases B and AA with the substrate CDNB is several times greater than that with DCNB (Table 1.6), while GSH S-transferases A and C both have considerable activity with DCNB. However their specific activity for CDNB is 10 times greater. GSH S-transferase A and C differ in their ability to catalyse reactions with the substrate **trans**-4-phenyl-3-buten-2-one, the specific activity of GSH S-transferase C is 20-fold higher than A (Guthenberg and Mannervik 1979). In addition to the broad specificity these proteins have for their substrates, Ketley **et al** (1975) have shown that they have overlapping binding of non-substrate ligands. The tightness of binding is dependent on both the nature of the ligand and the specific GSH S-transferase.

The amino-acid composition of rat GSH S-transferases A and C are similar. The difference index is 2.2 and can be interpreted as producing statistically significant proof of a close relationship between two proteins provided they are of similar length (Metzger **et al** 1968). A value of zero is equivalent to complete amino

acid homology, while 100 indicates absence of homology. Such comparisons of GSH S-transferase B with A, B with C, and B with AA yielded values of 9.5, 8.8 and 7.4 respectively (Jakoby **et al** 1976). Cornish-Bowden (1978) adopted an alternative approach, ie estimation of the number of sequence differences in the various GSH S-transferases. Rat liver GSH S-transferases A and C had an estimated sequence identity of about 95%, while the other transferases showed much less similarity. His finding that rat GSH S-transferases AA and B were far more similar than expected for unrelated proteins may be of particular importance as the results of our study become apparent.

Each GSH S-transferase has a distinct isoelectric point ranging from pH 7 to 10. Using preparative isoelectrofocusing Hales **et al** (1978) have separated transferases AA, B, A and C (in order of decreasing basicity). They also identified an additional GSH S-transferase with a pI of 6.6 which may be GSH S-transferase M, or may represent an as yet unidentified isoenzyme.

(iii) Rat GSH S-Transferases : Other Tissues

The capacity of different tissues to metabolise foreign compounds is important, particularly if that tissue is the site of entry or exit of foreign compounds and their metabolites from the body eg liver, lungs, gastrointestinal tract, kidneys and skin. Generally, hepatic tissues have greater GSH S-transferase activity than extrahepatic tissues, although evidence has accumulated that these enzymes are present in significant amounts in the extrahepatic tissues of the rat.

TABLE 1.5. RAT GSH S-TRANSFERASES (See Tables 1.1. and 1.2. for key)

SPECIES	TISSUE	TRANSFERASE FORMS	MOLECULAR WEIGHTS	SUBSTRATE	CATALYTIC PROPERTIES	REFERENCES
Rat	Lung	I (form absent in rat liver) (3b;6)		CDNB	sa=24	Guthenberg and Mannervik 1979
		II (arose from blood)		CDNB	sa=11	
		III (cross-reacted with rat liver T/F B)		CDNB	sa=18	
		IV (cross-reacted with rat liver T/F A + C)		CDNB tpBO	sa=22	
		V (cross-reacted with rat liver T/F A + C)		CDNB	sa=25	
Rat	Lung, Spleen	1 major form (2a) (identified as T/F AA)	s/u 25000=Yc			Scully and Mantle 1981
Rat	Epididymis, vas deferens	pk 1 (5) pI=8.9 corresponds to T/F B		CDNB		Hales et al 1980
		pk 2 pI=8.2 T/F A		CDNB,DCNB,tpBO		
		pk 3 pI=7.8 T/F C		CDNB,DCNB,tpBO		
		pk 5 pI=7.2 T/F M		CDNB,DCNB,tpBO		
		pks 4 & 6 pI=7.5 & 7.0 do not correspond to other T/Fs		CDNB,DCNB,tpBO		
Rat	Testis	pk 1 (2a) Corresponds to T/F D + E	s/u	(pk 1-5 have		Eidne and Kirsch 1982
		pk 2 T/F C	23500=Yb	CNDB activity)		
		pk 3 T/F B - (cross-reacts with rat liver Ya Ya ligandin antisera)	22+25000=YaYc	cumene.OOH		
		pk 4 T/F A	23500=Yb	cumene.OOH		
		pk 5 T/F AA	25000=Yc	cumene.OOH		
Rat	Liver mitochondria	1 (3c) pI=7.1 - 7.4 contributes 92% activity	45000 s/u 23000	CDNB	Km=0.7nmol/l	Kraus 1980
		2.1 " 0.7%	88000	CDNB	Km=1.3nmol/l	
		2.2 pI=4.8 " 7%	45000 s/u 23000	CDNB	Km=0.4nmol/l	

TABLE 1.6. RAT LIVER GSH S-TRANSFERASES* (See Table 1.2. for abbreviations)

PROPERTY	AA	A	B	C	D	E	M
M Weights	45000	46000	47000	47000	-	40000	-
s/u	25000 YcYc	23000 YbYb	22 + 25000 YaYc	23500 YbYb	-	-	-
Reaction with antibody	none	A, C	B	A, C	-	E	-
Isoelectric point	10	8.9	9.8	8.0	-	7.3	-
Relative concentration	0.14	0.22	1.0	0.52	0.02	0.1	-
Dif. index** between A and C	-	2.2	-	2.2			
Dif. index** between B; and AA, A and C	7.4	9.5	-	8.8			
Substrate specific activities (umol/min/mg)							
CDNB	14	62	11	10	-	0.01	-
DCNB	0.008	4.3	0.003	2.0	-	0	0.004
IM	1.4	0	0.59	0	-	8.9	0
EA	0.3	0	0.26	0.11	-	0	-
pNBC	0.09	11.4	0.1	10.2	-	4.1	0.5
tPBO	0	0.02	0.001	0.40	-	0	-
SO							
MS	-	0	0.004	-	-	-	0.1
BSP		0.53	0.006				
Prostaglandin A_1		0.013	0.005	0.021			
Δ^5-Androstene-3, 17-dione	.001	0.01	1.87	0.005			

* Data in this Table are summarised from references in Jakoby et al (1976), Jakoby and Habig (1980) and Habig and Jakoby (1981)

** Difference index, details in text.

Kraus and Kloft (1980) demonstrated that the steroidogenic organs displayed very high specific activity with varying substrates compared to the other tissues analysed. Specific activity for 1,2-epoxy-3-(p-nitrophenoxy)-propane was high in the testis, while both testis and adrenal cytosol displayed high specific activity for CDNB. The adrenal gland and the ovaries were also able to catalyse the nucleophilic attack of GSH on methyl iodide. Compared to the liver, testis preparations had substantially higher specific activities for CDNB, 1,2-epoxy-3-(p-nitrophenoxy)-propane and methyl iodide. These workers were unable to explain why the steroidogenic organs should have such high GSH S-transferase activities with these substrates, since there was no apparent link between steroid synthesis and conjugation with glutathione. They postulated that the transferases functioned as binding or transport proteins, or by protecting nucleophilic tissue components from denaturation by electrophilic reactants.

Bend and co-workers have demonstrated the ability of GSH S-transferases to metabolise reactive chemicals in the steroidogenic organs. In the rat, testicular GSH S-transferase activities with SO, CDNB, DCNB and tPBO as substrates vary from 40% to 90% of hepatic activity per mg of protein (Mukhtar **et al** 1978a), while ovarian GSH S-transferase specific activity towards SO is 67% of that seen in the liver (Mukhtar **et al** 1978b).

Rat and mouse sperm GSH S-transferase and delta^5-3-ketosteroid isomerase specific activity varied from 0.4 to 4.1% values seen in liver cytosol (Mukhtar **et al** 1978c). The epididymis, the site of spermatozoal maturation, was also shown to have GSH S-transferase conjugating activity (Hales **et al** 1980). Isoelectric focusing resolved cytosol from fractions of rat epididymis-vas deferens into six peaks of activity (pI 7.0-8.9) (Table 1.5). Four peaks were similar to rat liver transferases A, B and C and possibly M, while the other two peaks did not correspond to previously described transferases.

The epididymis, unlike the liver, is not thought to be involved in drug metabolism. However the GSH S-transferases may protect the epidymis and maturing spermatozoa from electrophiles or alternatively may catalyse some other reaction in this tissue.

The intestine is another organ which comes into contact with toxins and is a route of entry for many carcinogens into the body. Consequently an adequate detoxifying enzyme system is necessary. Rat duodenum and jejunum display considerable GSH S-transferase activity with CDNB as substrate. A total of 3% of the extractable protein from these tissues cross-reacts with antibody to GSH S-transferases A, B and E (Pinkus **et al** 1977). The major fraction of total intestinal transferase activity has been shown to be GSH S-transferase B, contributing 2% of rat intestinal extractable protein (Fleishner **et al** 1972). The highest level of GSH S-transferase activity in the rat gastrointestinal tract is found in the proximal small intestine. Middle and distal small intestinal tissues also display activity (Clifton and Kaplowitz, 1977).

Another major route of entry for foreign compounds is through the lungs. Studies of pulmonary GSH S-transferase activity have been carried out with the emphasis on epoxide substrates (Mukhtar and Bresnick, 1976a,b, de Pierre and Moron, 1979). Grover (1974) found that rat lung preparations possessed greater GSH S-transferase activity towards the K-region epoxide of benz(a)anthracene than corresponding liver preparations. This is an exception to previously reported studies which stated that hepatic tissues contribute more activity per mg of soluble protein than do extrahepatic tissues (Bend et al 1976). However Chasseaud et al (1980) confirmed Grover's findings with studies carried out on monkey lung and liver tissue. Guthenberg and Mannervik (1979) described 5 different forms of lung GSH S-transferases separable on hydroxyapatite following hexylglutathione affinity chromatography (Table 1.5).

Using immunodiffusion techniques ligandin was detected only in the liver, kidney and small intestinal mucosa of the rat (Fleischner et al 1976). However with the development of radioimmunoassay techniques (Bass et al 1977b) it became apparent that ligandin was widely distributed in the tissues of the adult rat, with the highest concentrations in liver, kidney, small intestinal mucosa, testis, ovary and adrenal gland. Lesser quantities were present in salivary gland, gastric mucosa of colon, pancreas, pituitary, lung, thyroid, bladder, heart and seminal vesicles.

Bannikov and Tchipyseva (1978) were the first to describe localisation of ligandin in the rat gonadal tissue using immunofluorescent microscopy. Subsequent immunohistochemical and immunofluorescent studies have identified and localised ligandin in most tissues in which it has been detected by radioimmunoassay. Rat serum also contains GSH S-transferase activity. Using SO and BPO as substrates, Mukhtar and Bend (1977) found levels of less than 1% of hepatic enzyme activity. Serum enzyme specific activity was less than 1% of hepatic enzyme activity.

(iv) Human GSH S-Transferases

Five cationic or basic GSH S-transferases have been isolated from human liver (Table 1.7). These are designated alpha, beta, gamma, delta and epsilon in order of increasing isoelectric point (pI 7.8 - 8.8) (Kamisaka **et al** 1975). These proteins have similar amino acid composition and molecular weight (48000 daltons) and consist of two identical subunits. The delta protein accounts for the major portion of the total purified hepatic transferases. The minor differences between the various cationic transferases have been attributed to a variable number of amide groups giving rise to differing charge isomers. These enzymes are thought to be products of a single gene. All five cationic human transferases react similarly with monospecific antibody prepared against each of the other four proteins and are termed ligandin(s) (Fleishner **et al** 1976).

In addition to the cationic transferases, acidic or anionic transferases have been demonstrated in human liver (Table 1.7).

Awasthi **et al** (1980b) were able to purify two such transferases the major form omega with a pI of 4.6, and a minor form psi (pI 5.4). Koskelo and Valmet (1980) have also described the presence of an acidic form (pI=4.63). Significant differences in catalytic properties of the cationic and anionic forms have been noted. Recently Warholm **et al** (1980) described the presence of another human liver GSH S-transferase which only occurs in some individuals. This transferase, which has been purified, has an isolectric point of 6 - 6.5 and has been termed transferase mu (Warholm **et al** 1981). It is a "neutral" transferase which has a 20-fold higher activity with benzo(a)pyrene-4,5-oxide and 100-fold higher activity with **trans**-4-phenyl-3-buten-2-one than the cationic transferases.

Another anionic GSH S-transferase has been purified; this time from human erythrocytes (Marcus **et al** 1978). The enzyme (pI=4.5), is named transferase rho and has an amino acid composition different from liver anionic transferases. Its molecular weight is 47500. GSH S-transferase activity has also been documented in human lymphocytes and peripheral leucocytes (Kaplowitz **et al** 1978). Human placenta appears to have a single form of GSH S-transferase with isolectric point, amino acid composition and substrate specificity similar to red cell transferase (Guthenberg **et al** 1979, Polidoro **et al** 1981a). Bilirubin and BSP competitively inhibit placental transferase activity (Polidoro **et al** 1981a), thus suggesting that this system may have a role in selective exchange of organic hydrophobic anions across the placenta during pregnancy.

TABLE 1.7. HUMAN GSH S-TRANSFERASES (See Tables 1.1. and 1.2. for key)

TISSUE	TRANSFERASE FORMS	pI		MOLECULAR WEIGHTS Enzyme	s/u	SUBSTRATE	CATALYTIC PROPERTIES	REFERENCE
Liver	alpha	7.8	(alpha to epsilon all cross-react with delta antisera)	48500	25000		sa 19	Kamisaka et al 1975
	beta	8.25		" "	" "		16	
	gamma	8.55		" "	" "		17	
	delta	8.75		" "	" "		37	
	epsilon	8.8		" "	" "		34	
Liver	omega	4.6	(major anionic species) cross-reacts with antisera)	46000	22500 24500	CDNB	26	Awasthi et al 1980b
	psi	5.4	(trace amounts)					
Liver	mu(3b)	6 - 6.5	(only present in some individuals)			CDNB, DCNB, tpBO, BPO, cumene.OOH		Warholm et al 1981
Liver		4.63	(similar to rho transferase of erythrocytes)	46000				Koskelo and Valmett 1980
Erythrocytes	rho (2c,6.1))	4.5	immunologically distinct	47500	24000	CDNB, EA	sa=66; Km=1mM sa=2.9	Marcus et al 1978
Foetal Erythrocytes	(one form similar to adult form) (2d)			50000 (1) 47-49000 (4)	25000	CDNB GSH	Km=1-1.36mM Km=0.1mM	Strange et al 1980
Placenta	-	4.65		45000	22500			Polidoro et al 1981a, Guthenberg et al 1979
	-	4.8		45000		CDNB, DCNB		

GSH S-transferase activity with epoxide substrates has been detected in human skin (Mukhtar and Bresnick 1976c), and in lung tissue (Greene and Jernstrom 1980, Moron **et al** 1979).

Established cell cultures derived from different species display GSH S-transferase activity towards CDNB, DCNB and 1,2-epoxy-3-(p-nitrophenoxy)propane (Wiebel **et al** 1980), rodent cell lines having greater activity than human cell lines.

CELLULAR LOCALISATION

Histological Studies

Several groups involved in the study of azocarcinogen binding in rat tissues have investigated the tissue and cellular distribution of ligandin using histological methods. Employing a fluorescent antibody procedure, Bannikov **et al** (1972, 1973) demonstrated the presence of ligandin in normal rat liver, kidney, small intestine, ovary and testis. Fleishner **et al** (1977) using a similar technique, found ligandin to be present in the cytoplasm of hepatocytes, proximal renal tubular epithelium and epithelial cells of the proximal, mid and distal small intestine of rat, hamster and man. These investigators did not detect ligandin in ovary and testis. Carruthers and Baumler (1979), who also employed the fluorescent antibody procedure, detected ligandin uniformly distributed in the cytoplasm of normal and 3'-methyl-4-dimethyl-aminoazobenzene-treated rat liver, but not in liver carcinomas. In highly differentiated hepatomas, ligandin was uniformly distributed, but was absent in poorly differentiated hepatomas, anaplastic carcinomas and in most adenocarcinomas (Bannikov **et al** 1973).

The effect of carcinogens on the ligandin content of normal and carcinogen treated rat liver, kidney and testis was investigated by Carruthers and Baumler (1980). Using the fluorescent antibody technique, liver and kidney from carcinogen-treated rats contained lower amounts of ligandin compared to untreated tissues, while the amount of ligandin in the testis of barbital-3'-methyl-p-dimethylaminoazobenzene-treated rats was significantly increased.

Ligandin was localised in the steroidogenically active cells using immunofluorescence and a monospecific anti-liver ligandin antibody. Testis of adult rats and rats at 1, 2, 7, 9, 11, 21 and 30 days after birth were examined and all typical Leydig cells were shown to contain ligandin, while the other cell types (seminiferous tubule cells, Sertoli cells and cells of blood vessel-like endothelium) did not (Bannikov and Tchipyseva 1979). The nuclei of the Leydig cells often showed very bright fluorescence indicating that ligandin could be present in these organelles. These workers localised ovarian ligandin to the theca interna cells, to thecal cells persisting in corpora lutea, to atretic follicles and to ovarian interstitial tissue. No ligandin-specific fluorescence was found in the primordial follicles, theca externa cells, granulosa cells, connective tissue of ovaries, or luteal cells of the corpus luteum. The same ligandin-specific fluorescence was found in pregnant ovaries and in the luteal cells of corpora lutea. Corpora lutea at the stage of involution, contained no ligandin. These studies demonstrated the presence of ligandin in gonadal cells in which active steroidogenesis occurs, ie Leydig cells, interstitial cells of ovaries, cells of theca interna and luteal cells in pregnant rats. The presence of ligandin in these steroidogenically active cells may be explained by postulating a protective role whereby ligandin prevents possible damage caused by high concentrations of steroids.

Bannikov and Tchipyseva (1979) were unable to detect any ligandin-containing cells in the adrenal gland or placenta of the rat. Using immunofluorescence and immunodiffusion Arias and co-workers

were similarly unable to detect any ligandin in the adrenal (Arias 1981).

With the application of the peroxidase-antiperoxidase procedure of Sternberger (1979) for light and electron microscopy, ligandin was localised to the cytosol and smooth endoplasmic reticulum of rat hepatocytes (Capron **et al** 1979). Campbell **et al** (1980), using the peroxidase-antiperoxidase method, demonstrated ligandin in human hepatocytes in renal proximal convoluted tubules and in the thick segment of Henle's loop of kidney. Ligandin was also present in oxyntic cells of human stomach, interstitial cells of testis, fully developed Graffian follicles of ovary and reticular-type cortical cells of the adrenal gland. Foetal adrenal cortex displayed intense staining compared to the other tissues investigated.

SUBCELLULAR LOCALISATION

The GSH S-transferases are located mainly in the cytosol as is glutathione. The mitochondrial matrix space has also been shown to contain activity (Wahllander **et al** 1979) while GSH S-transferase activity has been associated with the membranes of the endoplasmic reticulum (microsomes) (Glatt and Oesch 1977, Morgenstern **et al** 1979), a site particularly suited for GSH conjugation of reactive intermediates generated by the cytochrome P-450 system. Because this activity could be greatly stimulated by treatment of the microsomes with sulfhydryl reagents, while supernatant activities remained unaffected, and differences in substrate specificities and characteristics were observed, it was concluded that the microsomal GSH S-transferase activity was distinct from that of the cytoplasm (Morgenstern **et al** 1980).

Similarly Friedberg **et al** (1979) found differences in induction response in that phenobarbital treatment increased the specific activity of the soluble fraction two-fold, while microsomal specific activity remained unchanged. Three microsomal GSH S-transferases had similar isoelectric points to those of cytoplasmic GSH S-transferases A, B and C, also antiserum raised against transferase A (cross-reacting with transferase C) immunoprecipitated 50% of the microsomal activity. These findings suggested that the pattern of GSH S-transferases in the microsomal fraction is similar to that in the cytoplasm.

Three of the GSH S-transferases from rat liver mitochondria have been isolated. Two have similar molecular weights to that of the cytosolic forms, while the third has a molecular weight of 88000. The isoelectric points range from neutral to acidic for these mitochondrial forms (Kraus 1980). The physiological function of GSH S-transferases in these organelles remains obscure. GSH S-transferase activity in highly purified endomembranes and plasma membrane fractions was highest in rough endoplasmic reticulum, followed by microsomes and then smooth endoplasmic reticulum. Nuclei, nuclear envelope, plasma membrane, unbroken mitochondria and Golgi apparatus had much lower specific activities (Friedberg **et al** 1979).

STRUCTURAL AND SUBUNIT STUDIES

The structure and composition of ligandin and GSH S-transferase subunits have been subjected to intensive investigation in an attempt to understand some of the many different functions attributed to this protein.

(a) Subunits of Ligandin / GSH S-Transferase B

Initial SDS-PAGE of pure rat liver ligandin, employing a continuous system revealed a single band of 23000 dalton molecular weight (Ketterer **et al** 1975, Listowsky **et al** 1976). Using gel filtration chromatography ligandin had been shown to exist in solution as a 46000 dalton molecular weight protein. The protein was thus thought to exist as a dimer consisting of two identical subunits. However, with the introduction of discontinuous SDS-PAGE (2% Acrylamide spacer gel; 10 or 12% Acrylamide resolving gel), greater resolution was obtained, and rat liver ligandin was shown to consist of equal amounts of two non-identical subunits. The molecular weights of these two subunits are 25000 daltons and 22000 daltons (Bass **et al** 1977a).

The Y sulphobromopthalein-binding fraction of rat hepatic cytosol was shown by discontinuous SDS-PAGE to consist of three major bands, termed Ya (22000), Yb (23500) and Yc (25000) (Bass **et al** 1977a). Although the three bands were initially present in rat hepatic cytosol, the Yb subunit disappeared during the first anion exchange step of purification suggesting that Yb represents a relatively acidic species. Yb has recently been identified as a

subunit of GSH S-transferases A and C (Hayes **et al** 1980, Scully and Mantle 1980).

Phenobarbital pretreatment increased the concentration of Ya and Yb but had little effect on Yc (Bass **et al** 1977a). Ligandin could be separated using non-denaturing techniques into two forms, YaYa and YaYc. These findings led Bass **et al** (1977a) to suggest that ligandin was a heterogeneous species existing as two non-identical subunits which were the monomers of two distinct proteins. Bhargava **et al** (1978ab) disputed this hypothesis and presented results of electrofocusing and cross-linking experiments which indicated that the two subunits were part of a single heterodimeric protein rather than monomers of two different proteins. It is now accepted by most workers that there are two distinct species of ligandin, a homodimeric species, and a heterodimeric species. Carne **et al** (1979) and Hayes **et al** (1979) were able to separate the YaYa dimer from the YaYc dimer on CM-Sephadex. When compared with GSH S-transferase B (prepared by the method of Habig **et al** 1976) and ligandin (prepared as described by Bass **et al** 1977a) the CM-Sephadex elution volumes of ligandin and the YaYa dimer; and GSH S-transferases and the YaYc dimer were identical. This led Hayes **et al** (1979) to suggest distinct identities for ligandin (YaYa) and GSH S-transferase B (YaYc).

Antisera raised against either YaYa or YaYc both reacted monospecifically against purified YaYa and YaYc ligandin and also against liver supernatant fractions, suggesting that antigenic

similarity exists between the Ya and Yc subunits of ligandin (Bass et al 1977a). Amino acid analysis and limited proteolytic digests revealed a close relationship between the two species (Bhargava et al 1980b). Although isolation of the YaYa dimer could be achieved, separation of the Yc subunit from the Ya could not be carried out in non-denaturing systems (Bass et al 1977a). Bhargava et al (1980b) isolated Yc by extraction from SDS-polyacrylamide gels. This product did not retain catalytic activity. Urea (7M) denaturation of YaYc was able to dissociate the subunits (Hayes et al 1981) after which chromatography of the renatured products was carried out on CM-Sephadex. Sixty per cent of the original CDNB/GSH conjugating activity was recovered. Three peaks of enzyme activity eluted from CM-Sephadex, corresponded to the elution positions of ligandin (YaYa) GSH S-transferase B (YaYc) and GSH S-transferase AA (YcYc) (Hayes et al 1981). These experiments suggested that YaYc protein may be a hybrid of ligandin (YaYa) and GSH S-transferase AA (YcYc). However, this does not explain the antigenic cross-reactivity between Ya and Yc subunits (Bhargava et al 1980b), and their lack of immunologic identity to transferase AA (Habig et al 1976).

Amino acid analysis of the Yc subunit showed that although it contained most of the cysteine present in the intact dimer, the overall amino acid composition of each of the subunits was similar. This has been confirmed by peptide mapping studies (Bhargava et al 1980b). SDS-PAGE of **staphylococcus** U8/V8 pronase-treated subunits revealed similar patterns for Ya and for Yc. It is thought that the Yc contains an extra peptide of about 25 to 30

amino acid residues at the COOH-terminal end (Bhargava **et al** 1978a).

The Ya and Yc subunits are not linked by disulfide bridges. This was shown by demonstrating similarity of the subunits on SDS-PAGE in the presence and in the absence of beta-mercaptoethanol (Bass **et al** 1977a, Bhargava **et al** 1978a).

(b) Subunits of Other GSH S-Transferases

The molecular weight of rat liver GSH S-transferases is approximately 46000. All have been shown to exist as dimers of three possible subunits Ya (22000), Yb (23500) and Yc (25000). Hayes **et al** (1980) and Scully and Mantle (1980) have determined the monomer composition of the different GSH S-transferases by means of SDS-PAGE. Transferase AA exists as YcYc, while transferases A and C exist as YbYb. GSH S-transferase B and ligandin, as defined by Hayes **et al** (1979), exist as YaYc and YaYa dimers respectively.

The subunit and amino acid composition of GSH S-transferases AA and B suggest that they may be the product of a single gene. Transferases A and C may also be coded for by a separate single gene, especially since they cross-react with antisera raised against each other. Scully and Mantle (1981) have proposed that all the transferases are generated by proteolysis of the YcYc protein and suggest that the YaYa species is a purification artifact. This suggestion has been disputed by Hayes **et al** (1981), and although no spontaneous inter-conversion between YaYa and YaYc was observed on storage, their hybridisation studies

suggested that GSH S-transferase B (YaYc) is a hybrid of ligandin (YaYa) and GSH S-transferase AA (YcYc). Transferases D and E appear to contain both Yc and Yb bands on SDS-PAGE (Hayes **et al** 1980).

(c) Isoelectric Points of GSH S-Transferases

The isoelectric points for each of the rat GSH S-transferases is shown in Table 1.6. Gel isoelectric focusing yielded two bands of active enzyme for GSH S-transferases A (Pabst **et al** 1974) and E (Fjellstedt **et al** 1973). Two bands were also observed for transferases B and C (Habig **et al** 1974b). The multiple forms, or 'microheterogeneity' exhibited by ligandin, described by Ketterer **et al** (1976) and by Bass **et al** (1977a), have been attributed by Hayes **et al** (1979) to be due to the distinct YaYa and YaYc dimeric forms. The heterodimer YaYc described by Bhargava **et al** (1978a) showed a single band on electrofocusing. GSH S-transferase AA, which has a single band on isoelectric focusing, has an isoelectric point identical to one of the bands of GSH S-transferase B, ie a band which focuses at a pH of 10 (Habig **et al** 1976). In order to prove that GSH S-transferase AA was unique from the other GSH S-transferases, Habig **et al** (1976) compared enzyme activities of eluted sectioned gels and demonstrated distinct differences in behaviour between the bands of transferases AA and B.

(d) Subunit Binding Studies

The binding activity of ligandin has been extensively studied. Ligandin binds a variety of endogenous and exogenous hydrophobic compounds at a single site (Ketterer **et al** 1975, Tipping **et**

al 1976a,b, 1978). GSH binds at an adjacent site (Jakoby 1978). Thus in the case of GSH S-transferase activity, the hydrophobic ligands compete with the electrophilic substrates (Ketterer et al 1976). Although specific subunit binding of non-substrate ligands to the GSH S-transferases has not been investigated, Ketley et al (1975) have shown that bilirubin binds strongly to transferases B, C and A and poorly to AA.

Several studies of the binding and catalytic activities of the ligandin subunits have been carried out by Arias and co-workers. Their evidence indicates that primary high affinity binding of bilirubin to ligandin and GSH S-transferase activity are independent and occur at different domains in the protein (Bhargava et al 1978b). Several reports have described the presence of cysteine residues on the Yc subunit (Bhargava et al 1980b). Since binding occurs to cysteine residues (Ketterer et al 1976) it has been suggested that thiol groups are involved in the binding and catalytic sites of ligandin. Carne et al (1979) detected four thiol residues in ligandin. One of these residues appears to be associated with the hydrophobic binding site of ligandin. Bhargava et al (1978b) reported two binding sites for bilirubin to rat liver ligandin; a high affinity primary site:

$(K_{assoc}=5 \times 10^{7} M^{-1})$,

which may be present on the homodimer YaYa, and a secondary, lower affinity site:

$(K_{assoc}=3 \times 10^{5} M^{-1})$.

Their findings suggest that the high affinity site at which bilirubin is bound to ligandin is independent from the site at which catalytically reactive substrates bind.

Following photoactivation, BSP was shown to bind covalently to the Ya subunit only (Bhargava **et al** 1980b) suggesting that non-covalent binding of BSP, which preceded photolabelling, was probably mediated by Ya. Other reports (Arias 1979, Arias **et al** 1979, 1980) have suggested that the 25000 molecular weight subunit (Yc) was able to bind photolabelled BSP or activated carcinogen metabolites covalently. Yc binding to the labelled carcinogen exceeded that of Ya by 600-800%. The cysteine residues contained on the Yc polypeptide are thought to be responsible for this binding function. However, a more recent report suggests that the metabolites of 3'-methyl-**N,N**-dimethyl-4-aminoazobenzene bind exclusively to the Ya peptide (Ohmi **et al** 1981a).

The Ya monomer has been implicated in lithocholic acid (Fig. 1.4) binding and under non-equilibrium conditions the Yc monomer does not bind this bile acid (Hayes **et al** 1981). Under equilibrium conditions only the Ya subunit contains a high affinity binding site for cholic acid (Fig. 1.4) while both Ya and Yc subunits possess low affinity cholic acid binding sites (Hayes **et al** 1980).

(e) Translational Studies on the Subunits

Poly(A)-containing rat liver mRNA was isolated and translated into ligandin in a wheat-germ cell-free system (Daniel **et al** 1977). Ligandin synthesised **in vitro** dissociated into two polypeptides, the Yc subunit predominated immediately after synthesis converting into Ya upon ageing. These results suggest that Yc is the primary translational product of ligandin mRNA. Pickett **et al** (1981) found the primary translation product of rat liver GSH S-transferase B mRNA to be two distinct polypeptides.

The mRNA's coding for the two subunits were present in unequal concentration with the mRNA coding for Ya exceeding that for Yc. The amount of translationally active mRNA encoding for GSH S-transferase B is induced 3 to 4-fold by phenobarbital administration; in contrast to the findings by Daniel **et al** (1977) and, interestingly in view of Bass **et al**'s earlier work (1977a), only the mRNA for Ya is induced.

PURIFICATION METHODS

Purification procedures for the isolation of ligandin and GSH S-transferases have been numerous and varied. Most have made use of ion-exchange and molecular sieving chromatography, isoelectric focusing, polyacrylamide gel electrophoresis and affinity chromatography. Because of the importance of obtaining pure preparations before any conclusive studies can be carried out, this section will consider the merits and shortcomings of various methods of purification.

(a) Ligandin: Standard Purification Method

The standard purification procedure for rat liver ligandin was first described by Fleischner **et al** (1971). This method was used to purify rat renal ligandin and described in detail by Kirsch **et al** (1975). The technique makes use of the finding that ligandin has a higher pI than most cytoplasmic proteins. Fig. 1.6 details the procedure. A 100000 g supernatant fraction is passed over either a triethylaminoethyl (TEAE)- or a diethylaminoethyl (DEAE)-cellulose column equilibrated with a high pH, low ionic strength buffer. Gel-filtration of the unbound ligandin-containing fraction is then carried out (Sephadex G-100 at pH 7.4). Measurement of the binding activity of BSP, bilirubin, or GSH S-transferase catalytic activity with specific substrates enables the identification of the ligandin-containing peak. The final stage utilises QAE-Sephadex A.50 which combines both gel filtration and anion-exchange steps. Purity of the protein can be determined by SDS-polyacrylamide gel electrophoresis or gel isoelectric focusing.

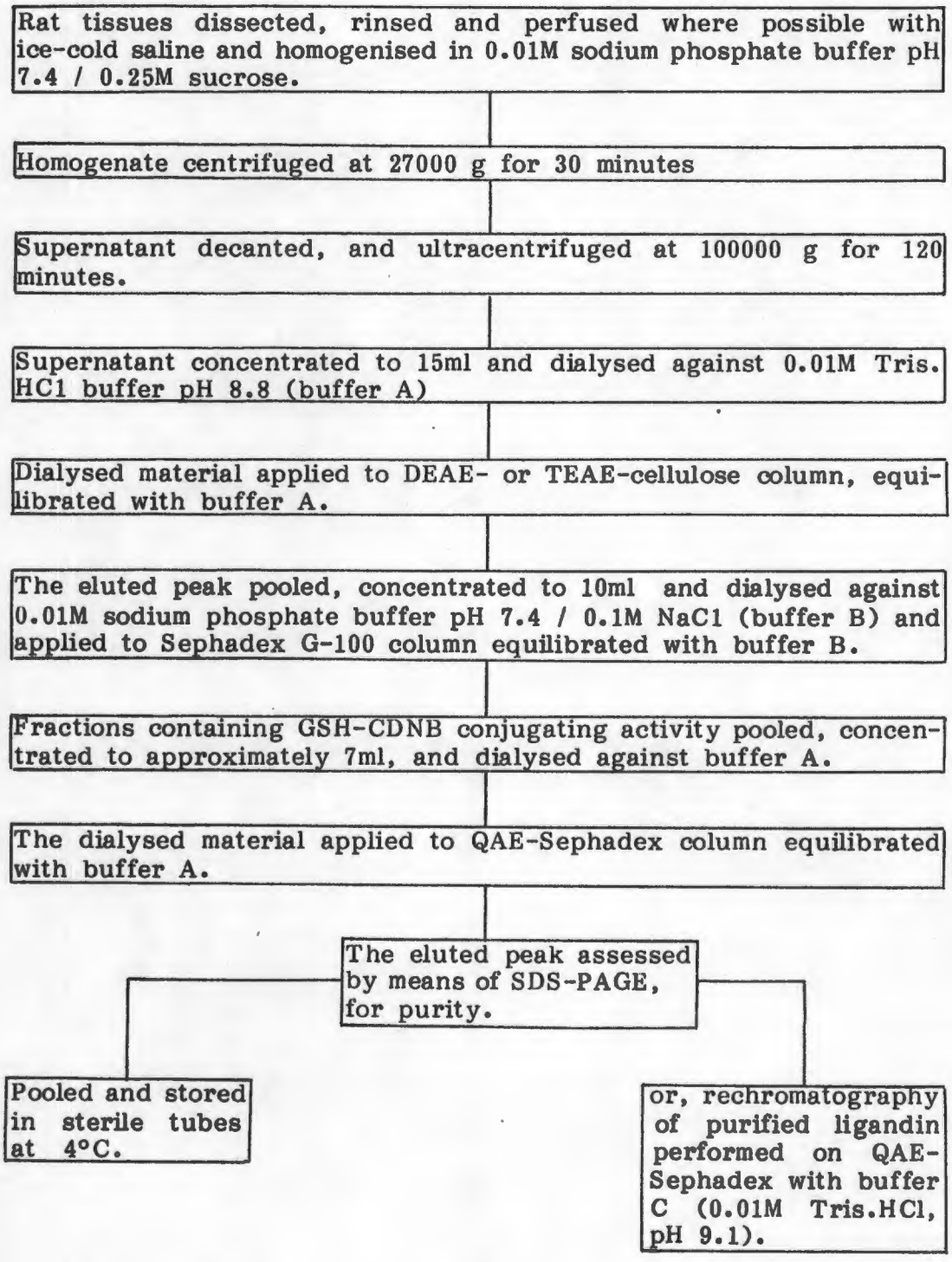


Fig. 1.6 - Standard Purification Procedure for Ligandin (Fleischner et al 1971 and Kirsch et al 1975)

This method has been used to prepare pure ligandin for many detailed structural and functional studies from rat liver (Bass **et al** 1977a, Bhargava **et al** (1978a,b, 1980a,b, 1980b) and from rat testis (Bhargava **et al** 1980a).

(b) GSH S-Transferases: Standard Purification Method

Rat GSH S-transferases have been purified to homogeneity by the use of entirely conventional procedures as is shown in Fig. 1.7 (Habig **et al** 1974a, 1976, Pabst **et al** 1974). This method makes use of the alkaline isoelectric point of these proteins and absorbs them to CM-cellulose. This is followed by their sequential elution with a salt gradient. The substrate CDNB is usually used to follow the course of purification. CM-cellulose chromatography is followed by repeated chromatography with hydroxyapatite. An independently derived but very similar purification technique for rat GSH S-transferases A and C has been reported which also takes advantage of this basic nature of the enzymes (Askelof **et al** 1975). Human liver GSH S-transferase purifications follow along similar lines to that described for rat except that gel filtration and preparative isoelectric focusing were used in the final steps (Kamisaka **et al** 1975). Purification of GSH S-transferases from guinea pig liver (Hayakawa **et al** 1977) and from monkey liver (Asoaka **et al** 1977) have been achieved with this standard technique.

Others have substituted the final hydroxyapatite steps of the procedure with isoelectric focusing, ie Awasthi **et al** (1980a) when isolating bovine lens GSH S-transferase, and Yeung and Gidari (1980) when purifying chicken liver GSH S-transferase. Preparative

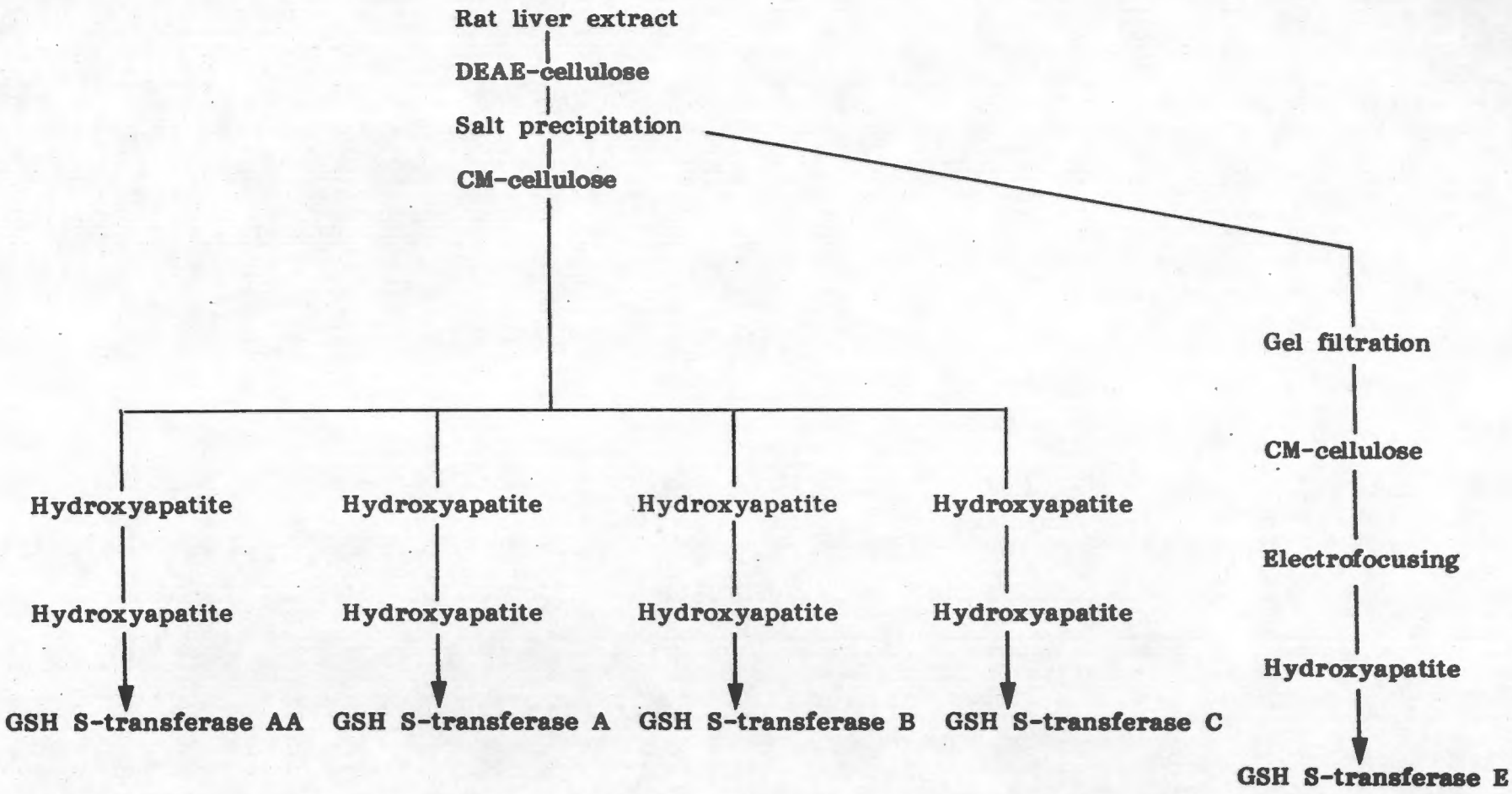


Fig. 1.7 Purification procedure for rat liver GSH S-transferases (Jakoby and Habig 1980)

isoelectric focusing has been used for obtaining preparations from rat liver and kidney (Hales **et al** 1978).

(c) GSH S-Transferases: Affinity Methods

More recently, another approach has been described which speeds the process of protein purification and holds much promise. This is affinity chromatography, where a number of compounds having affinity for the transferases can be coupled to a matrix and the GSH S-transferases selectively isolated from other contaminating proteins.

GSH S-transferases from rat liver have been successfully isolated by means of a thioether of BSP and GSH coupled to cyanogen-bromide activated agarose gel (Wolkoff **et al** 1979), this separates the proteins on the basis of their organic anion binding rather than their charge or size characteristics. The GSH S-transferases bound to BSP coupled matrix was eluted by increasing the buffer pH and ionic strength. This technique has been used to purify ligandin which binds BSP with high affinity. The final product on SDS-PAGE consists of two subunits (the smaller subunit present in relatively higher concentrations). GSH S-transferases from the larvae of the Greater Wax Moth has also been isolated by this technique (Clark **et al** 1977).

BSP coupled directly by cyanogen bromide to agarose was effective in isolating GSH S-transferases from porcine liver (Grahnen and Sjoholm 1977) while GSH coupled directly to epoxide activated agarose was used to isolate the GSH S-transferases from human liver (Simons and vander Jagt 1977). A combination of affinity

chromatography, anion exchange and molecular sieving chromatography and isolectric focusing has been used to purify the cationic forms of human liver GSH S-transferases (Awasthi **et al** 1980b), as well as the transferases from rat liver mitochondria (Kraus 1980) and from guinea pig liver (Irwin **et al** 1980).

The usual column matrix is either agarose or sepharose to which the affinity ligand is covalently bound via primary amino groups or similar nucleophilic groups in an irreversible manner by methods involving the use of cyanogen halides (Axen **et al** 1967).

Use of Spacer Molecules

The inter-position of a spacer group between ligands and the supporting matrix is important for the isolation of small ligands, for protein-ligand complexes of low affinity and for multi-subunit proteins. Successful separations using adsorbants which have groups varying in length from two to ten carbon-long aliphatic chains placed between the ligand and supporting matrix, have been described (O'Carra **et al** 1973). The purification of GSH S-transferases was facilitated by the use of an affinity support column with 1,6-diaminohexane as a spacer between the coupled GSH and the agarose matrix. This system was able to separate several of the rat liver GSH S-transferases (Lawrence **et al** 1978a). GSH S-transferase B appeared in the void volume, transferase AA was eluted after applying a 0-0.75M KCl gradient, while transferases A and C were eluted with an 0-20mM GSH gradient.

A major disadvantage of any affinity column is detaching the protein once it has been specifically interacted with the ligand on

the column. These difficulties encountered in elution of the adsorbed transferases from a matrix coupled to BSP-GSH were described by Guthenberg and Mannervik (1979). These workers have prepared pure GSH S-transferases from rat lung and liver using S-hexylglutathione as the ligand coupled to Sepharose 6B (Guthenberg and Mannervik 1979). This ligand provided a better compromise between good binding and elution properties. Following elution from the S-hexylglutathione Sepharose affinity column, the GSH S-transferases could be separated by means of hydroxyapatite chromatography. Although this technique is very useful for isolation of GSH S-transferases from sources in which they are less abundant than liver, not all the transferases have been recovered. Thus only GSH S-transferases A, B and C have been recovered while the other transferases, namely AA, D and E, are apparently unaccounted for. It is not known whether these forms are lost during affinity or hydroxyapatite chromatography.

A combination of affinity chromatography and ion exchange chromatography was used to prepare GSH S-transferases from human placenta (Guthenberg **et al** 1979) and liver (Warholm **et al** 1981), while S-carbamidomethyl glutathione linked to Sepharose CL-4B via lysyl spacers was used to isolate 6 forms of hepatic rat GSH S-transferases (Inoue **et al** 1981). These workers used several adsorbants but found those with a free carboxyl group within the spacer moiety to have a high specificity as well as a high capacity for GSH S-transferases. An affinity matrix coupled to cholic acid was used to isolate a bile acid binding protein with GSH S-transferase activity from rat liver cytosol (Pattinson 1981). This protein has been identified as GSH S-transferase C, and migrates as Yb on SDS-PAGE.

Binding of the transferases to the adsorbent can be used to study enzyme-ligand interactions. Therefore affinity chromatography may be employed to characterise ligand-binding sites (Lowe and Dean 1974) in addition to its use in enzyme purification.

SECTION B

ONTOGENESIS

LIGANDIN IN RELATION TO DEVELOPMENT OF THE RAT

The inability of neonatal mammals to handle drugs normally metabolised by adults of the same species is well documented. Thus neonates are sensitive to harmful effects of many drugs and xenobiotics, which are relatively safe in adults (Horning **et al** 1975). Deficiency of enzymes responsible for drug biotransformation is partly responsible for the increased toxicity of these substances seen in the neonate. The extent and type of biotransformation depends on the stage of development. While there has been much interest in the development of enzyme systems **in utero** and in the early neonatal period, most of the reported data concerns enzyme changes in the liver. In the adult organism, the liver is a major site of xenobiotic detoxication. However, many of the enzymes responsible for this process are widely distributed and are present in practically all other organs and tissues.

The purpose here is to review the knowledge of changes in ligandin and the GSH S-transferases during development in relation to some other xenobiotic metabolising enzyme systems. Factors which may affect these enzyme systems, with particular reference to the rat, will also be considered.

(a) Changes in Ligandin During Development

In the early 1960s various workers found hepatic uptake and conjugation of BSP and bilirubin to be significantly reduced in newborn rats and man (Combes and Stakelum 1962, Vest 1962). Low concentrations of hepatic carrier proteins for BSP and bilirubin were found in foetal and neonatal tissue and rapid increases with maturity were observed (Grodsky **et al** 1970).

Developmental studies of the bilirubin and BSP-binding proteins, Y and Z, in guinea pig liver showed that Y developed much more slowly than Z. Y concentration at birth was between 5% and 20% of adult concentrations and matured at about 15 days postpartum, while Z reached adult concentrations immediately prior to birth (Levi **et al** 1969b). Deficiency of ligandin in neonatal primates was claimed to be causally related to associated "physiologic" jaundice (Levi **et al** 1970, Grodsky **et al** 1970) of the newborn.

Klaassen (1973, 1975) demonstrated that liver of five-day-old rats possessed only 10% of adult values of Y protein. This amount increased slowly during the first 15 days of life, after which there was a rapid increase between 15 and 35 days, and a second, slower rise to adult levels at about nine-weeks.

Although the development of Y protein and hepatic organic anion uptake appear to correlate, Klaassen (1975) disputed a major role for ligandin in hepatic handling of organic anions. In the kidney Pegg and Hook (1977) studied GSH S-transferase activity and renal organic anion transport capacity in developing rats and rabbits, and suggested that these enzymes were not rate-limiting in renal organic anion transport.

Recently, an ontogenic study of rat liver ligandin (Karavanova **et al** 1980) using immunohistochemical measurements of ligandin (immunofluorescent technique), reported that between 16-17 days of gestation foetal liver contained ligandin and noted an increase during the two days prior to birth. This consisted of an increase in both the number of fluorescent cells as well as the intensity of fluorescence in each cell. At birth practically all hepatocytes fluoresced intensely, but on the 4th-6th day of extra-uterine life ligandin fluorescence diminished prior to increasing again to reach adult levels at 15-18 days postpartum. These results contrasted with all previous studies which showed that the liver of foetal and newborn rats contained less of ligandin than at any time post partum (Hales and Neims 1976a, Foliot **et al** 1973). Karavanova **et al** (1980) observed an even distribution of ligandin throughout the liver lobule in the period immediately before and after birth, contrasting with the normal adult pattern where ligandin is predominantly centrilobular.

(b) Changes in the GSH S-Transferases During Development

(i) In the Liver

Rat liver GSH S-transferase activity towards epoxide substrates was shown to be less than 10% of adult values at birth, and to increase gradually reaching maturity at 40 days of age (Figs. 1.8 and 1.9) (James and Pheasant 1978, Mukhtar and Bresnick 1976b). However, excretion of the corresponding mercapturic acid was not influenced by this maturation of enzyme activity. In fact Reed and Beatty (1980) have suggested that the availability of GSH in foetal and newborn animals may be rate-limiting in determining activity of GSH-dependent enzymes.

The different patterns observed in maturation of GSH S-transferase activity towards styrene oxide and towards CDNB suggest that the development of various hepatic GSH S-transferases is probably not homogenous (Baars **et al** 1980). The results of Hales and Neims (1976a) are in accordance with this suggestion. It is now known that there is preferential development of GSH S-transferase B during the first postnatal week, resulting in higher levels of GSH S-transferase B relative to the other GSH S-transferases. Weaning may result in further variation in maturation of GSH S-transferase species (Baars **et al** 1980).

(ii) In the Steroidogenic Tissues

Studies of xenobiotic-metabolising systems have been directed at liver and to a lesser degree kidney, intestine and lung. Little attention has focused on the ability of steroidogenic tissues to metabolise reactive chemicals in spite of the importance of potential interactions between reactive electrophiles and germinal cells.

However it is known that testis, ovary and adrenal glands have the ability to metabolise toxic chemicals, such as alkene or arene oxides (Mukhtar **et al** 1978a,b). The developmental patterns of GSH S-transferases in endocrine tissues has been described by these workers. The specific activity of GSH S-transferase in testes of 6-day-old rats was three times greater than the corresponding specific activity in the liver of the same rats (Mukhtar **et al** 1978a). Testicular GSH S-transferase activity (measured with styrene oxide as substrate) increased slowly with age (Fig. 1.8). The specific activity of adult rat testis GSH S-transferase was

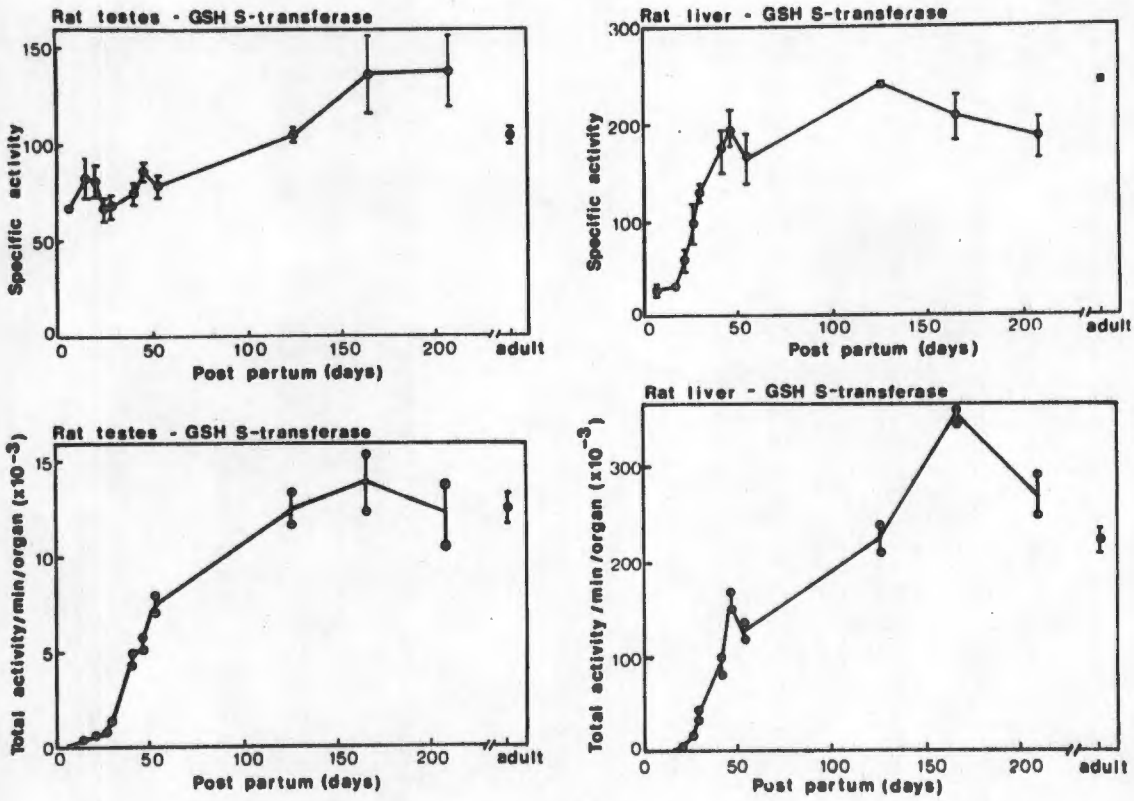


Fig. 1.8 Postnatal development of 100000 g supernatant fractions of GSH S-transferase activities in rat testis and liver with styrene oxide as substrate. Left top panel represents testicular specific activities (nmolproduct/min/mg protein, mean ± SD.). Left bottom panel represents total testicular enzyme activities (nmol product/min/organ, mean ± range). Right top and bottom panels represent corresponding hepatic specific activity and total organ activity respectively. From: Mukhtar et al (1978a).

high (about 50-60% of corresponding hepatic values) when compared to the specific activities of testicular microsomal epoxide metabolising enzymes (epoxide hydrase, cytochrome P.450, aryl hydrocarbon hydroxylase). In fact, the GSH S-transferase specific activity was 100 times greater than the specific activity of the other enzymes mentioned. A similar study of epoxide metabolising enzymes in rat ovary (Mukhtar **et al** 1978b) revealed significant GSH S-transferase activity at the earliest time point studied (12 days postpartum) (Fig. 1.9). Ovarian GSH S-tansferase specific activity (substrates SO and 4,5-BPO) increased to reach adult levels at about 35 days of age.

Relatively high specific GSH S-transferase activity was present in the adrenal glands of 1-day-old rats (95% of adult values), this specific activity remained relatively constant for the first 64 days of extrauterine life (Fig. 1.9).

(iii) In Other Tissues

The developmental pattern of GSH S-transferase activity in serum is identical to that found in rat liver. Rat serum transferase activity using either styrene oxide or benzo(**a**)pyrene 4.5-oxide as substrates is higher than that of rabbit and guinea pig (Mukhtar and Bend 1977).

GSH S-transferase activity towards styrene oxide or 3-methyl-cholanthrene-11, 12-oxide in rat foetal lungs is reduced. Lung enzyme activity reaches maximum levels 20-30 days after birth Fig. 1.9) (Mukhtar and Bresnick 1976a).

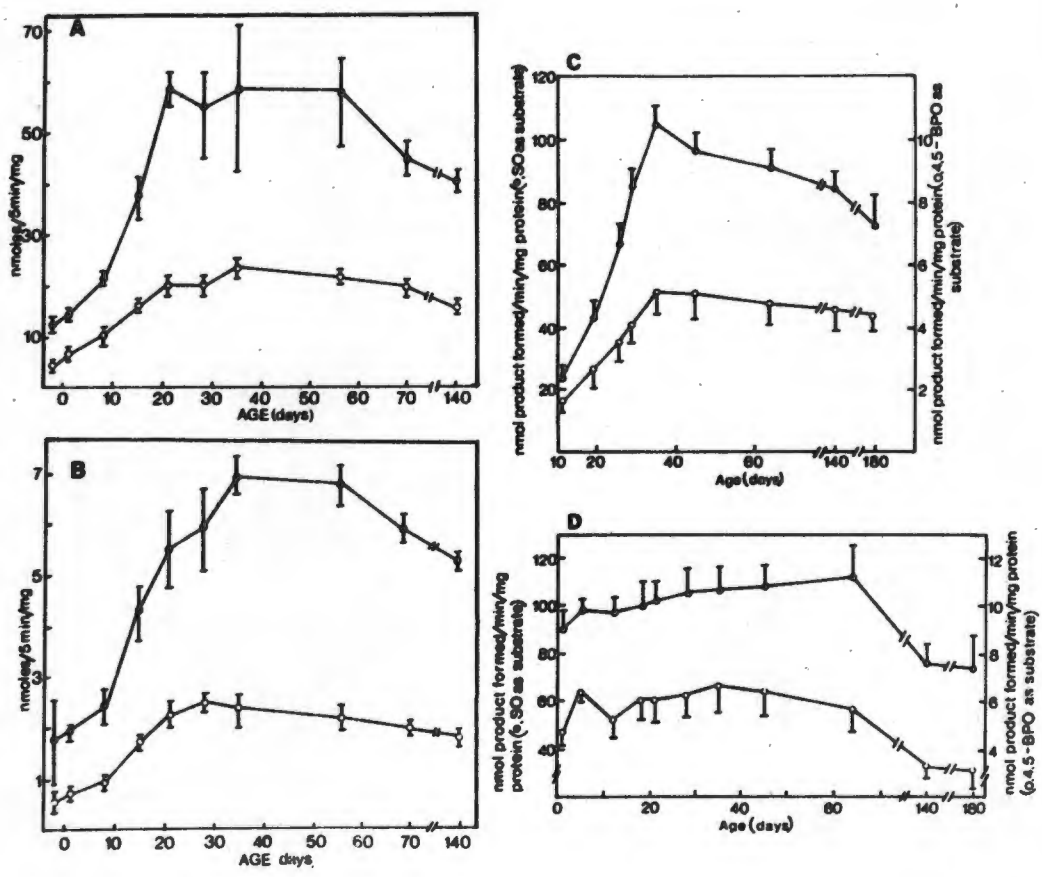


Fig. 1.9 Postnatal development of rat GSH S-transferase supernatant fractions of (A) liver (●) and lung (o) with styrene oxide and (B) with 3-methyl cholanthrene-11, 12-oxide as substrates, from: Mukhtar and Bresnick (1976). (C) represents the postanatal development of rat ovary GSH S-transferase activity measured with styrene 9.8-oxide (●) and benzo(a)pyrene 4,5-oxide (o) as substrates. (D) represents GSH S-transferase activity measured in adrenals with the same substrates as above, from: Mukhtar et al (1978b).

(iv) In the Foetus

Pelkonen (1980) found oxidative enzyme systems to be deficient in guinea pig and rat foetal liver with activity appearing soon after birth. Adult levels were attained within a few days in the guinea pig and within a few weeks in the rat. These observations are in contrast with data from GSH S-transferase enzyme systems which suggests that these enzymes in human foetal and adult liver have similar activity (Grover and Sims 1964, Boyland and Chasseaud 1969), although the concentration of immunoreactive hepatic ligandin in the human foetus near term was lower than that in adult liver (Fleishner **et al** 1976). It may be possible to explain these apparently conflicting data by invoking differences in foetal and adult enzyme phenotypes thus allowing a lower concentration of an enzyme with a high specific activity to have the same total activity for a particular substrate than a greater amount of enzyme with a lower specific activity. An alternative explanation is that GSH and not the GSH S-transferase may be rate-limiting.

The presence of inter-species and inter-organ differences in the maturation of GSH S-transferases was suggested by the studies of Juchau and Namkung (1974) who observed that GSH S-transferase activity with napthalene-1,2-oxide as substrate was higher in soluble fractions from primate foetal tissue than in the soluble adult rat liver fractions. This activity was notably higher in foetal adrenal glands of humans (72 days gestation) and pigtail monkeys (60 days gestation) than in simultaneously studied liver.

Foetal adrenal gland is known to be more efficient than its adult counterpart in metabolising of xenobiotics and possesses

considerable sulphate (Namkung **et al** 1977) and GSH conjugating activity (Jachau and Namkung 1974). Similarly, considerable xenobiotic metabolising activity has been detected in foetal gonads and sex organs. Mukhtar **et al** (1981) recently reported that GSH S-transferase activity (with CDNB as substrate) of foetal liver, kidney, lung, muscle, heart, adrenal gland, pancreas and stomach is higher than the corresponding activity of each organ in the adult.

FACTORS AFFECTING THE DEVELOPMENT OF THE GSH S-TRANSFERASES AND OTHER XENOBIOTIC METABOLISING SYSTEMS

Several factors may influence the maturation of xenobiotic-metabolising enzyme systems. These include maternal influences, premature or normal birth, weaning, puberty, pregnancy and senescence. The actual mechanisms responsible for the development of these enzyme systems are not known.

(a) Birth.

At birth there is a pronounced increase in the activity of many hepatic enzymes (eg enzymes of the monooxygenase system of mammalian liver (Neims **et al** 1976). Prior to birth maternal enzymes metabolise and detoxify foreign compounds for both mother and foetus. Although demonstration of metabolites of diazepam (Erkkola **et al** 1974) and chlorpromazine (Pelkonen **et al** 1973) in the foetus suggests detoxication enzyme activity **in vivo**, the possibility of placental transfer of these metabolites has not been excluded. The increase in enzyme activity, usually initiated at birth, is not affected by premature delivery nor prolongation of the gestation period (Dauterman 1980). While it is thought that separation of maternal-foetal connections trigger and activate maturation responses in the cell, the mechanism by which these changes occur is not known.

(b) Weaning

Feuer and Liscio (1969) found that early weaning in rats increased enzymes such as 4-methylcoumarin hydroxylase. It has been proposed by Henderson (1978) that this increase was due to withdrawal of enzyme inhibitors present in milk. This effect has not been found for GSH S-transferase activity which, if anything, is suppressed by early weaning (Baars **et al** 1980).

(c) Puberty

Conney (1967) found that a number of xenobiotic metabolising systems which were barely detectable before puberty, increased dramatically with the onset of sexual maturity. Several testicular enzymes follow a similar ontogenetic pattern including epoxide hydrase (Mukhtar **et al** 1978a), gamma-glutamyltranspeptidase (Hodgen and Sherins 1973), S-adenosyl-L-methionine decarboxylase (Macindoe and Turkington 1973), uridine diphosphatase (Xuma and Turkington 1972), sorbitol dehydrogenase and hyaluronidase (Lee and Shen 1975) which have all been reported to increase at puberty. Some enzymes, eg lactic dehydrogenase are present in high concentrations in neonatal testes, decline to a nadir during the second or third week of life and increase dramatically at puberty to reach adult levels (Hodgen and Sherins 1973).

Greengard (1969, 1971) first provided information suggesting that endocrine events could trigger the changes in formation of enzymes. Initiation of pubertal onset is dependent on a complex process of neuroendocrinological events which involve maturation of the hypothalamic-pituitary-gonadal axis. The onset of puberty has been presumed to be associated with increased follicle stimulating

hormone (FSH) and luteinising hormone (LH) release from the pituitary (Donovan and van der Werff ten Bosch 1965). These gonadotropins are present at low levels during the prepubertal period of female rats, after which there is an increase and change in relative production before the first ovulation. This surge of gonadotropin is accompanied by an increase of plasma prolactin levels (Ojeda **et al** 1976, Ojeda **et al** 1980). Puberty in the female rat may be defined as vaginal opening and the first ovulation. These events occur from 36-39 days of age. Vaginal opening and the initial ovulation are preceded by increases in blood concentrations of estradiol, progesterone and testosterone (Parker and Mahesh 1976). The biosynthetic precursors of these hormones also increase at the time of puberty. Puberty in the male rat is more difficult to define. When relative weight of body, testes, seminal vesicles and prostates as well as amount of serum testosterone have reached a certain level, sexual maturation usually occurs (Glass and Swerdloff 1980). FSH induction of gonadal sensitivity to LH may be a cause of sexual maturation in the male rat (Odell **et al** 1973).

Changes in Circulating Hormonal Levels

In a study of serum LH, FSH and prolactin levels of male and female rats from birth to puberty, Dohler and Wuttke (1974) found that neonates of both sexes had high FSH and LH levels. These levels fell after the first week of life, then sporadically rose and fell until about day 37 when a preovulatory surge was noted in females. Following this the rats displayed normal cyclic activity.

For the first three weeks of life prolactin levels are low in both male and female rats, after which they increase gradually in both sexes and attain peak values between days 37 and 45. Cyclic fluctuations were observed in the females after day 37 had been reached.

Meijs-Roelofs **et al** (1975) and Parker and Mahesh (1976) showed that serum estradiol levels increase before the preovulatory surge of gonadotropins and prolactin. It has been suggested that enhanced estradiol secretion of the ovary may be responsible for triggering secretion of prolactin and gonadotropins. Serum progesterone levels are low in both newborn males and females, remain low for the first 10 days of life, increase slowly for the next 10 days after which they rise rapidly during the 3rd and 4th week of life to reach adult levels.

Prolactin has also been implicated in the maturational process which leads to puberty and is said to have a dual effect, part of which is exerted by increasing the LH receptor content of granulosa cells, and part mediated through the hypothalamus where it results in decreased prolactin and increased pituitary FSH secretion (Clemens **et al** 1969). Prolactin administration has been shown to advance the onset of puberty by about six days when given at 20 days of age, and by two days when given at 25 days of age. Administration of other anterior pituitary hormones (FSH, LH, TSH and GH) had no effect. The effect of prolactin administration was abolished in hypophysectomised rats (Wuttke **et al** 1976, Advis and Ojeda 1978). Precocious puberty may also be produced by electrical stimulation of the hypothalamus. This increases plasma FSH levels (Meijs-Roelofs 1972). Adrenalectomy has been found to

delay vaginal opening and ovulation by 4-5 days (Ramaley and Campbell 1977).

(d) Pregnancy

The activity of a large number of enzymes is decreased during pregnancy. For example glucuronide conjugation of xenobiotics is reduced during late pregnancy as a result of increases of progesterone and pregnanediol, which are known inhibitors of glucuronyl transferase **in vitro.** Similar inhibition of glucuronide conjugation has led to high levels of unconjugated bilirubin in the blood of breast-fed infants. This jaundice has been claimed to be associated with the presence of pregnane-3-alpha-20beta-diol in the mother's milk (Dauterman 1980).

The activities of liver microsomal metabolising enzymes are also reduced during pregnancy. Rat hepatic cytochrome P.450 is decreased by 25% and is uninducible by pre-treatment of the pregnant rats with phenobarbital (Dauterman 1980). Although activity of glucuronyl transferase is reduced in pregnant rabbits, cytochrome P-450 concentration remains unchanged.

In contrast to these findings, GSH S-transferase specific activity towards epoxide substrates has been reported to occur in the ovaries of pregnant rats at mid-pregnancy (Mukhtar **et al** 1978d). The different rat GSH S-transferases appear to be induced at various stages of the gestation period (Polidoro **et al** 1981b). At mid-pregnancy (10-11 days) only hepatic GSH S-transferase activity towards 1,2-epoxy-3-(p-nitrophenoxy)propane

was increased (82% of control values). However, in late pregnancy (19-20 days) hepatic GSH S-transferase activity with CDNB is significantly increased (57%) while those for DCNB are decreased.

(e) Senescence

Senescent animals are less responsive to changes in their environment than younger animals. A slower rate of enzyme induction has been observed in old versus young animals (Adelman **et al** 1972). Significant decreases in hepatic drug-metabolising capacity occur with senescence (Schmucker and Wang 1980). A significant decrease in BSP uptake by isolated hepatocytes with advanced age has been observed (Knook 1980). This has been attributed to an age-related decrease in the BSP-storage capacity of the liver.

Although studies in ageing tissues have generally indicated a decreased ability to metabolise drugs, hepatic cytosol from senescent rodents do not appear to have altered GSH S-transferase activity when metabolising epoxides (Birnbaum and Baird 1979). The testis, ovaries and adrenals show an apparent reduction in GSH S-transferase activities (measured with SO, 4,5-BPO) after the rats had reached about 140 days of age (Mukhtar **et al** 1978a,b). In addition these workers were able to show a decline in hepatic GSH S-transferase specific activities measured with these substrates.

Plasma testosterone levels of mammals decrease markedly during senescence (Ghanadien **et al** 1975), while ageing Leydig cells appear to have impaired steroidogenic capacity (Lin **et al** 1980).

INDUCTION OF LIGANDIN AND GSH S-TRANSFERASES IN DEVELOPING ANIMALS

Rat liver ligandin content can be increased by up to 100% after administration of microsomal enzyme inducers such as phenobarbitone (Fleischner **et al** 1976). Similar changes in GSH S-transferase activity can be induced in developing rats by phenobarbitone treatment (Hales and Neims 1976a). The increase in GSH S-transferase activity following induction in neonatal rats results in adult levels which then persist. Male and female rats exposed perinatally to polychlorinated biphenyls had higher levels of GSH S-transferase activity (mainly towards CDNB) than controls at all ages examined (Lamartiniere **et al** 1979). Administration of the glucocorticoids, dexamethasone acetate or corticosterone acetate to neonatal rats stimulated the development of adult levels of hepatic GSH S-transferase activity (assayed with SO, CDNB, or 4.5-BPO as substrates). Glucagon administration did not affect GSH S-transferase activity (Mukhtar **et al** 1979).

SEX DIFFERENCES OF LIGANDIN AND THE GSH S-TRANSFERASES

Sex differences in mammalian liver xenobiotic metabolism appear with the onset of puberty and are maintained throughout life (Dauterman 1980). Sex differences in enzyme activity are also dependent upon which substrates are used and which species are investigated. Although activity of hepatic GSH S-transferase levels continue to rise in animals of both sexes after weaning, DCNB conjugation to GSH is two to three-fold higher in adult males than in adult females (Darby and Grundy 1972). Activity towards CDNB in rat liver did not exhibit sexual differentiation (Hales and Neims 1976a,b).

Ligandin levels in male rat liver supernatants were significantly lower (3.3% of male liver supernatant protein) than those from female rat liver (4.5% of female liver supernatant protein). Hypophysectomy of the females abolished this sex difference (Hales and Neims 1976b). Male mammals (rat and mouse) exhibited relatively lower GSH S-transferase activity (measured with CDNB) in the brain than did female mammals, while several species of male birds exhibited comparatively higher activity of brain GSH S-transferases than did the correspoinding females (Das **et al** 1981). No significant sex differences in GSH S-transferase activity towards CDNB specimens was detected in human liver (Darby 1973).

Recent work by Lamartiniere (1981) suggests that the sexual differentiation of hepatic GSH S-transferases is due to a hypothalamic inhibiting factor present in the male, but not in the

female. This factor acts on the pituitary, postpubertally, preventing the secretion of a pituitary inhibiting factor, resulting in higher GSH S-transferase activities in adult males than in females. Adult females autonomously secrete this pituitary inhibiting factor. Thus, hepatic GSH S-transferases may be under hypothalamic-hypophyseal-gonadal regulation in the rat.

SECTION C

GLUTATHIONE PEROXIDASE

Another quite unexpected role for ligandin and certain GSH S-transferases was discovered in the late 1970s. This activity, ie glutathione peroxidase, is present in many tissues of the rat in varying amounts. The role of the GSH S-transferases/GSH peroxidases as multi-funtional proteins involved in hydroperoxide metabolism as well as their relationship with selenium-dependent GSH peroxidases is considered in this chapter.

HISTORICAL ASPECTS

Glutathione peroxidase (GSH peroxidase) (Hydrogen peroxide:GSH oxidoreductase EC 1.11.1.9), was first detected in erythrocyte lysates where it protects haemoglobin from oxidative breakdown (Mills 1957). In 1959 (Mills 1959) GSH peroxidase was isolated from bovine erythrocytes and shown to be distinct from both catalase and haemoglobin. Cohen and Hochstein (1963) initially showed that GSH peroxidase, and not catalase, protects haemoglobin in intact erythrocytes from being oxidised to methaemoglobin by hydrogen peroxide. As a result of these experiments with red cells the detoxification of H_2O_2 has generally been ascribed to GSH peroxidase. More recent work with the isolated perfused rat liver (Oshino and Chance 1977) indicates that within the peroxisome catalase does function as the primary H_2O_2 scavenger, while GSH peroxidase plays an important role in other subcellular fractions

such as mitochondria and cytosol. In addition GSH peroxidase has been shown to have added importance in protecting isolated hepatocytes from H_2O_2 produced as a result of microsomal oxidative demethylation in which the formate produced can inhibit catalase and significantly reduce its contribution to H_2O_2 removal (Jones **et al** 1978).

GSH peroxidase activity is sustained by that of another glutathione dependent enzyme, namely glutathione reductase. This enzyme maintains the continuous reduction of oxidised glutathione (GSSG) in a $NADPH_2$-dependent reaction. In erythrocytes the major source of $NADPH_2$ is from hexose shunt activity, while in other tissues an alternative source could be from isocitric or malic dehydrogenase activity. Erythrocytes are unable to maintain this activity due to an incomplete Krebs cycle.

SELENIUM DEPENDENT GLUTATHIONE PEROXIDASE / GLUTATHIONE PEROXIDASE I

Several groups have studied GSH peroxidase I and purified it to homogeneity. This protein is unusual in that it contains a selenium moeity which has been shown to participate in the catalytic cycle of the enzyme. The protein is made up of four identical spherical (r=19Å) monomers each containing a selenium atom (Ladenstein **et al** 1979). Selenoproteins are apparently very rare and interesting studies have been carried out in order to ascertain how the selenium is incorporated into GSH peroxidase I (Sunde and Hoekstra 1980a). The Se moiety in the reduced form of GSH peroxidase I is thought to be a selenocysteine. The selenocysteine moiety was identified as the catalytic site in GSH peroxidase I (Forstrom **et al** 1978) which was shown to fit into the electron density of residue 35 of the 180 amino acid residue subunit. The molecular weight of each subunit purified from rat liver was determined to be approximately 19000 (Nakamura **et al** 1974, Tappel 1978).

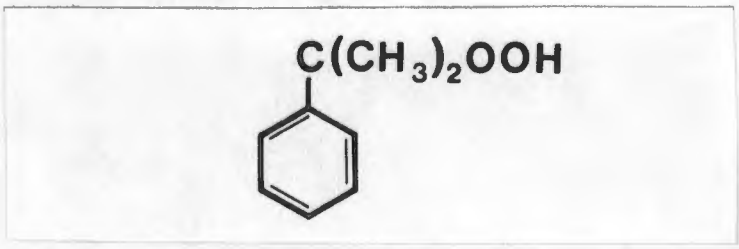
Rat liver GSH peroxidase I was found to be very stable under denaturing conditions. In the presence of other proteins it exists in an aggregated form becoming dissociated upon isolation and storage, thus suggesting a strongly hydrophobic nature which would support the enzyme's ability to interact with hydroperoxides at the hydrophobic membrane surface (Tappel 1978, Stults **et al** 1977). There also appears to be a charge phenomenon in that the protein initially isolated is neutrally charged but becomes negatively charged on storage. A disulfide interchange could be responsible for this. Of all sulfhydryl compounds tested only GSH was able to

serve as a substrate for this enzyme (Flohe **et al** 1971). Cyanide inhibits GSH peroxidase I resulting in severe loss of enzyme activity and release of selenium from the enzyme (Prohaska **et al** 1977a). Cadmium and other heavy metals were also shown to have an inhibitive effect on GSH peroxidase I (Splittgerber and Tappel 1979). At physiological concentrations of GSH and peroxides, GSH peroxidase I is in a reduced form (Flohe **et al** 1973) and cyanide has no effect. After purification the enzyme presumably exists in a more oxidised form and cyanide is able to inhibit catalytic activity. An explanation for this observation could be that the active centre of the enzyme alternates between oxidised and reduced forms, this steady-state mechanism having been well established as a UNI UNI BI UNI PING-PONG mechanism.

Immunohistologic studies suggest that GSH peroxidase I is located in cytoplasm of hepatocytes. More intense staining was seen in the periportal areas than the central zone of the lobule (Yoshimura **et al** 1980).

DISCOVERY OF THE NON-SELENIUM DEPENDENT GLUTATHIONE PEROXIDASE / GLUTATHIONE PEROXIDASE II

In 1970 Little **et al** carried out kinetic analysis of partially purified GSH peroxidase preparations from pig's blood. Using the organic hydroperoxide, cumene hydroperoxide (see structural formula below) as substrate, nonlinear Lineweaver-Burk plots were obtained, and as high levels of substrate were able to enhance enzyme activity the possibility that there were two enzymes with different Vmax and Km values was considered.



It was not until 1976 that the first evidence for the existence of a second GSH peroxidase species was obtained by Lawrence and Burk using cumene hydroperoxide as substrate. These workers observed that GSH peroxidase activities were 42% of control values in liver supernatant of rats fed a Se-deficient diet for two weeks, while activity measured with H_2O_2 was only 8% of control values. Fractionation of liver cytosol on Sephadex G-150 columns revealed two peaks of activity. The first peak (absent in Se-deficient rat liver supernatants) could be detected with H_2O_2, while the second peak, eluting at a molecular weight corresponding to 39000 daltons, could only be detected with cumene hydroperoxide. This peak persists in severe selenium deficiency. Heating experiments demonstrated that peak 2 was heat labile while peak 1 was more heat resistant. Dissociation experiments subsequently proved that the second peak was not due to subunits of peak 1 dimers.

Comparison of the two distinct GSH peroxidase species (Prohaska and Ganther 1977b) showed that the second species was not active with H_2O_2 and was not a selenoenzyme (there was no incorporation of ^{75}Se **in vivo**). This non-selenoenzyme showed zero order dependence on GSH concentration and was not inhibited by cyanide, while the selenoenzyme showed 1st order dependence on GSH and was inhibited by cyanide. The non Se-dependent GSH peroxidase (GSH peroxidase II) activity of testis cytosol accounted for more than half of the total GSH peroxidase activity when chromatographed on Sepadex G-75 columns (Fig 1.10).

The question arose as to what the nature of this second species of GSH peroxidase activity was. Prohaska and Ganther (1977b) suggested that the GSH S-transferases, known for their affinity for hydrophobic substrates and their ability to catalyse GSH to electrophilic centres, might be responsible. They believed the GSH S-transferases could bind organic hydroperoxides and GSH in close proximity, thus promoting a nucleophilic attack on electrophilic oxygen. Partial purification of rat liver GSH peroxidase II on CM-cellulose yielded 5 peaks of GSH S-transferase activity when assayed with CDNB. When these peaks were assayed for peroxidase activity with cumene hydroperoxide as substrate, GSH S-transferase AA was found to have the highest specific activity when compared to the other transferases. Transferase B also exhibited considerable activity while activity of transferases A and C was much lower (Prohaska and Ganther 1977b, Irwin **et al** 1980). Transferases D and E which do not bind to the column had measurable GSH peroxidase II activity but was contaminated by large amounts of GSH peroxidase I, recognised by its activity with H_2O_2.

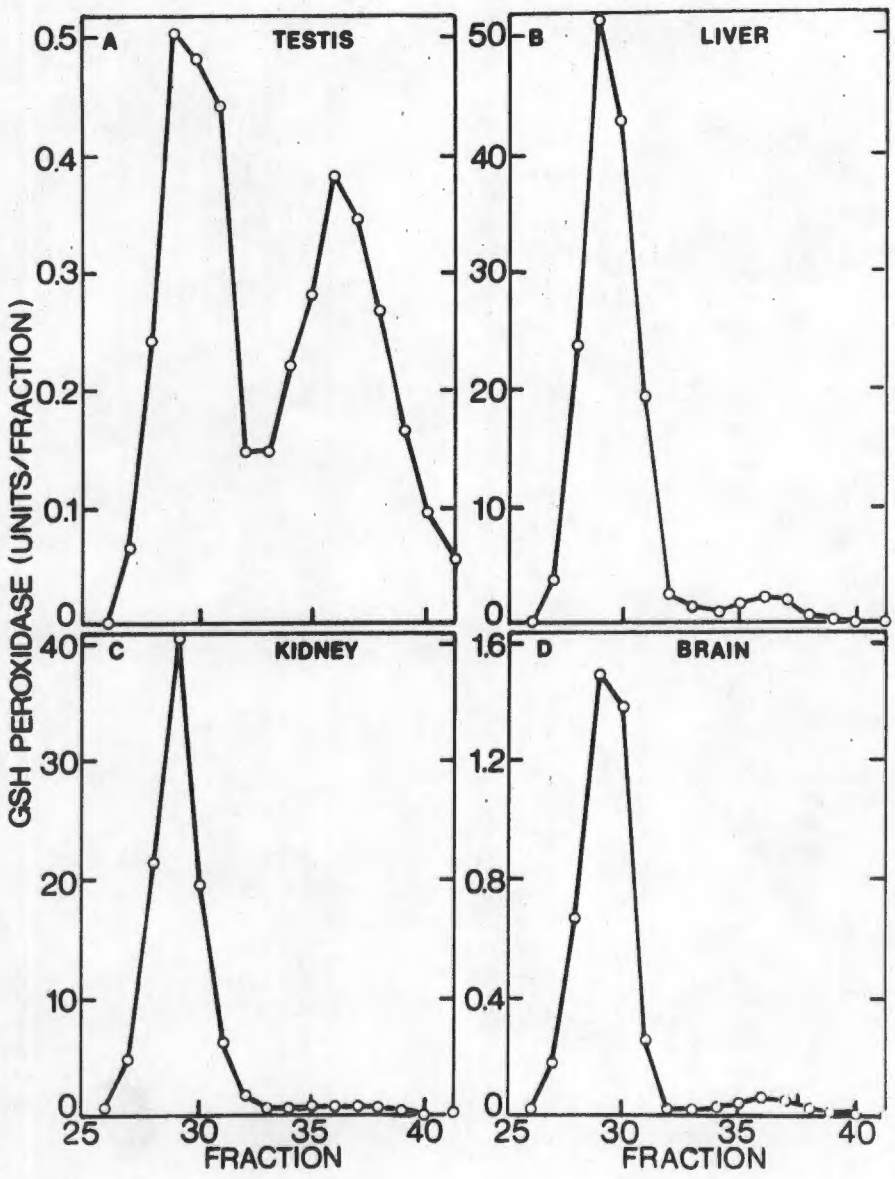


Fig. 1.10 Sephadex G-75 chromatography and GSH peroxidase activity of the 100000 g supernatant fractions from rat (A) testis, (B) liver, (C) kidney and (D) brain. Column fractions were assayed for GSH peroxidase activity with 0.1mM cumene hydroperoxide. From: Prohaska and Ganther (1977b).

Guinea pig liver transferases separated by CM-cellulose and assayed for peroxidase activity showed a different elution profile to that of rat liver transferases (Irwin **et al** 1980). The final peak of guinea pig liver transferase activity eluting from CM-cellulose exhibited the majority of peroxidase activity. In fact this represented the primary functional GSH peroxidase since there is very little of GSH peroxidase I in guinea pig liver (Lawrence and Burk 1978). Similarities between this CM-cellulose peak and rat liver GSH S-transferase AA have been noted.

All five human liver cationic GSH S-transferases (alpha, beta, gamma, delta and epsilon) display GSH peroxidase II activity. Anionic species (psi and omega) do not exhibit peroxidase activity (Awasthi **et al** 1980). The GSH peroxidase II activity of the cationic GSH S-transferases from human liver is limited to cumene hydroperoxide. H_2O_2 and **tert**-butylhydroperoxide ($[CH_3]_3$ COOH) are unsuitable substrates for these enzymes.

Lawrence **et al** (1978b) purified rat liver GSH S-transferase B and demonstrated GSH peroxidase II activity towards cumene hydroperoxide and **tert**-butylhydroperoxide. This peroxidase activity was shown to be competitively inhibited by BSP. Induction in rats with phenobarbital increased GSH peroxidase II activity but had no effect on GSH peroxidase I.

OCCURRENCE AND DISTRIBUTION

(a) Species Distribution

There is a great deal of inter-species variation with regard to the relative proportions of GSH peroxidases I and II. GSH peroxidase II is the predominant form in liver soluble fractions of guinea pigs, humans and sheep while GSH peroxidase I predominates in hamsters and rats (Lawrence and Burk 1978) (see Table 1.8A):

TABLE 1.8A - From Lawrence and Burk 1978

SPECIES	% GSH Peroxidase II activity / Total GSH peroxidase activity
Rat	35
Hamster	43
Sheep	81
Pig	67
Chicken	70
Human	84
Guinea Pig	100

(b) Tissue Distribution

GSH peroxidase enzyme activity has been found in most tissues of mammals including liver, kidney, stomach, spleen, erythrocyte, heart, lung, lens, brain, adrenal and testis (Flohe and Gunzler 1976). Variable amounts of GSH peroxidase II are present in

tissues of higher animals (Lawrence and Burk 1978). Thus spleen, heart, lung, thymus and intestinal mucosa had very little GSH peroxidase II, adrenals, liver and kidney had more than one-third of total activity attributed to GSH peroxidase II, while brain and fat tissue had less than one-third. In testis GSH peroxidase II accounted for 91% of total activity (see Table 1.8B). No data is available demonstrating the organ distribution of GSH peroxidase in man.

TABLE 1.8B - From Lawrence and Burk 1978

TISSUE	% GSH Peroxidase II activity / Total GSH peroxidase activity
Liver	35
Kidney	31
Fat	23
Brain	26
Intestinal mucosa, spleen, thymus lung and heart	0
Adrenal	38
Testis	91

(c) Subcellular Distribution

Subcellular distribution of GSH peroxidase differs from that of catalase. GSH peroxidase is absent in peroxisomes but present in the supernatant fraction and in matrix space of mitochondria (Flohe

and Schlegel 1971). Mitochondrial extracts from rats fed a selenium-deficient diet have virtually no measurable GSH peroxidase activity (Sies and Moss 1978) indicating that the non Se-dependent species is not important in these organelles. Cardiac mitochondria lack selenium-dependent GSH peroxidase activity which is present in the mitochondrial matrix of liver (Katki and Myers 1980). This membrane bound activity of cardiac mitochondria differs from GSH S-transferases which exhibit peroxidase activity in that it is capable of utilising both cumene hydroperoxide and H_2O_2 as substrates.

A non-selenium dependent enzyme species exhibiting GSH S-transferase activity has been demonstrated in rat liver microsomal fractions (Reddy **et al** 1981).

MECHANISM OF THE GLUTATHIONE PEROXIDASE ACTIVITY OF THE GLUTATHIONE S-TRANSFERASES

The kinetic properties of purified preparations of rat liver GSH S-transferases AA and B were compared using both cumene hydroperoxide and tertbutylhydroperoxide substrates (Prohaska 1980). The peroxidase activity of transferase AA with either substrate was shown to be similar to that of B. Transferase AA has high maximum velocity with both hydroperoxides when compared to that of other substrates.

Keen et al (1976) suggested that the mechanism of GSH transferases involves nucleophilic attack by GS^- on some electrophilic atom (C, S or N) in the substrate. A similar mechanism could be suggested for the transferases when they act upon organic hydroperoxides (ROOH) in which GS^- attacks an electrophilic oxygen, resulting in the formation of the unstable sulfenic acid of glutathione (Prohaska 1980).

$$ROOH + GSH \rightleftarrows [GSOH] + ROH$$

$$[GSOH] + GSH \rightleftarrows GSSG + H_2O$$

This intermediate [GSOH] could be formed during the oxidation of GSH by cumene hydroperoxide, catalysed by the transferases (AA or B).

ROLE OF THE GLUTATHIONE PEROXIDASE ENZYME SYSTEM IN:

(a) Oxygen Toxicity

Molecular oxygen is toxic to virtually all forms of life. Exposure to concentrations greater than the ambient fifth of an atmosphere causes toxicity, thus the margin of safety is narrow. In addition normal products of aerobic life such as reactive oxygen intermediates (O_2^-), singlet oxygen (O_2^*) and hydrogen peroxide (H_2O_2) are able to initiate free radical chain reactions that lead to organic and lipid peroxide formation. GSH peroxidases in conjunction with superoxide dismutases and catalase, function in the detoxification of hydroperoxides. These defences against oxygen toxicity although sufficient to meet ordinary demands are easily overwhelmed. There are several ways in which to achieve this. One is to apply hyperbaric oxygen (Deneke and Fanburg 1980), and another is to use compounds which increase the extent of the monovalent pathway of oxygen reduction, eg paraquat (Bus **et al** 1975, Smith **et al** 1979).

This defense system against oxygen toxicity operates as follows: the first in the line of defence is superoxide dismutase which converts superoxide radicals into H_2O_2 and molecular oxygen (Fig. 1.11); second is catalase and selenium (Se)-dependent GSH peroxidase / GSH peroxidase I which share the task of reducing H_2O_2 to H_2O. Catalase has the advantage of not consuming any reductant other than H_2O_2, while GSH peroxidase uses GSH as a reductant and H_2O_2 or alkyl hydroperoxides as oxidant.
Third and last in the line of defence, and required only if a polyunsaturated membrane fatty acid has been peroxidised, are the GSH peroxidases (I and II). This is the only enzymatic system

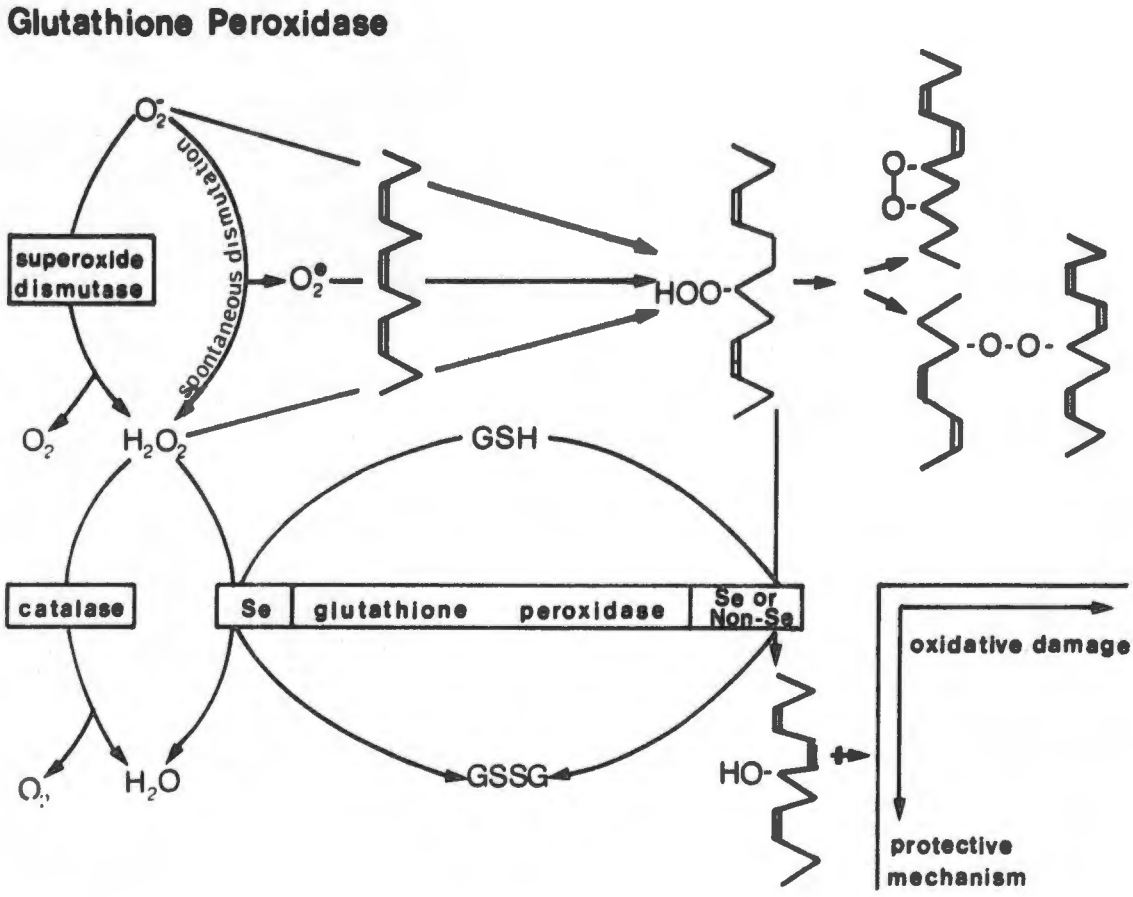


Fig. 1.11 General illustration of the involvement of selenium-dependent (GSH peroxidase I) and non-selenium dependent (GSH peroxidase II) glutathione peroxidases in the detoxication of hydroperoxides. Adapted from Wendel (1980).

capable of preventing further propagation of a radical chain reaction that leads to lipid peroxidation, deterioration of membrane lipids and severe impairment of energy-related membrane functions. Loss of membrane integrity leads to cell death.

GSH peroxidase was thought to catalyse the following reaction:

$$2GSH + H_2O_2 \rightleftharpoons GSSG + H_2O.$$

In the late 1960s this enzyme was also shown to catalyse the reduction of organic hydroperoxides formed from unsaturated fatty acids (Christopherson 1968). Hence the above reaction may be generalised to:

$$2GSH + ROOH \rightleftharpoons GSSG + ROH + H_2O.$$

Catalase was found to have no effect on the lipid peroxides (Little and O'Brien 1968). The lipid products produced by the decomposition of lipid peroxides by GSH peroxidase were identified (Christophersen 1968) and the possibility that this enzyme functioned as an anti-oxidant in breaking the autocatalytic chain reaction of lipid peroxidation was considered.

(b) Lipid Peroxidation

Lipid peroxidation is a unique form of cellular injury implicated in the genesis of liver necrosis and evoked by hepatotoxins such as carbon tetrachloride, alpha-amanitin and yellow phosphorus. It is a multiphasic process involving initiation, propagation and termination reactions. The initiation step, the mechanism of which is not yet understood, is represented by the abstraction of hydrogen from unsaturated fatty acids during free radical attack yielding free radicals of lipids (Fig. 1.12). The hydrogen atoms on methylene carbons separating double bonds in polyenoic fatty

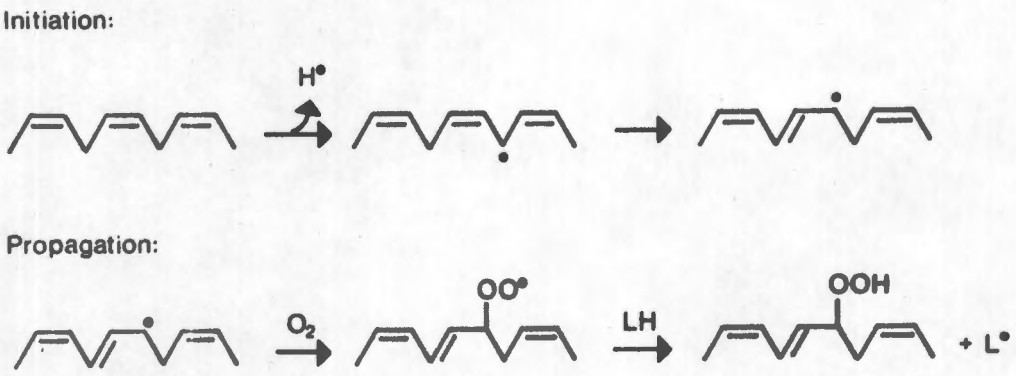


Fig. 1.12 Initiation, propagation and termination reactions in lipid peroxidation. From: Bus and Gibson (1979).

acids are highly susceptible to this free radical attack. Oxygen, singlet oxygen, superoxide anion and/or hydroxyl radical have all been proposed as the initiators of lipid peroxidation.

The free radicals generated from fatty acids during initiation are unstable and undergo a series of transformations reacting rapidly with molecular oxygen to form peroxy-free radicals. These species then abstract from one fatty acid chain the methylene hydrogen of a neighbouring unsaturated fatty acid yielding a hydroperoxide and a new radical. Thus the propagation step is represented by an autocatalytic chain reaction where lipid peroxidation spreads linearly. Unstable hydroperoxides decompose to form additional free radicals. Termination of lipid peroxidation occurs when the substrate is depleted yielding non-radical products that stop the reaction. The obvious targets for lipid peroxidation are the subcellular membranes rich in unsaturated fatty acids, resulting in loss of both structural integrity and function in the affected organelles. In addition to this localised damage the breakdown products of lipid peroxides, such as aldehydes, migrate far from their site of production causing damage at distant loci. Several of these lipid peroxides are known for their extremely high toxicity including carbon tetrachloride, chloroform, 1,1- dichloroethylene and thioacetamide, all of which produce liver necrosis **in vivo** or **in vitro.** Oxidation of membrane lipids and proteins may result **inter alia** in membrane dysfunction and altered membrane permeability.

Methods of measuring lipid peroxidation include determination of the levels of malondialdehyde an end-product of oxidative chain

cleavage of polyunsaturated fatty acids formed during peroxide breakdown. The microsomal NADPH oxidase system promotes lipid peroxidation in microsomes during catalytic activity forming stable malondialdehyde which can thus be used as a measurement of lipid peroxidation.

The GSH peroxidase enzyme system has been claimed by several workers to be responsible for inhibition of lipid peroxidation. Flohe and Zimmerman (1970) originally considered that GSH peroxidase exerted its inhibition by converting the lipid hydroperoxide precursors of malondialdehyde to hydroxy fatty acids. Christophersen (1968) proved that GSH peroxidase could catalyse this reduction reaction **in vitro.** It was generally assumed that the same phenomenon accounts for **in vivo** GSH peroxidase protection against lipid peroxidation in biological membranes. The picture was complicated when McCay **et al** (1976) presented results showing that membrane lipid peroxides are not reduced to lipid alcohols, and that GSH peroxidase activity exerts its effect by preventing free radical attack on polyunsaturated membrane lipids. Thus **in vivo** GSH peroxidase may inhibit initiation of lipid peroxide formation rather than catalysing their reduction. Although the **in vivo** mechanism is still under debate it can be concluded that GSH peroxidase serves a critical function in inhibiting the lipid peroxidative process in biological membranes.

Following McCay **et al**'s (1976) observation that dialysed, unfractionated liver cytosol (105000 g supernatant) and GSH combined could block malondialdehyde production by the NADPH microsomal lipid peroxidation system, Burk **et al** (1980) set out to ascertain which of the two peroxidase species, was responsible

for this function. Partially purified GSH peroxidase I, free of GSH S-transferase activity, but very active with H_2O_2 or cumene hydroperoxide, was completely inactive in inhibiting lipid peroxidation. In contrast a broad peak of protective activity was present following Sephadex G-100 gel filtration coinciding with the GSH S-transferases.

A possible explanation for this observation is that GSH peroxidase I metabolises much of the H_2O_2 produced by metabolic processes in the cells. Selenium deficiency may allow sufficient H_2O_2 to accumulate in cells to initiate reactions which attack membrane lipids. The capacity of GSH peroxidase I may, therefore, lie in its ability to prevent significant levels of H_2O_2 from occurring. GSH peroxidase I does not appear to prevent the peroxidative damage to lipids in membranes initiated by either microsomal NADPH oxidase activity or by ascorbate. The glutathione-dependent cytosolic factor which inhibits lipid peroxidation may be due to one or more of the GSH S-transferases.

Chapter 2

THE AIMS OF THIS STUDY

AND EXPERIMENTAL DETAILS

THE AIMS OF THIS STUDY AND EXPERIMENTAL DETAILS.

This thesis investigated the nature and character of ligandin in the steroidogenically active tissues of the rat. A comparative study was undertaken to establish whether ligandin in the testis did in fact differ from ligandin in the liver. Different methods of purification were used to investigate the multiple forms of ligandin and its relationship with the GSH S-transferases in steroidogenic tissue (Section A).

The development of ligandin in the testis, ovary and adrenal was studied and compared with that of liver and kidney. The effect of puberty, pregnancy and induction on ligandin concentrations were also studied. A quantitative relationship between steroid hormone secretion and ligandin in steroidogenic tissues was considered (Section B).

The peroxidase antiperoxidase (PAP) immunocytochemical technique was used to localise ligandin in the liver, testis, ovary and adrenal of developing rat, and the ovary and placenta during pregancy (Section C).

SECTION A

IDENTITY OF LIGANDIN IN RAT TESTIS AND LIVER AND STUDIES ON THE DIFFERENT GSH S-TRANSFERASE FORMS IN STEROIDOGENIC TISSUES

(a) Introduction

The main aim of this work was to determine whether ligandin in the steroidogenic tissues of rat testis, ovary and adrenal gland was in fact different in structure and function from that found in the non-steroidogenic tissues such as liver and kidney. Comparative studies were carried out with ligandin obtained from both testis and liver of rat.

This section deals with the purification of testis ligandin and its subsequent characterisation. In order to perform these studies, various techniques had to be developed and applied. Cross-linking studies were employed in order to study the subunit structure of testis ligandin. The application of dimethylsuberimidate (DMS), a bifunctional imidoester highly specific for the epsilon-amino group of lysine residues (Davies and Stark 1970), followed by SDS-PAGE, was carried out in an attempt to resolve the arrangement and stoichiometry (homodimer vs heterodimer) of the subunits. The rationale being that where two polypeptides are in close contact, the reactive groups of each protomer may approach each other closely enough for the bifunctional reagent to "cross-link" the two chains. SDS-PAGE analysis would then reveal a single band

migrating with the combined molecular weights of the two polypeptides.

The production of antisera to ligandin was necessary for immuno- affinity chromatographic studies of ligandin from liver and testis. The techniques applied and the results obtained are presented in this section. In addition, antisera was required for radioimmunoassay and immunohistochemistry, which will be detailed in this Chapter, Sections B and C.

Following the standard purification procedure for liver ligandin (Bass **et al** 1977a) (Fig. 1.6) the preparation obtained from testis material will be referred to here (unless specified otherwise) as testis ligandin.

(b) **Materials**

All reagents used were of analytical grade and were purchased as detailed below:

NADPH, agarose, bovine serum albumin (Fraction V) and human serum albumin (crystalline) from Miles Laboratories (Pty) Ltd, Goodwood, South Africa.

Glutathione reductase (Type IV) prepared from yeast, Coomassie Brilliant Blue R, Dansyl chloride and N-ethylmorpholine were obtained from Sigma Chemical Co., Saint Louis, Missouri, USA.

Amido Black, Sodium azide and Tris-(hydroxy-methyl)-aminomethane (Tris) and 1-Iodohexane from E. Merck, Darmstadt, Germany.

Cumene hydroperoxide (70% in cumene) was from Fluka AG, Buchs, Switzerland.

Complete Freund's Adjuvant and Agar Noble from Difco Laboratories Detroit, Michigan USA.

Acrylamide, N,N'-methylene-bisacrylamide and sodium dodecyl sulphate (were specially purified for electrophoresis); ammonium persulphate, N,N,N',N'-tetramethylethylene diamine (TEMED), bilirubin, reduced glutathione (GSH) and Hydriodic Acid (55% HI), from BDH (British Drug Houses) Chemicals Ltd, Poole, Dorset, England.

Cyanogen bromide-activated Sepharose 4B, epoxy-activated Sepharose 6B, Thiol-Sepharose, QAE-Sephadex A-50 and molecular weight standards for electrophoresis from Pharmacia, Uppsala, Sweden.

Dimethylsuberimidate from Pierce Chemical Co. Rockford, Illinois, USA.

Spectrapor membrane tubing 2 (molecular weight cut off 12000-14 000) from Spectrum Medical Industries INC, Terminal Annex, Los Angeles, USA.

Undistilled anaesthetic ether from Natal Cane By-Products Ltd., Merebank, Natal, South Africa.

TLC-micropolyamide sheets from Schleicher and Schull, D-3354 Dassel, Postfach 4, Germany.

Buffer Compositions

Composition	pH	Temperature
0.01M Sodium phosphate containing 0.25M sucrose	7.4	4°C
0.01M Sodium phosphate containing 0.1M NaCl	7.4	4°C
0.05M sodium sulphate containing 0.5M NaCl and 0.00M EDTA	8.0	4°C
0.01M Potassium phosphate	7.5	20°C
0.1M Potassium phosphate	6.5	20°C
0.1M Potassium phosphate	6.7	4°C
0.01M Tris/HCl	8.8	4°C
0.01M Tris/HCl	9.1	4°C
0.01M Tris/HCl containing 0.001M EDTA	7.8	4°C
0.05M Triethanolamine containing 0.05M KCl and 0.4M NaCl	8.0	4°C

Table 2.1: **Buffer compositions and temperatures at which they were prepared.**

(c) Methods

Preparation of Tissue The livers, testes and adrenals of adult male Long-Evans rats (250-300g) fed **ad libitum** or the testes from male cadavers as soon after death as possible (less than 12 hours) were used. Ovaries from adult female Long-Evans rats (200-250g), also fed **ad libitum**, were used.

Male Long-Evans rats (250-300g) housed under controlled conditions of temperature and humidity were used as organ donors. Rats were allowed free access to food and water unless otherwise stated. Ovaries were obtained from Long-Evans rats (200-250g). Human testis was obtained within 12 hours of death from subjects who died as a result of trauma.

Preparation of Cytosol Rats were anaesthetised with ether. Liver and kidney were rapidly removed and immediately perfused via the hilar vessels with ice cold 0.14M NaCl. Following excision of all surrounding tissue and other adherent tissue, the testes, ovaries and adrenals were immersed in saline at 0°C prior to being blotted, weighed and homogenised. Following initial fragmentation with scissors, homogenisation was carried out in 0.01M sodium phosphate buffer pH 7.4 / 0.25M sucrose, with sufficient buffer added to yield a 25% (w/v) homogenate, except for the adrenals and ovaries where 2ml of buffer was routinely added. All steps were carried out at 4°C.

NOTE: read ug, ul and uM as µg, µl and µM

The initial centrifugation was at 27000 g for 30 minutes in a Sorvall RC-5 superspeed centrifuge. The supernatant fraction was carefully removed without disturbing the floating lipid layer and re-centrifuged at 100000 g for 120 minutes in a Beckman model L5.35 ultracentrifuge. The supernatant was removed as before and stored on ice.

Ultrafiltration and Dialysis Cytosol (prepared as previously described) was concentrated using an Amicon model 202 ultrafiltration cell with a PM-10 membrane. Dialysis was carried out either in the Amicon cell, where buffer change was achieved by flushing through dialysing buffer until the correct pH was obtained, or by using dialysis tubing (Spectrapor molecular weight cut off 12000-14000) presoaked in distilled H_2O.

Standard Purification Procedure of Ligandin (Fig. 1.6) Ligandin was purified from the liver, testes, ovaries and adrenals of Long Evans rats (250-300g) as described by Kirsch **et al** (1975) and Bhargava **et al** (1980a). Cytosol prepared by centrifugation at 100000 g was obtained from 100g of rat tissue (only 10g of ovaries or adrenals were obtained) and was dialysed against 0.01M Tris.HCl pH 8.8 (buffer A) and chromatographed on a column of either TEAE- or DEAE-cellulose in buffer A. The single protein peak eluted was pooled, concentrated (Amicon cell, PM-10 membrane) and chromatographed on a column of Sephadex G-100 in 0.01M sodium phosphate buffer, pH 7.4 / 0.1M NaCl. Fractions exhibiting enzyme activity with CDNB were pooled, concentrated, dialysed against buffer A and chromatographed on a column of QAE-Sephadex A-50 in buffer A.

The single protein peak eluted was pooled and stored in sterile tubes at 4°C. In certain studies the purified ligandin was subjected to a further chromatographic step with QAE-Sephadex using buffer C (0.01M Tris. HCl pH 9.1).

Polyacrylamide gel electrophoresis Polyacrylamide gel electrophoresis in 0.1% SDS was performed on vertical slab gels (Laemmli 1970). The system comprised a 3% (w/v) stacking gel (3 x 14 x 0,2cm) in 0.14M Tris.HCl buffer pH 6.8, and a 5% - 25% (w/v) gradient gel (20 x 14 x 0.2cm) in 0.56M Tris.HCl buffer, pH 8.8. Samples were prepared as described by Maizel (1971). Gels were fixed and stained for 2.5 hours in 10% trichloroacetic acid, 1.0% Coomassie Brilliant Blue R in methanol/water (1:2 v/v) and destained in a solution of 5% methanol/7% acetic acid (v/v) molecular weight estimation. Molecular weights of ligandin preparations, the aggregates and dimethylsuberimidate cross-linked material were determined by comparison with standards of known molecular size (phosphorylase b 94000; albumin 67000; ovalbumin 43000; carbonic anhydrase 30000; trypsin inhibitor 20100; alpha-lactalbumin 14400). The relative mobility of each protein was calculated from the distance migrated of each protein compared with that of the bromophenol blue "tracking-dye". These values were plotted against the log standard protein molecular weight (Fig. 2.1). Linear graphs were obtained and the unknowns read from this plot.

Coelectrophoresis To compare the subunits of purified rat liver and testis ligandin, dilute samples were coelectrophoresed on SDS-polyacrylamide gels with rat testis and rat liver ligandin samples in adjacent lanes.

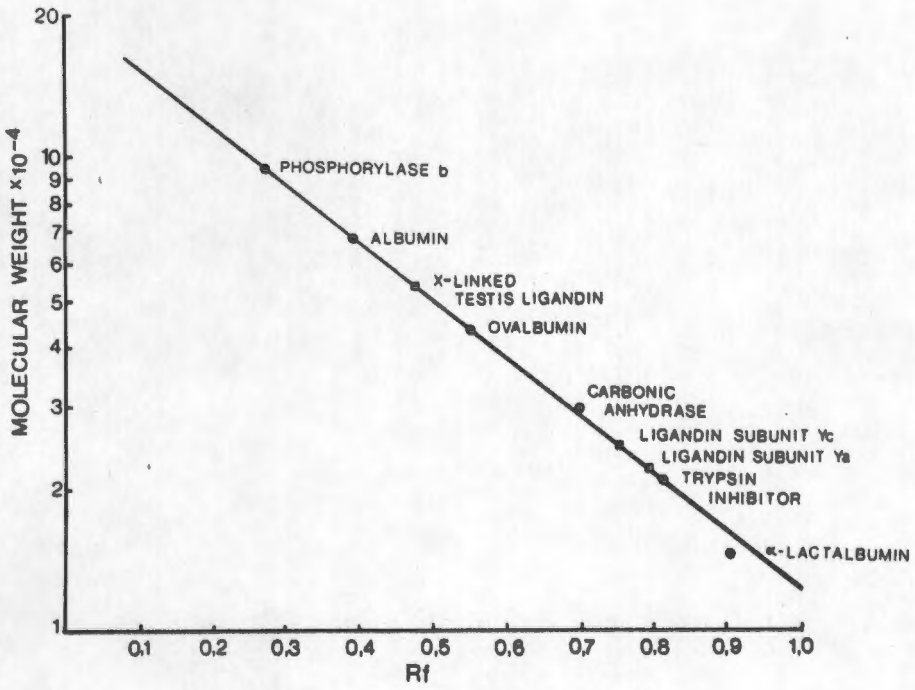


Fig. 2.1 Determination of the comparative molecular weights of rat testis ligandin Ya and Yc subunits and dimethylsuberimidate cross-linked testis ligandin material by discontinuous gradient PAGE in SDS. Details of these procedures as well as the assumed molecular weights of the standard proteins are given in Chapter 2. Section A.

Dansylation of Proteins: N-terminal analysis Protein labelling was achieved using the method of Gray (1972) modified as described below. Test tubes (6 x 50mm) were acid cleaned and heated in 100°C oven overnight before use. 50-250ug protein was dissolved in 50ul 1% SDS by heating in a boiling water bath for 2-5 minutes. When the solution had cooled, 50ul of distilled N-ethylmorpholine was added and mixed. Immediately before use 50mg dansyl-chloride was dissolved in 1ml water-free acetone, 50ul dansyl-chloride solution was added to the protein solution and mixed. The reaction test tube was covered with tin foil and incubated for 30 minutes at 45°C. The protein was precipated with acetone. Following centrifugation the supernatant was carefully decanted and discarded while the pellet was washed with 80% acetone and recentrifuged. After the supernatant was discarded the pellet was dried under vacuum. 20ul of 5.7M HCl was added and the tubes were sealed in a gas flame and incubated at 105°C for 6-15 hours. The shorter hydrolysis time gives a better recovery of proline but less complete protein hydrolysis. Following hydrolysis the mixture was was freeze-dried, the residue re-dissolved in 5ul of 95% ethanol and spotted onto a 5 x 5 cm micropolyamide sheet. One spot of a mixture of standard amino acids was spotted onto the reverse side of the polyamide sheet. Development was carried out sequentially in four solvent systems; solvent 1, 1.5% formic acid in water (v/v); solvent 2, Benzene:acetic acid (9:1 v/v); solvent 3, ethyl acetate: acetic acid: methanol (20:1:1 v/v); solvent 4, 50mM Na_3PO_4 in 25% aqueous ethanol. After drying the sheet was examined under an ultraviolet lamp. A blue dansyl-OH spot was seen on all chromatograms. Dansyl-derivatives of tyrosine fluoresced an intense yellow-brown colour, while dansyl derivatives of other amino acids yielded a yellowish-white colour. Blocked NH_2

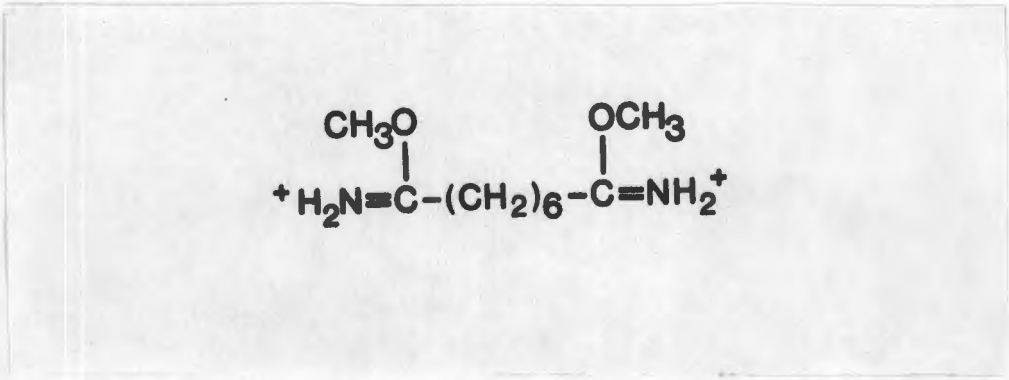
terminals failed to produce fluorescence corresponding to any of the standard amino acids.

Amino Acid Analysis (Moore and Stein 1963) The protein was dialysed into distilled H_2O. After determination of protein concentration 3 measured aliquots were transferred into chromic acid-washed pyrex hydrolysis tubes and freeze-dried. 1ml of 6N HCl was added to each tube and the contents frozen in liquid N_2. Excess HCl was evacuated from the tube on an oil-vacuum pump to less than 50 microns after which the tubes were flushed with N_2 and vacuum-sealed. Hydrolysis was carried out in a 110°C oven for 24 hours.

Analysis of amino acids was carried out on a Beckman (121 MB) amino-acid analyser. Chromatographic peaks were identified and integrated by an on-line computer and results expressed as residues per mole.

Cross-Linking Studies To prevent the aggregation of ligandin which may occur under low salt conditions, cross-linking reactions were carried out at a salt concentration of 0.4M NaCl / 0.05M KCl, and to favour cross-linking within the protomers over that between the protomers (Davies and Stark 1970), low protein concentrations were used (0.3mg/ml). A reaction buffer of 0.05M triethanolamine containing 0.4M NaCl / 0.05M KCl adjusted to a pH of 8 was used. Immediately before use a 30mg/ml solution of dimethylsuberimidate (DMS) (see structural formula below) was prepared in 0.05M triethanolamine / 0.4M NaCl / 0.05M KCl, pH 8.0 and immediately readjusted to pH 8 with 0.5M NaOH. Aliquots of this solution were added to liver and testis ligandin (200ul) in 0.01M Tris HCl buffer, pH 8.0. After standing on ice for various time periods

the reaction was quenched by addition of an equal volume of 0.3M ethanolamine. Thereafter the SDS-PAGE sample application buffer containing 3% beta-mercaptoethanol was added in equal volume to the cross-linked solution, incubated for 6 minutes at 100°C and electrophoresed in SDS on polyacrylamide gradient gels.



Difference Spectroscopy of Bilirubin and Ligandin Stock solutions of bilirubin were prepared (10uM) in 10mM NaOH and adjusted to a pH of 8.8 with 0.02M Tris. HCl buffer pH 7.0. The concentration of the stock solution was determined spectrophotometrically by using an ε_{400} value of 52000 litre.mol^{-1}.cm^{-1}. An Aminco (DW 2) dual wavelength ultra-violet/visible spectrophotometer or a Varian 635 spectrophotometer was used to compare difference spectra of bilirubin with liver ligandin and of bilirubin with testis ligandin. The methods used were described by Tipping **et al** (1976a). The temperature of the cell compartment was 24°C. 1 ml of a 10uM solution of bilirubin was added to one compartment of both reference and sample cells, while an equal volume of protein was added to the other compartment of both cells. A base line was obtained and, after mixing the solutions in the sample cells,

measurements were obtained immediately and after 5, 10 and 15 minutes.

Separation of Glutathione S-transferases Rat liver and testis supernatants were chromatographed on a DEAE-cellulose column (2.6 x 35cm), equilibrated with 0.01M Tris.HCl buffer, pH 8.0. Fractions exhibiting absorbance at 280 nm were pooled and concentrated to a volume of 10 ml (Amicon cell, PM-10 membrane). After dialysis into 0.01M potassium phosphate buffer pH 6.7 (buffer C), the preparation was applied to a CM-cellulose column (2.6 x 30 cm) previously equilibrated with buffer C (Habig **et al** 1974b). A 600 ml linear gradient, 0-120mM KCl in buffer C was applied to the column and 3ml fractions were collected and stored at 4°C prior to assay.

In a separate experiment, testis ligandin prepared according to Bhargava **et al** (1980a) was chromatographed on a CM-cellulose column (1.6 x 10cm) as described above.

Assays Glutathione S-transferase activity was measured by following the conjugation of GSH with either 1-chloro-2,4-dinitrobenzene (CDNB) at 340nm or 2,4-dinitrochlorobenzene (DCNB) at 345nm (Habig **et al** 1974b). Reaction rates were corrected for the small amount of non-enzyme catalysed conjugation of GSH with either CDNB (ε=9600) or DCNB (ε=8500). Specific activities (umol/min/mg protein) were calculated from the equation:

$$\frac{\text{OD/min}}{(\varepsilon)} \times \frac{\text{final assay vol}}{\text{sample vol}} \times \frac{10^3}{\text{protein conc.}}$$

Glutathione peroxidase activity was assayed, as described by Prohaska and Ganther (1977). 50ul of sample was added to a mixture in a cuvette of 0.55ml 0.1M potassium phosphate buffer pH 7.0 / 0.1M KC1; 0.1ml 10.03M EDTA; 0.1ml 0.01M GSH; 0.1ml 0.0011M NADPH and 0.1ml GSH reductase (4ug GSH reductase). A blank cuvette contained all the above solutions excepting for the sample. The cuvettes were incubated at room temperature for 10 minutes. A stock solution of 0.2g (w/v) cumeric hydroperoxide (70% in cumene) in 10% (v/v) ethanol was made up and 10ul of this stock added to both blank and sample cuvettes. The change in absorbance was read at 340nm which measured the disappearance of NADPH (ε=6000). Results were expressed as umoles/min, calculated from the following equation:

$$\frac{OD/min}{(\varepsilon)} \times 10^6$$

One enzyme unit is defined as 1 umol of NADPH oxidised per minute.

Protein Determinations Protein concentrations were determined by the method of Lowry et al (1951), using bovine serum albumin as standard.

Sulphobromopthalein Assay Sulphobromopthalein (BSP) concentrations were measured by adding 100ul 2M NaOH to 250ul aliquots of column fractions and reading absorbance at 580nm (Levi et al 1969a).

Immunological studies Antisera to liver and testis ligandin were raised by injecting 50ug of purified proteins in Freund's complete adjuvant into popliteal lymph nodes of male albino rabbits. Thereafter, 50ug of ligandin in adjuvant was injected into multiple subcutaneous sites at three-week intervals. Animals were bled 10 days after booster inoculations. The separated sera were tested for specific antibody production by immunodiffusion and immunoelectrophoresis (Bass **et al** 1977a). Radial immunodiffusion used 1.2% agar gels (Ouchterlony 1958) and immunoelectrophoresis 1% agar gels (Bass **et al** 1977a).

(i) Immunodiffusion

1.2g Agar-Noble, 0.1g sodium azide and 100ml of 0.05M sodium phosphate buffer pH 7.4 / 0.15M NaCl were heated in a conical flask until the solution clarified. The mixture was not allowed to boil. After clarification the flask was placed in a 60°C water bath. 10ml of molten agar solution was poured onto warmed glass plates (9.2cm x 8.2cm) positioned on a horizontal platform. The plates were allowed to cool for 1 hour. 4mm diameter wells were cut using an LKB 2117 Multiphor template and bores. 10ul antiserum was placed into the central well and 10ul of antigens into the peripheral wells. The plates were kept horizontal in an air-tight moisture box at room temperature for 48 hours after which they were washed for 3 days with several changes of 0.9% saline / 0.1% sodium azide solution. The plates were stained in 1.0% Amido Black (made up in destaining solution) for 3 hours, and destained in 5% Acetic Acid. An alternative method which avoided this delay due to washing was also employed. After immunodiffusion the plates were covered with six layers of blotting paper soaked in distilled

H_2O, eight layers of dry blotting paper and placed inbetween 2 glass sheets. A 1kg weight was placed evenly on top of the glass plates and left for 1 hour. The blotting paper was replaced after 30 minutes. The weights were removed and the gel dried down to a fine film with a hair dryer. Staining and destaining were carried out as before.

(ii) Immunoelectrophoresis

100ml of 0.05M veronal buffer pH 8.7 / 0.2% sodium azide was added to 1g of Agar-Noble. A similar procedure as that described for immunodiffusion was followed until the agar was set and cooled on the glass plates. Using the LKB 2117 Multiphor template for immunoelectrophoresis 4mm diameter wells were cut. A sharp scalpel blade was used to cut the outline of troughs without removal of the agar. Gel plates were placed onto LKB Multiphor apparatus, the tanks filled with 0.05M veronal buffer, and moistened filter paper wicks placed in contact with ends of gel plate and the buffer tanks. 10ul of sample was placed into the wells and a current of 12mA/plate was applied for 1½-2 hours. The agar was carefully removed from the troughs and 30-50ul of antiserum added. Immunodiffusion was then carried out as described before.

(iii) Preparation of IgG

IgG was prepared by ammonium sulphate precipitation according to the method of Hebert **et al** (1973). A volume of serum was gently stirred whilst an equal volume of a 70% ammonium sulphate solution was slowly added. The reaction mixture was set aside at room temperature for 4 hours and then centrifuged. The supernatant

was discarded and the pellet resuspended in 0.9% NaCl, pH 8.0 to a final volume equal to the original volume of serum. This procedure was repeated twice, after which the resuspended precipitate was dialysed into 0.9% Nacl, pH 8.0 with frequent changes of the buffer. To test for the presence of residual sulphate, a small volume of saturated barium chloride was added to an equal volume of the saline-dialysate. If no cloudiness resulted, the dialysed fraction was considered to be substantially free of sulphate. The IgG fraction was checked for albumin contamination by means of cellulose acetate electrophoresis (Kohn 1960).

Affinity Chromatography Techniques

(i) Immunoaffinity Chromatography

The procedure for immunoaffinity chromatography of proteins as described by Livingston (1974) formed the basis of these studies. Affinity reagents consisting of the gamma globulin fractions of either anti-rat liver ligandin antiserum, or anti-rat testis ligandin antiserum were prepared as previously described in this chapter. 3g of cyanogen bromide (CNBr)-activated Sepharose 4B was swelled and washed in 600ml 0.01M HC1 on a sintered glass filter in order to remove dextran and lactose additives. Approximately 100mg of anti-rat liver YaYa ligandin IgG or anti-rat testis ligandin IgG was dissolved in 10 ml of coupling buffer (0.01M potassium phosphate buffer pH 6.8 / 0.5M NaC1) and mixed with the swollen gel in a glass-stoppered test tube and rotated end-over-end overnight at 4°C. An aliquot was immediately removed, centrifuged and absorbance at 280nm recorded. The remaining active groups were reacted with 1M ethanolamine at pH 8 for 2 hours, after which

several washing cycles (0.1M acetate buffer / 1M NaCl pH 8.0 and 0.1M borate buffer / 1M NaCl pH 4.0) were carried out to remove non-covalently adsorbed protein. A second aliquot was removed and the absorbance at 280nm recorded.

$$\% \text{ coupling} = \frac{\text{1st } A_{280} \text{ reading} \times 100}{\text{2nd } A_{280} \text{ reading}}$$

98.4% of liver antibody and 94.2% of testis antibody was coupled. Partially purified rat tissue ligandin (TEAE eluate, Fig. 2.2) was applied to a 10 x 0.9 cm column of affinity material. After unbound protein had been washed from the column, immunoreactive material was eluted with 0.1M glycine pH 3.0. The pH of all fractions was immediately adjusted to pH 7.4 with 1M Tris buffer.

(ii) Activated Thiol-Sepharose

Ligandin YaYc was dialysed into 0.05M sodium phosphate buffer pH 8.0 / 0.5M NaCl / 0.001M EDTA. 0.05M L-cysteine was made up in the same buffer. Buffers were deaerated before use in order to avoid oxidation of free thiol groups. 4g of activated Thiol-Sepharose 4B was swollen and washed with 0.3M NaCl pH 7.0. The swollen gel was packed into a column (10cm x 15cm) and equilibrated with starting buffer (0.05M sodium phosphate buffer pH 8.0 / 0.5M NaCl / 0.001M EDTA) at a flow-rate of 5ml/hour. The ligandin sample was applied to the column and the unbound material washed off with the starting buffer. Bound fractions were eluted after application of L-cysteine buffer to the column. Fractions containing enzyme activity were pooled and desalted on a Sephadex G-25 (Fine) column (2.6cm x 60cm).

(iii) Hexylglutathione Affinity Chromatography

The method described by Guthenberg and Mannervik (1979) was used. Supernatant fractions of liver, testis, ovary and adrenal were passed through a Sephadex G-25 column (bed volume; 5 times sample volume) in 0.01M Tris HCl buffer pH 7.8. The enzyme active peak was pooled, concentrated in an Amicon ultrafiltration chamber (PM.10 membrane) and applied to the hexylglutathione affinity column (1cm x 10cm) (see below for method) equilibrated in the Tris buffer. The column was washed with 0.01M Tris.HCl buffer pH 7.8 / 0.2M NaCl until no more protein eluted from the matrix. GSH S-transferases were eluted from the affinity column with 0.005M S-hexylgluthione in 0.01M Tris.HCl buffer pH 7.8 / 0.2M NaCl. All fractions displaying GSH-CDNB conjugating activity were pooled and desalted on the Sephadex G-25 column equilibrated in 0.01M potassium phosphate buffer pH 6.8 / 0.001M EDTA.

S-hexylglutathione was synthesised according to method A of Vince **et al** (1971). 2mmoles of GSH were dissolved in 2ml H_2O. 4 mmoles of 2N NaOH (freshly made up) was added with stirring at room temperature. 12-15ml of absolute ethanol was added "dropwise", with stirring to the 'cloud point', after which equimolar (2mmoles) 1-Iodohexane was added "dropwise" over a period of 30 minutes, with stirring. The mixture was stirred vigorously at room temperature for 3 hours. If any precipitation occurred, a few drops of ethanol were added. The pH was then reduced to 3.5 by "dropwise" addition of 47% hydrogen iodide (approximately 15 drops) and the mixture chilled to 4°C. The crystals formed were separated by filtration and washed with 20ml H_2O. Recrystallisation steps were carried out. The sample was dried and stored in a dessicator at -20°C. The yield obtained was approximately 65%.

A 0.005M solution of S-hexylglutathione for eluting bound material was prepared by homogenising crystals in 0.01M Tris.HCl buffer pH 7.8 / 0.2M NaCl. 1M Tris. stock was added "dropwise" until the crystals were dissolved. The solution was then made up to volume with 0.01M Tris.HCl buffer pH 7.8 / 0.2M NaCl.

For preparation of the hexylglutathione affinity column, 6g of epoxy-activated Sepharose 6B was washed in a sintered glass funnel with 800ml of H_2O for 1 hour. 94mg of S-hexylglutathione was then dissolved into 12 ml of 0.1M $NaHCO_3$ / NaOH buffer pH 10.6, with stirring. Approximately 7 drops of 1 M NaOH was required to readjust pH back to 10.6. The ligand solution was added to the gel suspension and shaken gently for 30 hours at 30°C.

The gel suspension was then washed on a sintered glass funnel with each of the following:

(a) 200ml 0.1M $NaHCO_3$ / NaOH buffer pH 10.6,
(b) 200ml 0.1M sodium borate buffer pH 8.0 / 0.5M NaCl,
(c) 200ml 0.1M sodium acetate buffer pH 4.0 / 0.5M NaCl, and
(d) 400ml H_2O.

Chromatofocusing (Sluyterman and Elgersma 1978) Following concentration of material which bound to the hexylglutathione affinity column, the sample was applied to a chromatofocusing column (0.9cm x 20cm) of PBE 94 gel equilibrated with 15 column volumes of 0.025M ethanolamine.HCl pH 9.4. The GSH S-transferases were eluted with 250 ml of Polybuffer 96, (diluted 1:10), pH 8.0.

Following SDS-polyacrylamide electrophoresis of fractions eluting from the chromatofocusing column, the gels were washed in 11% Trichloroacetic acid for 24 hours, before staining with Coomassie Blue.

(d) **Results**

PURIFICATION AND CHARACTERISATION OF RAT TESTIS LIGANDIN

Standard Purification Procedure Table 2.2 shows the details of purification of testis ligandin following the standard purification procedure described by Kirsch **et al** (1975) and Bhargava **et al** (1980a). Specific activity using CDNB and GSH as substrates was determined at each stage. The mean specific activity of the final product of four separate testis preparations was 15.6 umol/min/mg. The elution profile from TEAE-cellulose, Sephadex G-100 and QAE-Sephadex chromatography steps of the purification are shown in Fig. 2.2. The patterns correspond with those obtained for the purification of rat liver ligandin (Bass **et al** 1977a) and for rat renal ligandin (Kirsch **et al** 1975). A single peak comprising the basic cytosol proteins eluted from DEAE-cellulose. Several purifications were carried out using either TEAE- or DEAE-cellulose in the first step and no difference was apparent when either of these cellulosic-anion exchangers were used.

Following concentration and chromatography on Sephadex G-100, three peaks were obtained, the CDNB-GSH conjugating activity was confined to the second peak. QAE-Sephadex chromatography of the enzyme active peak resulted in a single peak being eluted.

Subunit Composition SDS-PAGE of each stage of testis ligandin purification is shown in Fig 2.3. The final preparation of testis ligandin has more Yc than Ya (lanes 6, 7 and 8). Ligandin prepared from rat liver using the same purification method had equal amounts

	Total Activity * (umol/min/ml)	Protein (mg/ml)	Volume (ml)	Specific Activity * (umol/min/mg)
Testis cytosol	50	46.3	23.5	1.1
TEAE-cellulose	10.3	2.6	9.4	3.9
Sephadex G-100	2.1	0.2	25.0	9.1
QAE-Sephadex A50	1.7	0.1	31.0	15.9

Table 2.2 - **Summary of Purification of Rat Testis Ligandin**
* Determined with 1-chloro-2,4-dinitrobenzene as substrate.

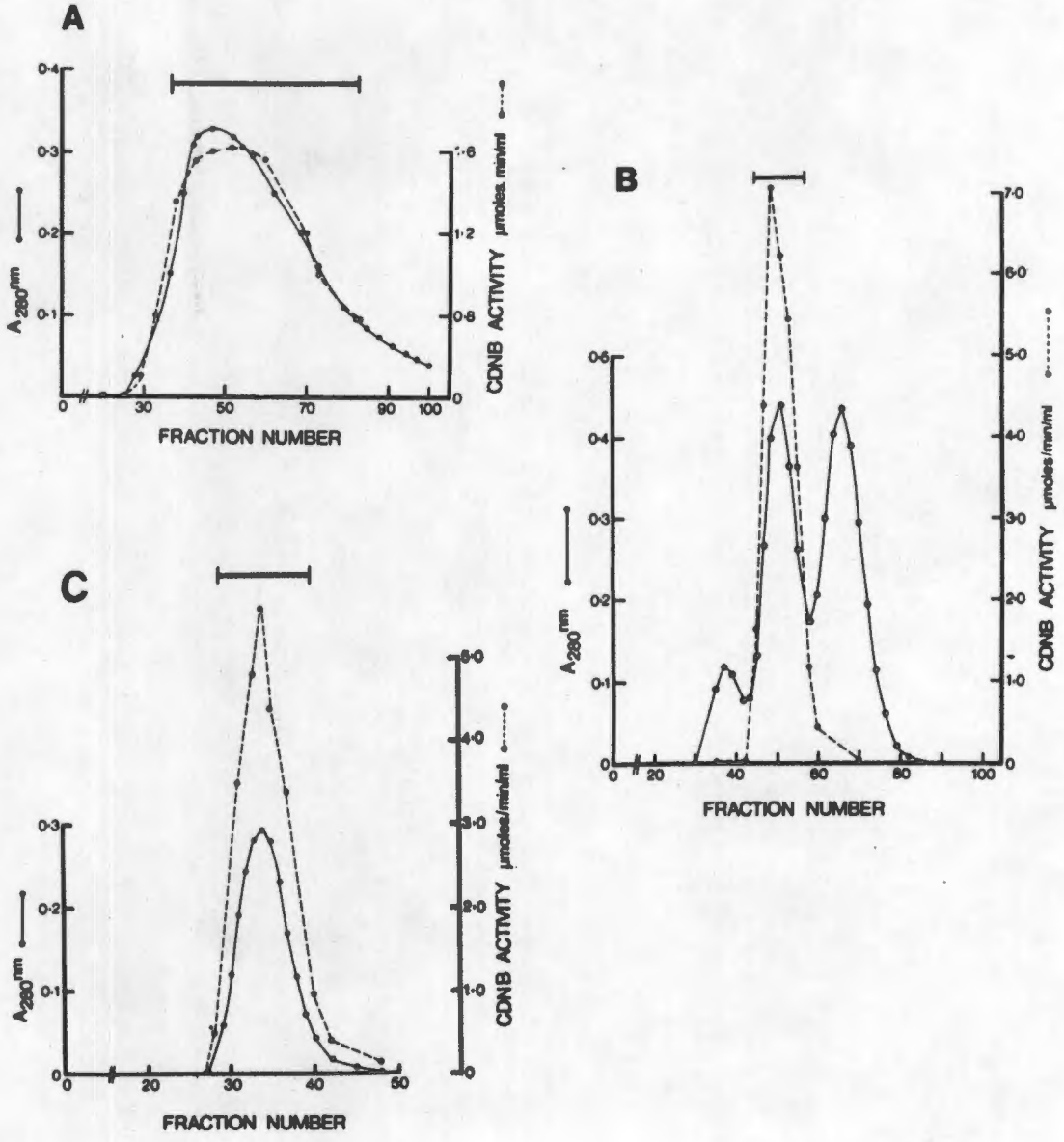


Fig. 2.2 **Purification of ligandin from the 100000 g supernatant of rat testes.** (A) Elution of protein from DEAE-cellulose in 0.01M Tris.HCl buffer pH 8.8. (B) Elution of protein from Sephadex G-100 in 0.01M sodium phosphate buffer pH 7.4 / 0.1M NaCl. (C) Purification of the CDNB-enzyme active peak on QAE-Sephadex A.50 in 0.01M Tris.HCl buffer pH 8.8. Fractions pooled at each stage of the purification are indicated by the horizontal bars (see Chapter 2, Section A, Methods and Results, for details).

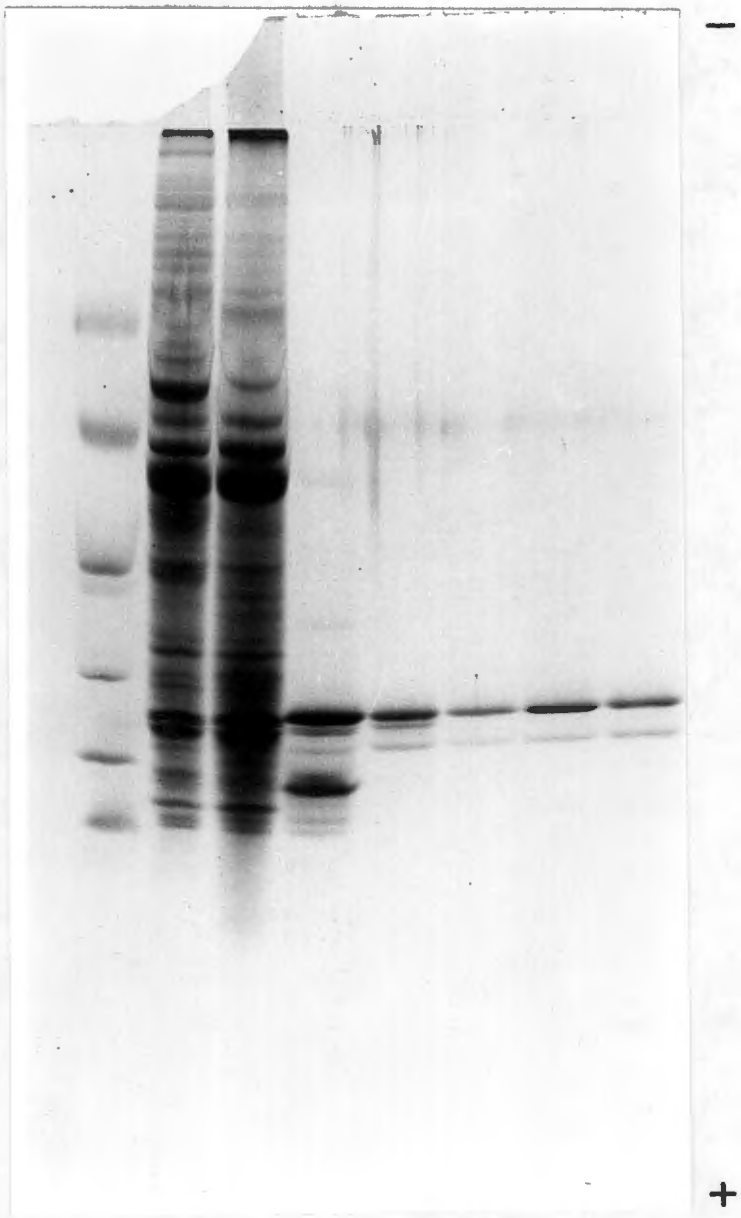


Fig. 2.3 **SDS/Polyacrylamide 5 - 25% (w/v) gradient gel electrophoresis at each stage of rat testis ligandin purification.** (1) molecular weight markers 94000; 67000; 43000; 30000; 20100; 14400. (2), (3) testis cytosol (100000 g supernatant), (4) protein peak after TEAE-cellulose chromatography, (5) peak exhibiting GSH-CDNB conjugating activity after Sephadex G-100 chromatography, (6) fraction 30 from QAE Sephadex A-50 eluate, (7) fraction 35, (8) fraction 38 . Between 10 and 30 ug of protein were loaded per sample. Samples were prepared as described by Maizel (1971).

of Ya and Yc subunits, similar to that described by Bhargava **et al** (1978a), while livers from rats pre-treated with phenobarbital contained more Ya than Yc. The estimated molecular weight of Yc was 25000 daltons and of Ya, 22000 daltons (Fig. 2.1). Coelectrophoresis of purified liver and testis ligandin revealed that the Yc and Ya subunits of the two preparations migrated identically on SDS-PAGE.

N-Terminal Analysis Dansyl-chloride labelling of testis ligandin, liver YaYc ligandin and liver YaYa ligandin was carried out according to the method previously described. No NH_2-terminal residues were detected for freshly isolated preparations of ligandin suggesting that the NH_2-terminus is blocked. All three ligandin preparations (testis YaYc, liver YaYc and YaYa) gave a similar result. Dansyl derivatives of tyrosine and lysine were noted, indicating the presence of large amounts of internal tyrosine and lysine residues. Samples stored at 4°C for a period of three months showed proline to be the NH_2-terminal amino acid in all three ligandin preparations. This was possibly due to degradation of the proteins. Bhargava **et al** (1978a) presented similar findings for liver ligandin preparations.

Amino Acid Analysis The amino acid composition of testis ligandin prepared according to the standard purification procedure is presented in Table 2.3. In addition, the amino acid composition of liver YaYa ligandin, the range of values quoted by several workers for liver YaYc ligandin and the amino acid composition of liver GSH S-transferase AA are shown.

AMINO ACID	[a]TESTIS LIGANDIN	[b]GSH S-TRANS-FERASE AA (ex Habig et al (1976)	GSH S-TRANS-FERASE B (ex Jakoby 1978)	[b]LIVER YaYa LIGANDIN (ex Bass 1977)	LIVER YaYc LIGANDIN (ex Ketterer et al (1976)
Lysine	34	34	36	35	34
Histidine	7	7	6	4	-
Arginine	26	26	22	20	22
Aspartate	32	41	37	31	34
Threonine	7	8	11	12	12
Serine	15	12	12	14	14
Glutamate	40	47	46	40	40
Proline	24	25	20	17	16
Glycine	30	23	21	16	18
Alanine	42	33	31	26	26
½-Cystine	1	2	4	nd	6
Valine	14	31	25	17	18
Methionine	14	10	8	12	14
Isoleucine	7	14	18	18	46
Leucine	45	49	50	46	20
Tyrosine	17	19	13	12	12
Phenylalanine	16	15	17	15	18
Tryptophan	nd	2	9	1	2

Table 2.3 **Amino Acid Analysis of Testis ligandin; Liver GSH S-transferases AA and B; YaYa and YaYc Liver Ligandin.**
nd = not determined
[a]Residues per mole (47000g)
[b]Residues per mole (45500g)

Testis ligandin had similar amino acid composition to GSH S-transferase AA.

Covalent Cross-linking of Testis Ligandin SDS-PAGE analysis of material after cross-linking had been allowed to proceed for 2.5 hours at 4°C showed 2 distinct bands migrating in the 50000 molecular weight region (Fig. 2.4). DMS cross-linked liver YaYa or YaYc ligandin could not be resolved into more than 1 band, agreeing with the findings of Bhargava **et al** (1978a).

Degradation of Ligandin and Appearance of Aggregates after Storage at 4°C Although ligandin preparations (liver and testis) contained 0.1% sodium azide and were stored at 4°C under identical conditions, it appeared on SDS-PAGE that the testis Ya subunit degraded more readily (3 weeks after preparation) than was observed for the Ya subunit of liver ligandin. Testis ligandin preparations also revealed the presence of aggregates between 62000 and 66000 daltons on SDS-PAGE following storage at 4°C for approximately 2-3 weeks. Similar patterns of aggregates were observed with stored liver YaYc and YaYa ligandin and ovary and adrenal ligandin preparations. Incubation at 100°C for 10 minutes following reduction with beta-mercaptoethanol appeared to remove these aggregate forms, suggesting that the highly charged proteins may form disulfide bridges during ageing and subsequent denaturation.

Bilirubin Binding Earlier studies of bilirubin binding using difference spectra with liver ligandin and bilirubin, and testis ligandin and bilirubin showed liver ligandin to have binding

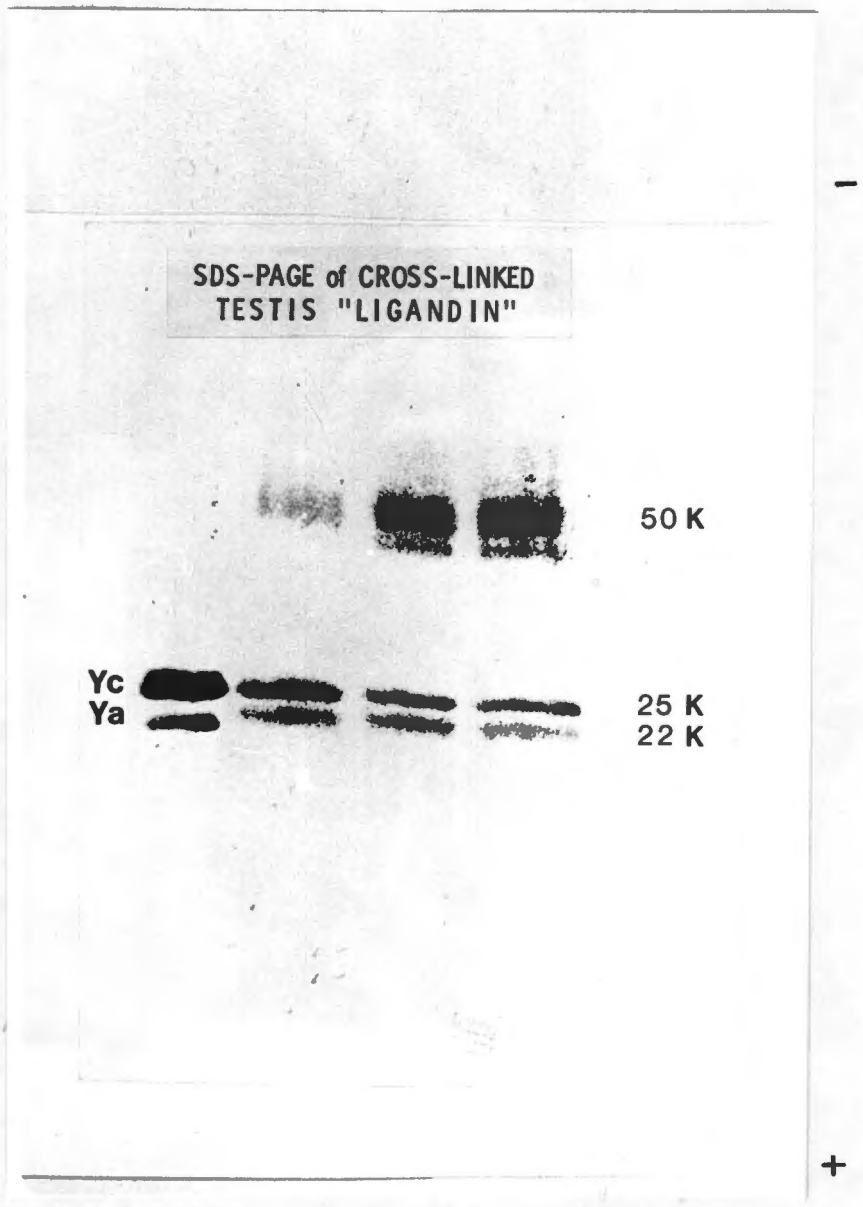


Fig. 2.4 **SDS-PAGE of "pure" testis ligandin cross-linked with dimethylsuberimidate.** The 1st lane contains testis ligandin purified by standard procedures, no DMS added; the 2nd lane, testis ligandin after addition of DMS and cross-linking reaction allowed to proceed for 1.2 h under conditions described in Methods section; the 3rd lane, after cross-linking for 2.5 h, and the 4th lane, after cross-linking for 3.5 h.

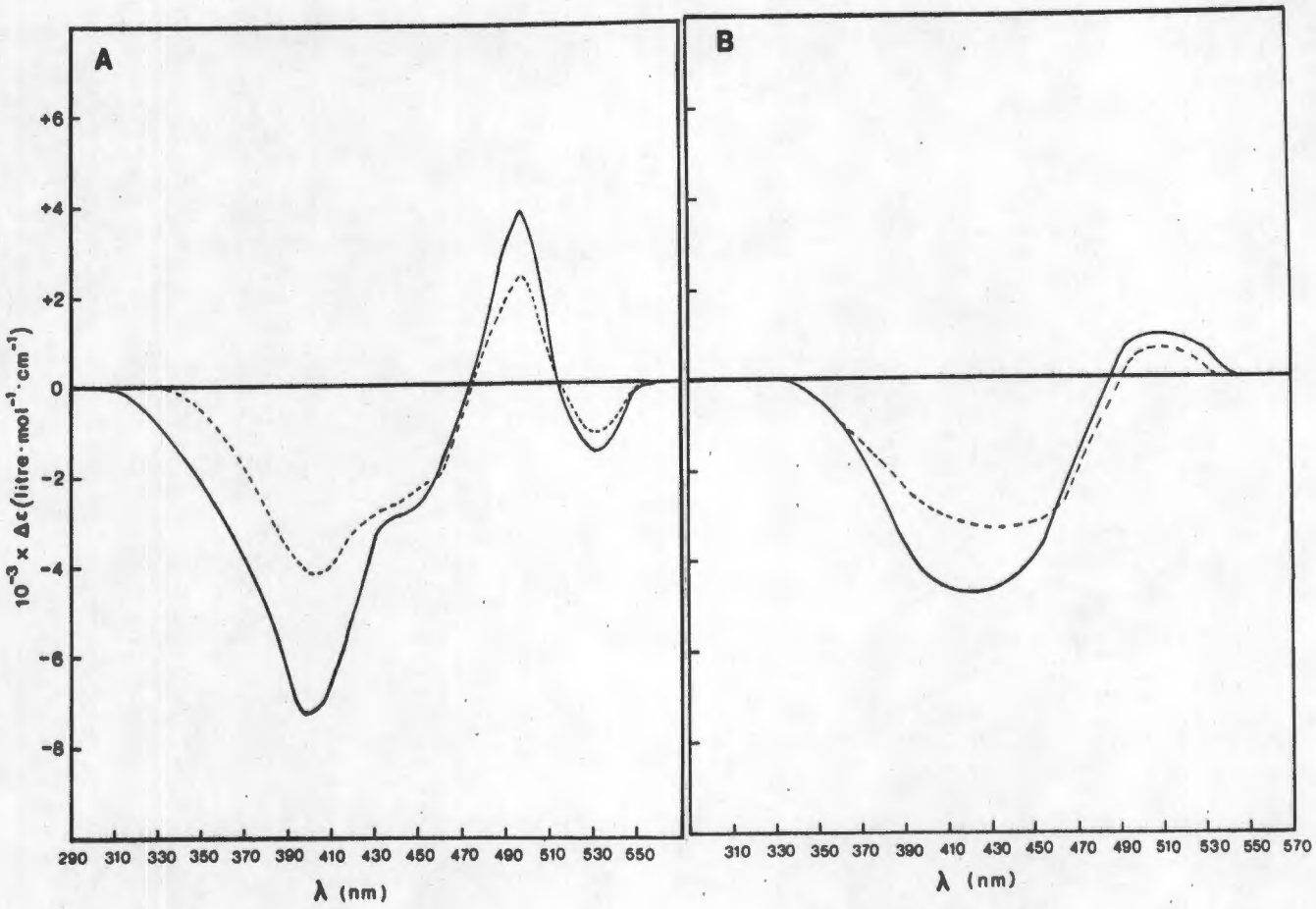


Fig. 2.5 Difference spectra resulting from the binding of bilirubin by liver ligandin (——) and bilirubin by testis ligandin (-----) (A) The sample cell compartments contained 1.0ml of a 50uM protein solution. The reference cell compartments contained 1.0ml 10uM bilirubin. The following spectra were obtained after mixing the solutions of sample cell cuvette. Details are described in Methods and Results. (ε) refers to total bilirubin. (B) Difference spectra resulting from the binding of 1:10 dilutions of bilirubin with liver ligandin and 1:10 dilutions of bilirubin with testis ligandin.

characteristics similar to those previously reported (Tipping **et al** 1976a) while testis ligandin appeared to have a lower affinity for bilirubin. This suggested that the primary high affinity binding site present on liver ligandin was lacking in testis ligandin preparations. More recently however, experiments carried out using concentrated (1.8mg/ml), freshly isolated testis ligandin demonstrated binding characteristics more reminiscent of liver ligandin than the earlier testis preparations (Fig 2.5A). 1 in 10 dilutions of bilirubin incubated with these ligandin preparations gave rise to a red shift in the absorption maximum, accompanied by a smaller difference-spectra result (Fig 2.5B).

Immunological Properties Testis ligandin and liver cytosol revealed a single line of identity when placed in adjacent wells and subjected to immunodiffusion against anti-liver YaYa ligandin serum. Antiserum to liver YaYc ligandin gave a similar result. Anti-liver YaYa ligandin IgG (prepared as described in the Methods section) gave single precipitin lines as well. These results were in accord with those published by Bhargava **et al** (1980a).

Antisera raised against testis ligandin (prepared using the standard technique) revealed a single line of identity when immunoelectrophoresed against testis cytosol and adrenal cytosol. However, a faint second line was noticed on immunoelectrophoresis against liver cytosol (Fig 2.6).

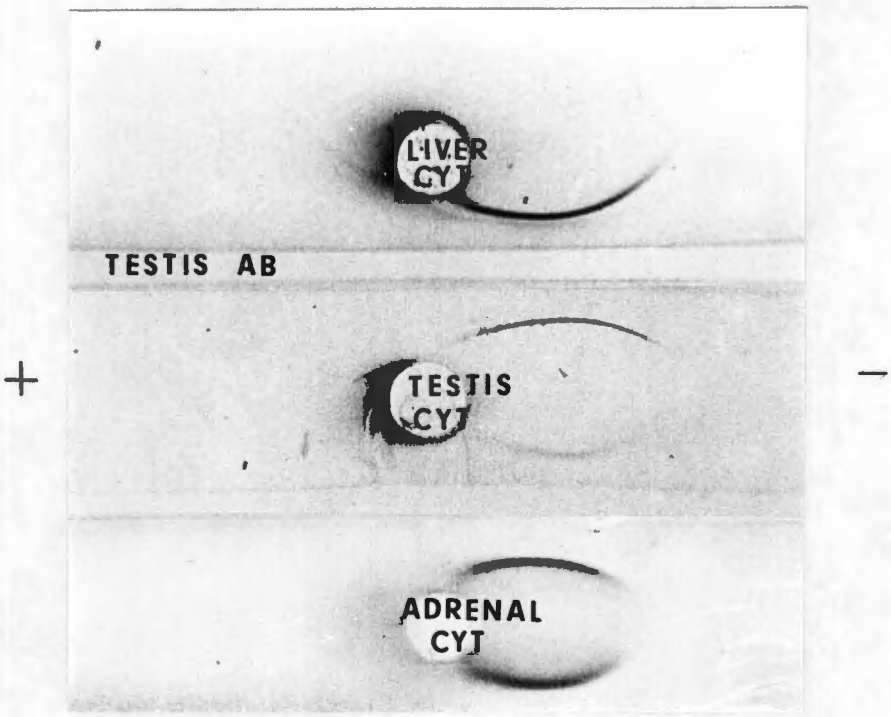


Fig. 2.6 **Immunoelectrophoresis of testis ligandin antibody against liver, testis and adrenal cytosols in 1 % agar gel.** The wells contain liver cytosol, testis cytosol and adrenal cytosol (1ug in 10ul). The troughs each contain 10ul of rabbit antiserum to testis ligandin. Electrophoresis was performed in 0.07M veronal buffer, pH 8.7, at 12 mA for 2 h. The precipitates were stained with Amido Black.

Affinity Chromatography Purification Procedures

(i) Anti-liver Ligandin Immunoaffinity Chromatography

The profile obtained when the testis protein peak (TEAE-cellulose eluate) was applied to an anti-liver YaYa ligandin affinity column is shown in Fig. 2.7. Fractions were analysed by SDS-PAGE (Fig. 2.8). The starting material and TEAE-cellulose eluate had more Yc subunit than Ya. In contrast, the material recognised by the matrix-coupled anti-liver ligandin IgG (elution volume 85ml) had equal quantities of Ya and Yc. Unbound fractions (elution volume 15 ml) contained no Ya subunit, while later fractions (elution volume 30ml) contained only traces of this species.

(ii) Anti-Testis Ligandin Immunoaffinity Chromatography

An affinity column of anti-testis ligandin antibody was prepared in a similar manner to the anti-liver ligandin affinity column, and the same testis starting preparation was applied to the column. SDS-PAGE (Fig. 2.9) revealed more Yc than Ya in the immunoreactive peak (elution volume 85ml) suggesting that the anti-testis ligandin antibody recognised more of the Yc subunit than the anti-liver ligandin antibody.

(iii) Thiol-Sepharose Affinity Chromatography

Liver ligandin prepared according to the standard procedure from phenobarbital pre-treated rats was applied to a Thiol-Sepharose column. Unbound material consisted of the YaYa dimer on SDS-PAGE (Fig. 2.10 - lane 1) while bound fractions obtained after elution

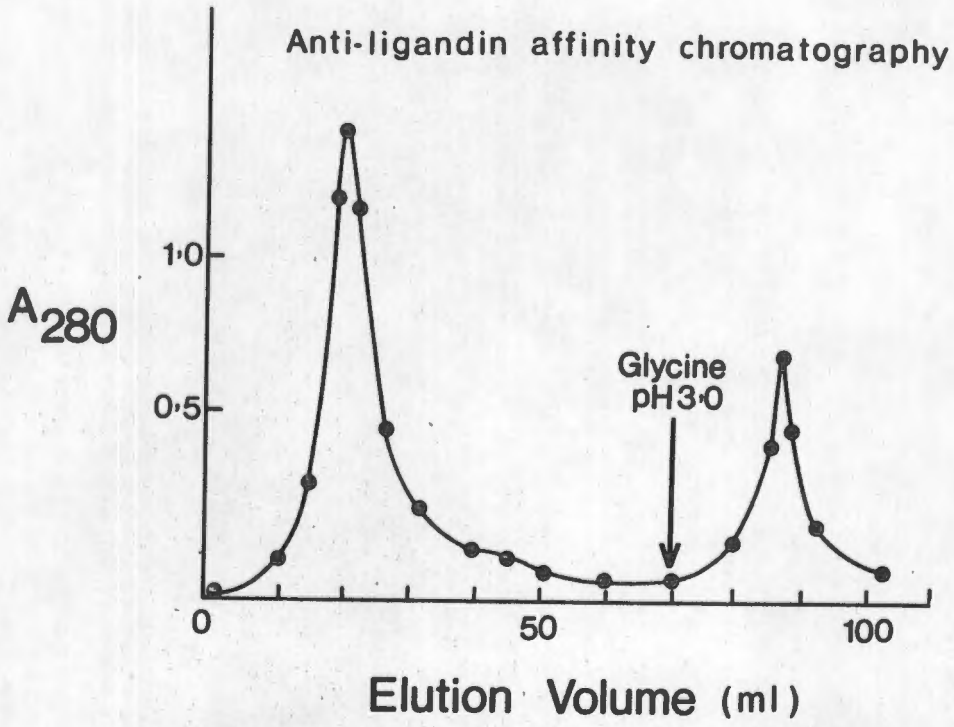


Fig. 2.7 Chromatography of partially purified (TEAE-eluate) rat testis ligandin on anti-ligandin Sepharose 4B affinity columns. The elution profiles for anti-liver ligandin and anti-testis ligandin affinity columns are identical. Unbound material eluted in the first peak (elution volume 10 - 40ml). Bound material was eluted with 0.1M glycine pH 3,0 (elution volume 80 - 95ml).

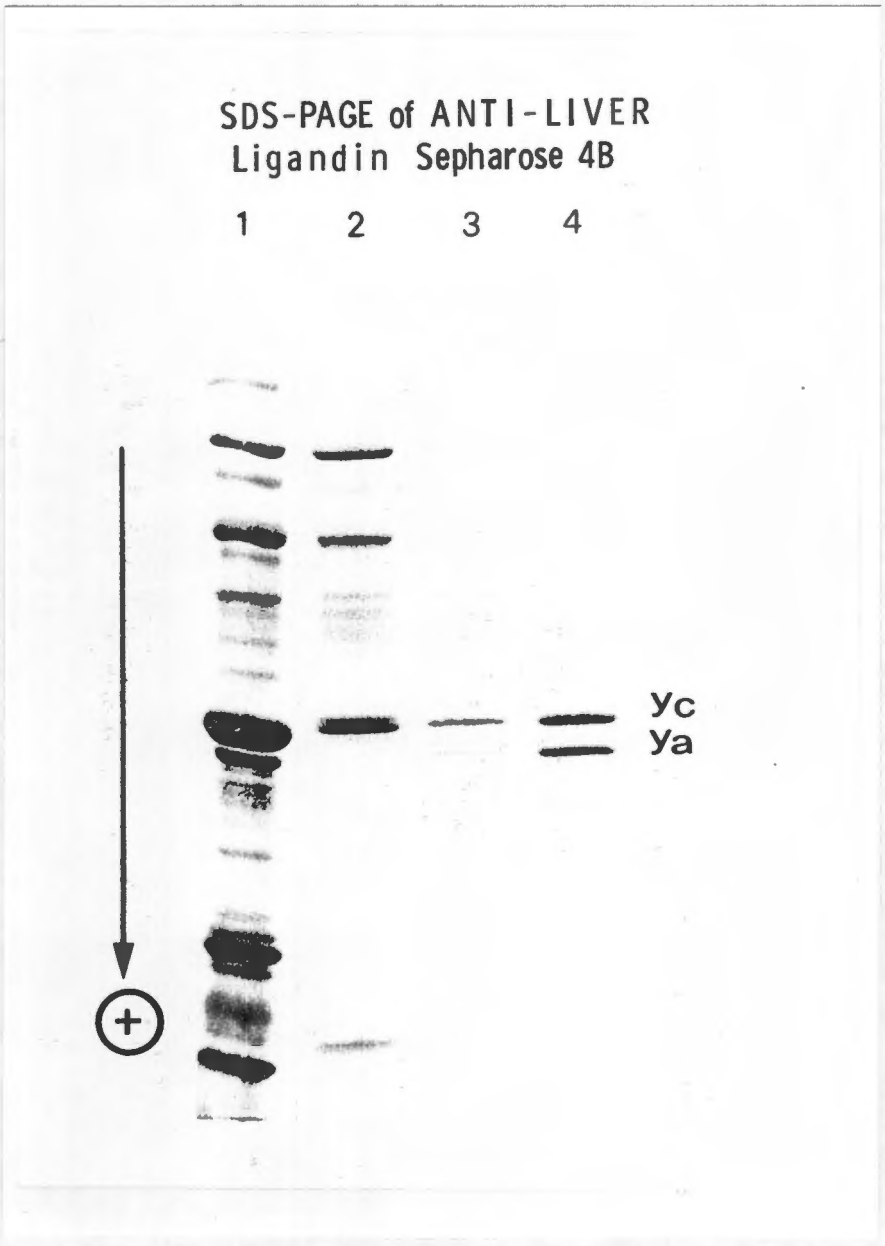


Fig. 2.8 SDS-PAGE of peaks eluted after anti-liver ligandin Sepharose 4B affinity chromatography. (1) testis TEAE-eluate (starting material), (2) material at elution volume 15ml, (3) material at elution volume 30 ml, (4) material after elution with 0.1M glycine pH 3.0. See Fig. 2.7 for details.

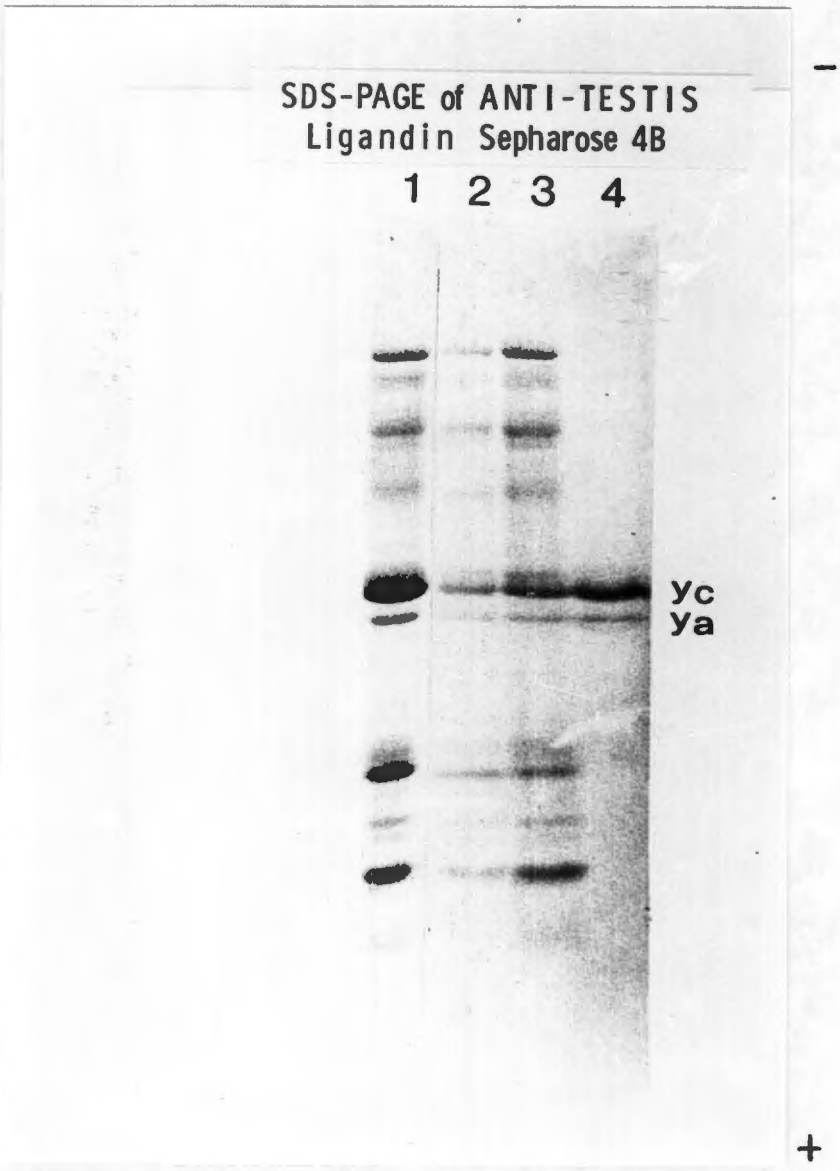


Fig. 2.9 SDS-PAGE of eluate from anti-testis ligandin Sepharose 4B affinity column. (1) testis TEAE-eluate (starting material), (2) material eluting at 15 ml, (3) material eluting at 25 ml, (4) material eluted after application of 0.1M glycine buffer pH 3.0 (elution volume 80 - 95 ml). See Fig. 2.7 for details.

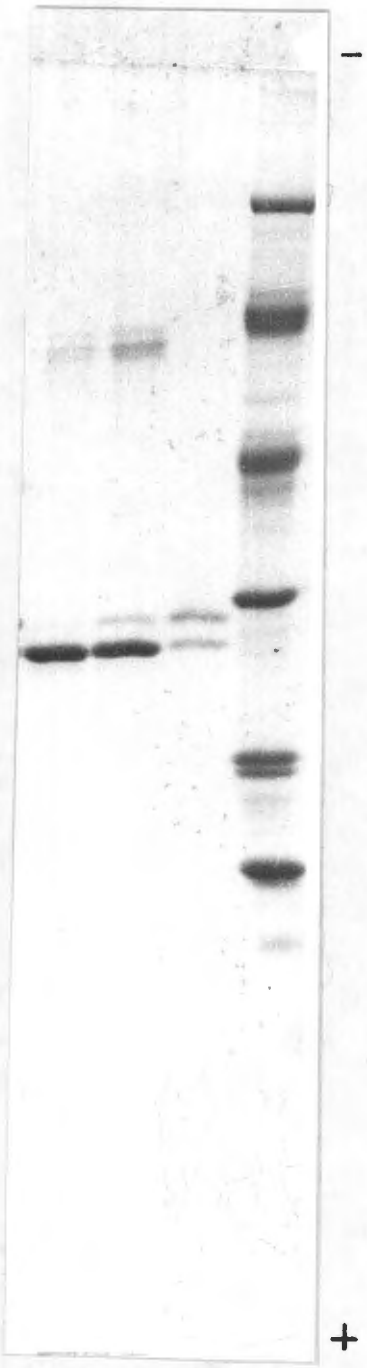


Fig. 2.10. **SDS-PAGE of eluate from a Thiol-Sepharose affinity column.** Lanes, (2) liver YaYc ligandin from phenobarbital pretreated rats (starting material), (1) liver YaYa ligandin homodimer, material which did not bind to the column, (3) liver YaYc ligandin heterodimer, eluted after application of L-cysteine buffer, (4) Molecular weight markers (see Chapter 2, Section A, Methods and Results for details).

with L-cysteine contained equal amounts of Ya and Yc (lane 3). The starting material contained more Ya than Yc (lane 2). Appearance of aggregrates, molecular weight between 62000 and 66000, was noted. Separation of testis ligandin subunits could not be achieved in this way (see Discussion).

(iv) Hexylglutathione Affinity Chromatography

Fig. 2.11 shows the elution profile of testis cytosol on an hexylglutathione affinity column. The first peak (unbound material) displayed relatively little enzyme activity with CDNB as substrate, while the second peak which bound to the column, contained much higher levels of GSH S-transferase activity. SDS-PAGE analysis of this material revealed the presence of Ya, Yb and Yc (Fig. 2.21).

COMPARISON OF RAT TESTIS LIGANDIN WITH OVARY AND ADRENAL LIGANDIN

The subunit composition of ligandin purified from rat ovary and adrenal tissue was investigated and comparisons made on SDS-PAGE with testis ligandin subunits. The same procedure described for testis ligandin was used to isolate ligandin from rat ovaries and adrenals. The protein preparations obtained were compared on SDS-PAGE with testis ligandin preparations (Fig. 2.12). The Yc subunit species of both ovary and adrenal preparations were predominant. Aggregates between 62000 and 66000 daltons were noted for all three preparations.

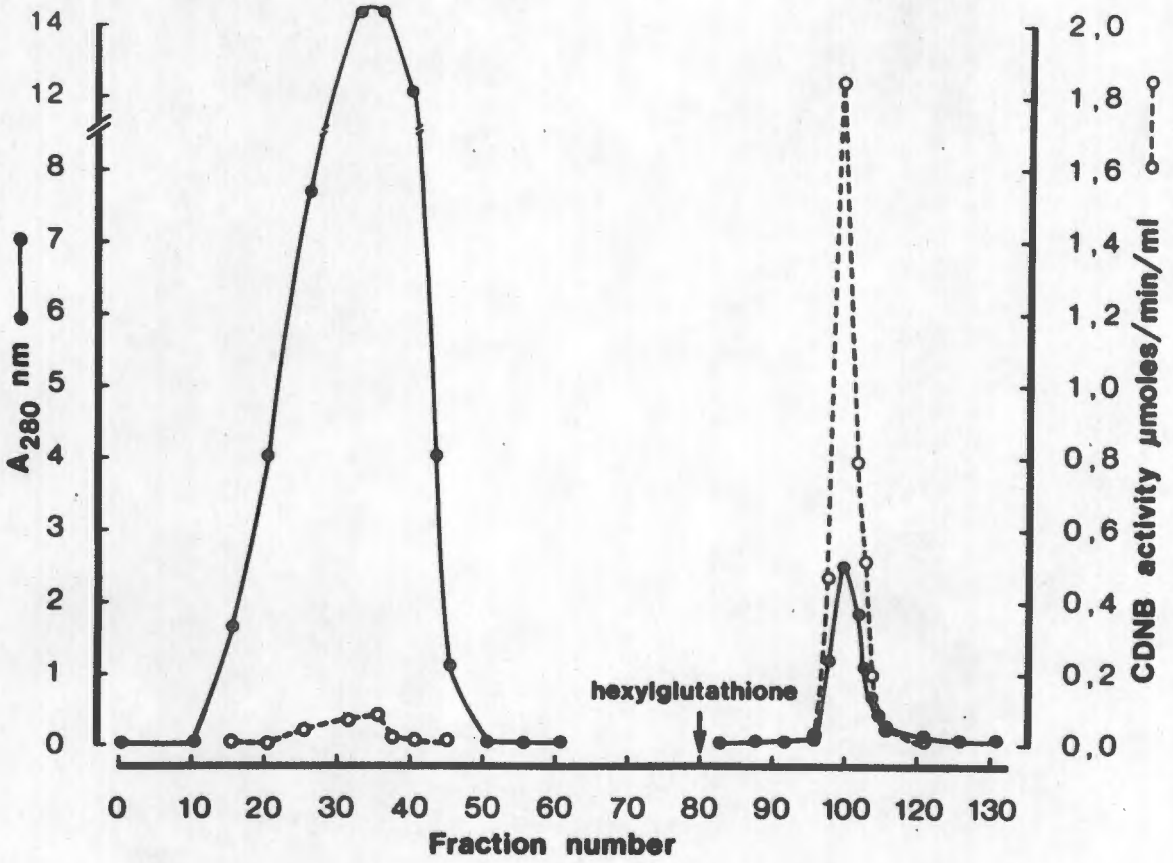


Fig. 2.11 **Chromatography of rat testis cytosol (Sephadex G-25 eluate) on hexylglutathione affinity columns.** Unbound material eluted in the first peak (elution volume 10-50ml). Bound material was eluted with 0.005M hexylglutathione (elution volume 96-118ml).

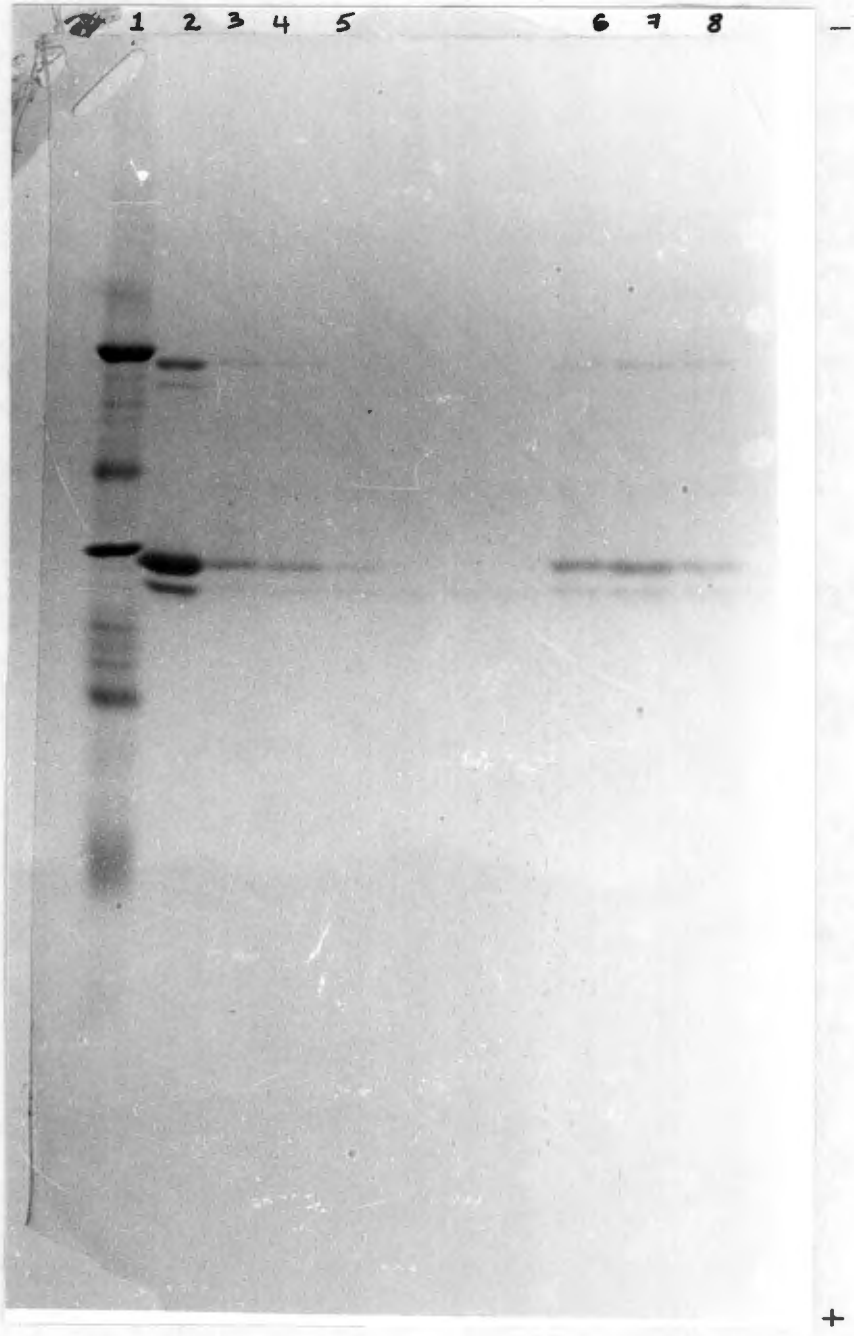


Fig. 2.12 **SDS-PAGE of rat adrenal and ovary ligandin, purified according to the standard procedure.** Lanes (1) molecular weight markers, (2) testis ligandin, (3), (4) and (5) adrenal ligandin (6), (7) and (8) ovarian ligandin. See Chapter 2, Section A, Methods and Results, for details. 8, 5 and 3 ug of protein were loaded in lanes (3), (4) and (5) respectively, as well as in lanes (6), (7) and (8).

Both ovary and adrenal ligandin exhibited GSH S-transferase activity with CDNB as a substrate. Due to the low concentration of ligandin in these tissues, the amount of protein recovered from these tissues was usually less than 0.1mg/ml.

COMPARISON OF HUMAN TESTIS LIGANDIN WITH HUMAN LIVER LIGANDIN

In order to determine whether the subunit composition of human testis ligandin differed from that found in human liver. Ligandin was isolated from the testis and liver of a 40 year-old cadaver, following the standard purification procedure. The elution profiles from the chromatographic steps are shown in Fig. 2.14.

The purification technique used was similar to that for the rat except that the pH of the 0.01M Tris.HCl buffer was 8.67 instead of 8.8. DEAE-cellulose chromatography of the cytosol resulted in a broad peak of enzyme activity. After chromatography on Sephadex G-100, the CDNB-GSH conjugating fractions were applied to a QAE-Sephadex A.50 column which yielded a single peak of enzyme activity. Comparison of purified human testis and human liver ligandin by means of SDS-PAGE showed that both consisted of identical subunits with molecular weights of 23500 (Fig. 2.15).

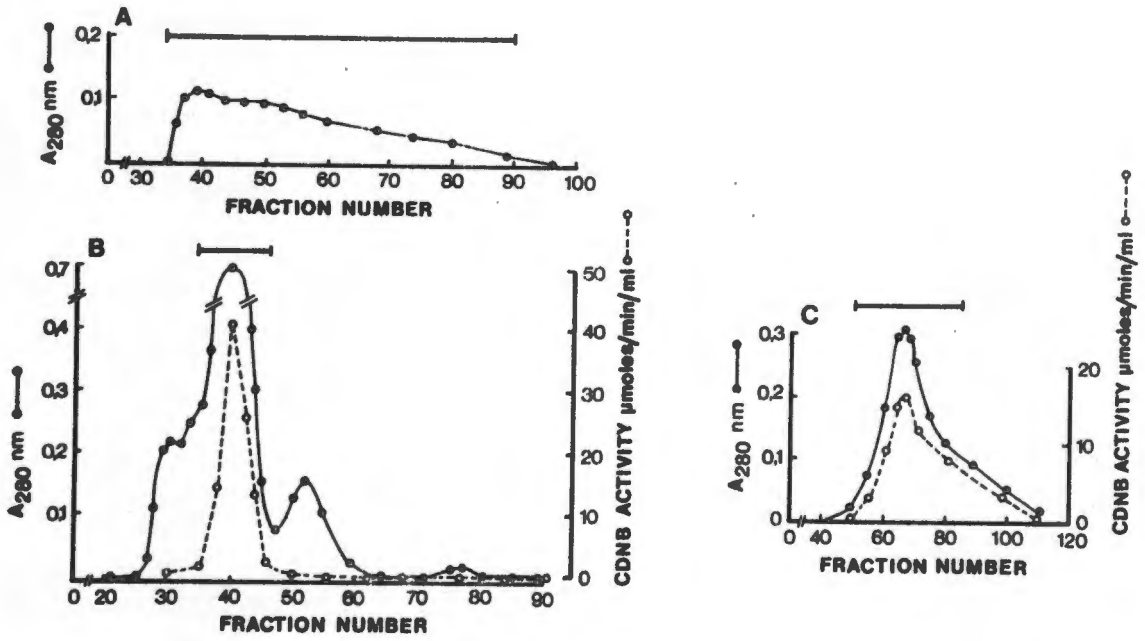


Fig. 2.14 **Purification of ligandin from the 100000 g supernatant of human testis.** (A) Elution of protein from DEAE-cellulose in 0.01M Tris.HC1 buffer pH 8.67. (B) Elution of protein from Sephadex G-100 in 0.01M sodium phosphate buffer pH 7.4 / 0.1M NaCl. (C) Purification of the CDNB-enzyme active peak on QAE-Sephadex A-50 in 0.01M Tris.HCl buffer pH 8.67. Fractions pooled at each stage of purification are indicated by the horizontal bars (see Methods and Results for details).

Fig. 2.15 **SDS-PAGE at each stage of human testis ligandin purification.** (1) Rat liver ligandin, (2) rat testis ligandin, (3) human testis cytosol (10000 g supernatant), (4) protein peak after DEAE-cellulose chromatography, (5) peak exhibiting GSH-CDNB conjugating activity after Sephadex G-100 chromatography, (6) and (8) testis protein peak after QAE-Sephadex A-50 chromatography, (7) human liver ligandin. Samples were loaded and prepared as described in Fig. 2.3.

THE DIFFERENT GSH S-TRANSFERASE FORMS OF RAT LIVER, TESTIS AND OVARY

The finding that ligandin from the steroid-producing tissues consists predominantly of the Yc subunit remains unexplained. The possibility that a substance other than ligandin may contribute to this electrophoretic finding and to the differences in function attributed to testis ligandin was investigated. The other GSH S-transferases are closely related to ligandin in the rat liver, with similar molecular weights and overlapping substrate specificities. Therefore, these proteins were the most likely to provide a contaminating factor during purification. Prior to this study, the different GSH S-transferase forms which might be present in the testis, ovary and adrenal gland had not been investigated.

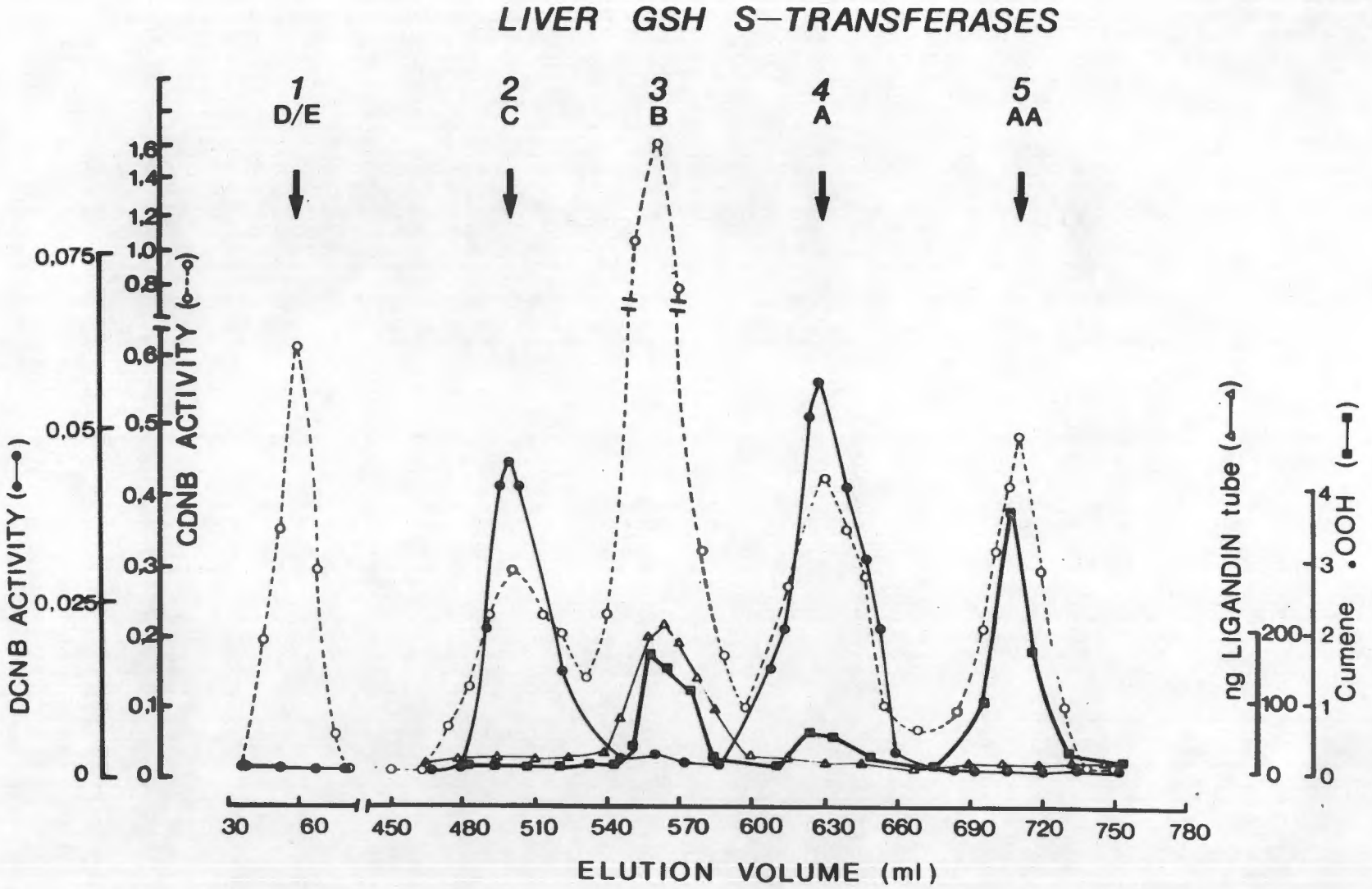
The different GSH S-transferase and GSH peroxidase II forms found in testis and ovary will be detailed in this section. In order to compare the different testis and ovary GSH S-transferases with the GSH S-transferase forms found in liver, a similar procedure, described by Habig **et al** (1974b) was adhered to.

Evidence is presented here which suggests that testis ligandin may be contaminated with another GSH S-transferase, namely GSH S-transferase AA.

Elution of Rat Liver GSH S-transferases from CM-cellulose

CM-cellulose chromatography resolved liver DEAE-cellulose eluate into five peaks of GSH S-transferase activity (GSH-CDNB conjugating activity) similar to those described by Habig **et al** (1976). Fig. 2.16 shows the peaks obtained. Peak 1 (elution volume

Fig. 2.16 **Elution pattern from CM-cellulose of liver DEAE-cellulose eluate at pH 6.7.** GSH S-transferase activity was assayed with 1-chloro-2.4-dinitrobenzene (CDNB,O) and 1.2-dichloro-4-nitrobenzene (DCNB, ●). Enzyme activity is expressed as umol/min per ml. GSH peroxidase II activity was assayed with cumene hydroperoxide (cumene.00H, ■) as substrate. Results were expressed as umol of cumene.OOH/min, by using the absorbtion coefficient (ε) for NADPH of 6 x 10^3 litre.mol^{-1}.cm^{-1}. Ligandin as measured by anti(liver ligandin) radioimmunoassay is expressed as ng of ligandin/tube (Δ). The KC1 gradient was started after peak 1 had been eluted (elution volume 0-180ml).

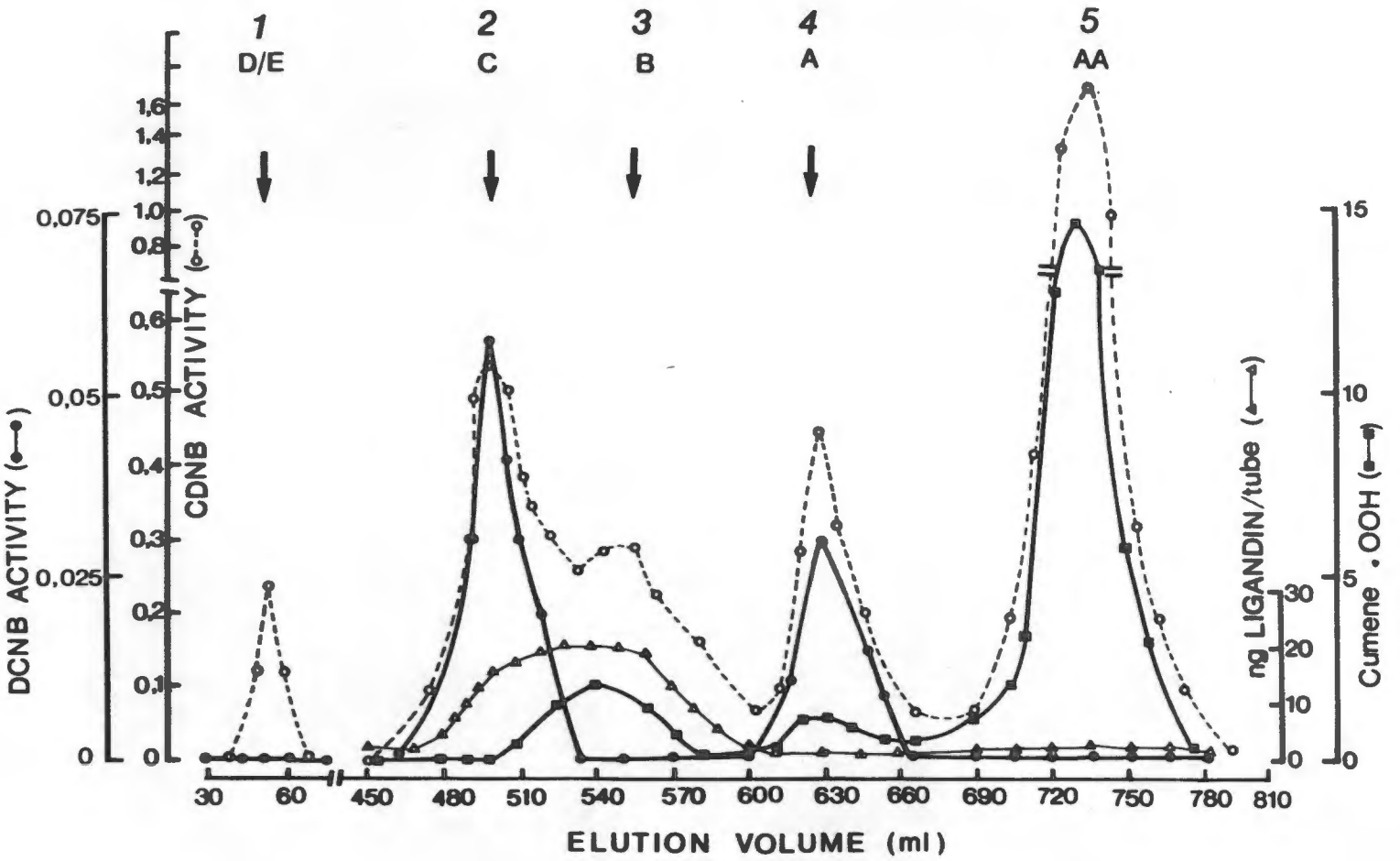

LIVER GSH S-TRANSFERASES
1
D/E
2
C
3
B
4
A
5
AA
DCNB ACTIVITY (●—●)
CDNB ACTIVITY (o--o)
0,075
0,05
0,025
0
1,6
1,4
1,2
1,0
0,8
0,6
0,5
0,4
0,3
0,2
0,1
0
ng LIGANDIN tube (o—o)
Cumene •OOH (■—■)
200
100
0
4
3
2
1
0
30
60
450
480
510
540
570
600
630
660
690
720
750
780
ELUTION VOLUME (ml)

32-70ml) represents material that did not bind to the column at pH 6.7. Following initiation of the salt gradient (0-120mM KCl) a further four peaks were obtained; peak 2 (elution volume 465-530ml), peak 3 (530-600ml), peak 4 (600-660ml) and peak 5 (680-735ml). Only peaks 2 and 4 had GSH-DCNB conjugating activity. GSH peroxidase II activity with cumene.OOH as substrate was present in peak 5 and to a lesser extent in peaks 3 and 4. GSH peroxidase I activities were not measured in these experiments. Immunoreactivity determined by radioimmunoassay with antibody raised against liver ligandin was limited to peak 3. Details of the radioimmunoassay procedure will be presented in Section B of Chapter 2.

Using the terminology of Jakoby **et al** (1976) based on elution volume from CM-cellulose and substrate specificities, peaks 1, 2, 3, 4 and 5 are believed to be GSH S-transferase D and E, C, B, A and AA respectively. Hayes **et al** (1980) have determined the monomer composition of rat liver GSH S-transferases eluted from CM-Sephadex by means of SDS-PAGE. Transferases D and E contain proteins migrating as Yc and Yb, transferases A and C subunits migrated as Yb, transferase B as both Ya and Yc, while transferase AA migrate as Yc only. SDS-PAGE analysis of the peaks eluting from CM-cellulose revealed similar subunit compositions (results not shown).

Elution of Rat Testis GSH S-transferases from CM-cellulose Fig. 2.17 shows the results of a similar experiment with testis DEAE-cellulose eluate. Peak 1 (elution volume 40-50ml) represents GSH-CDNB conjugating activity that does not bind to the column at pH 6.7. Four peaks of GSH-CDNB conjugating activity were obtained

Fig. 2.17 **Elution pattern from CM-cellulose of testis DEAE-eluate at pH 6.7.** Details are the same as described in Fig. 2.16.


TESTIS GSH S–TRANSFERASES
1
D/E
2
C
3
B
4
A
5
AA
DCNB ACTIVITY (●—●)
CDNB ACTIVITY (○---○)
ng LIGANDIN/tube (▲—▲)
Cumene • OOH (■—■)
0,075
0,05
0,025
0
1,6
1,4
1,2
1,0
0,8
0,6
0,5
0,4
0,3
0,2
0,1
0
15
10
5
0
30
20
10
0
30
60
450
480
510
540
570
600
630
660
690
720
750
780
810
ELUTION VOLUME (ml)

after initiation of the salt gradient; peak 2 (elution volume 460-530ml), peak 3 (530-600ml), peak 4 (600-660ml) and peak 5 (690-780ml). DCNB activity was limited to peaks 2 and 4. GSH peroxidase II activity (cumene.00H as substrate) was present largely in peak 5, with additional activity measured in peaks 3 and 4. Immunoreactivity determined by radioimmunoassay using anti-liver YaYa ligandin was present in fractions eluted between 480 and 600ml. No significant immunoreactivity was detected in peak 5. SDS-PAGE of the immunoreactive peak revealed equal amounts of the Ya and Yc subunits. Peak 5 contained a major band in the Yc region.

Chromatography of 'Purified' Testis Ligandin on CM-cellulose

Chromatography of 'purified' testis ligandin yielded two peaks of GSH-CDNB conjugating activity. Only peak 1 contained immunoreactive material as determined by radioimmunoassay by using antiserum raised against liver YaYa ligandin. SDS-PAGE of peak 1 material revealed equal amounts of Ya and Yc subunits; peak 2 consisted entirely of Yc subunit (Fig. 2.18).

Elution of Rat Ovary GSH S-transferases from CM-cellulose

CM-cellulose chromatography of 100000 g supernatant fraction of rat ovary could be resolved into 4 peaks and a shoulder of GSH-CDNB conjugating activity (Fig. 2.19). Peak 1 (elution volume 8-25ml) had considerable GSH-CDNB conjugating activity and represents material that does not bind to the column at pH 6.7. Following initiation of the salt gradient (0-120mM KCl) peak 2 eluted as a shoulder of peak 3 (total elution volumes 122-142ml), peak 4 eluted between 142 and 165ml, and peak 5 eluted between 165 and 190ml. GSH-DCNB conjugating activity was present in fractions eluting

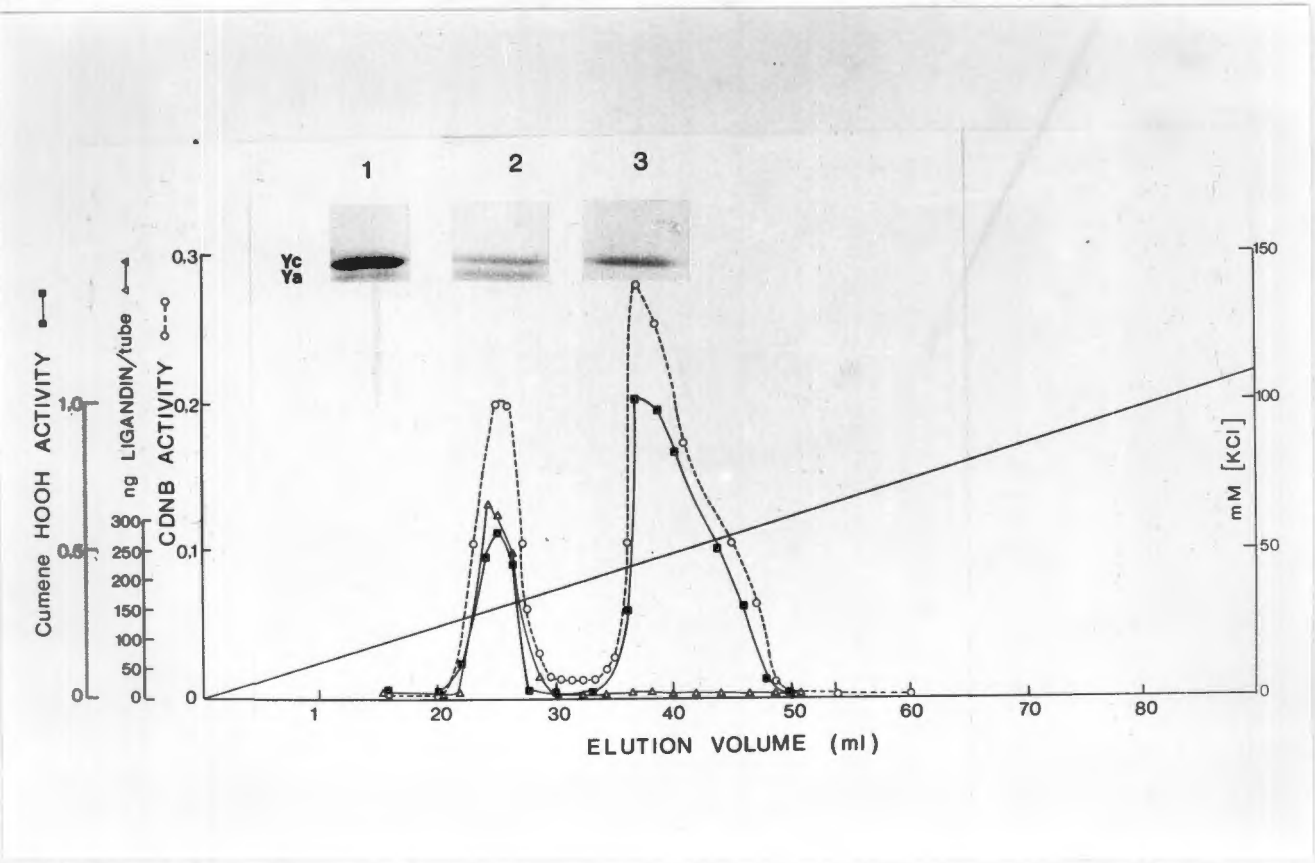
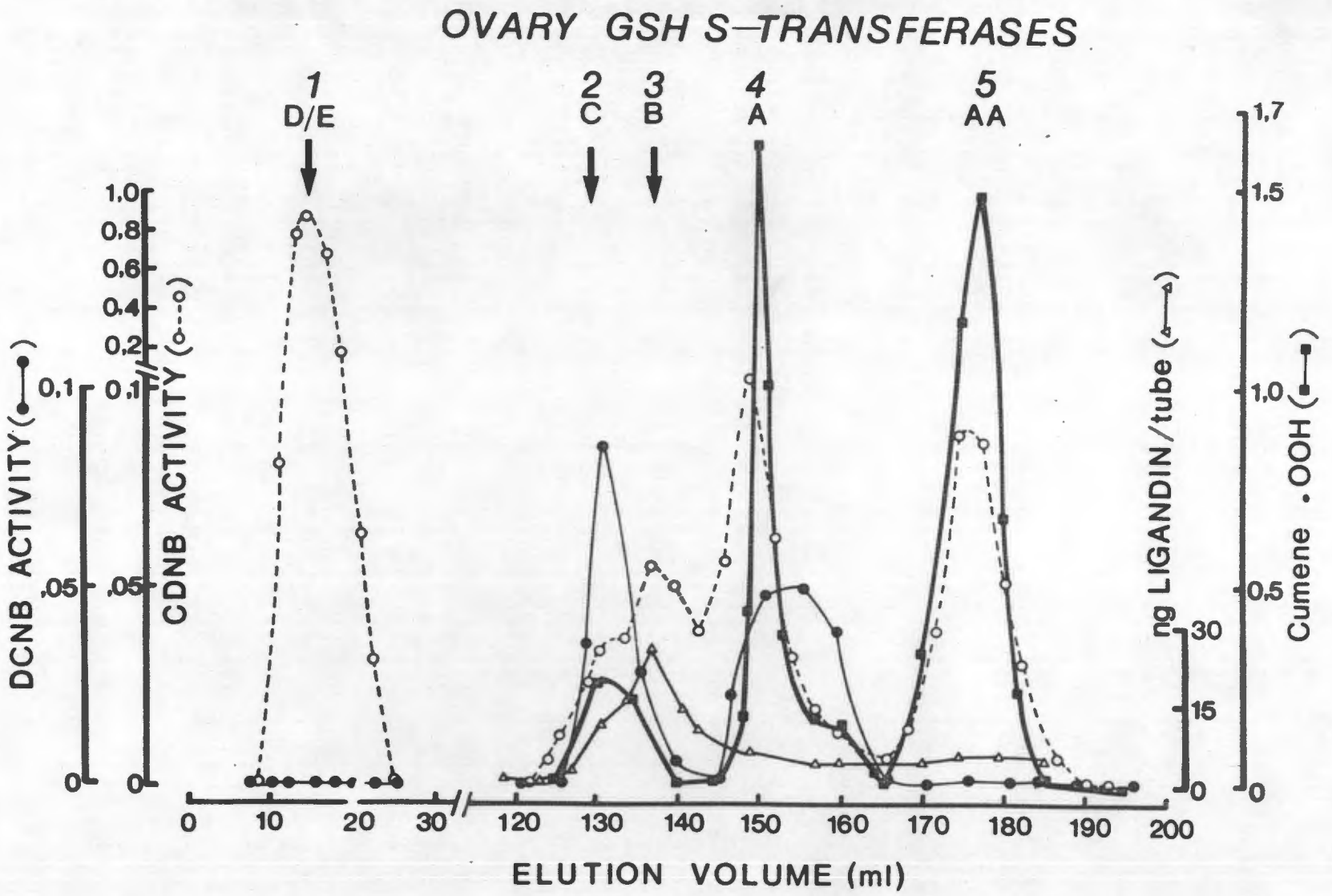


Fig. 2.18 **CM-cellulose chromatography of "pure" testis ligandin at pH 6.7.** The KCl gradient (0 - 120 mM) was started at the beginning of the column run. GSH S-transferase activity was assayed with CDNB (O----O) and expressed as umoles/min/ml. GSH peroxidase II activity was assayed with cumene hydroperoxide (cumene.OOH) and expressed as umoles/min (■—■). Radioimmunoassay with anti-liver ligandin measured ng ligandin/tube (△——△). Comparisons of the standard preparation of testis ligandin (1), and fractions from each peak (2), (3), on SDS-PAGE is shown.

Fig. 2.19 **Elution pattern from CM-cellulose of rat ovary cytosol at pH 6.7.** Details are the same as described in Fig. 2.16.


OVARY GSH S–TRANSFERASES
1
D/E
2
C
3
B
4
A
5
AA
DCNB ACTIVITY (●—●)
CDNB ACTIVITY (○---○)
ng LIGANDIN/tube (△—△)
Cumene • OOH (■—■)
ELUTION VOLUME (ml)

between 127 and 140ml and between 145 and 165ml.

GSH peroxidase II activity (cumene.00H as substrate) was measured in peaks 4 and 5 and to a lesser extent in fractions eluting between 126 and 140ml. Immunoreactive material (measured with anti-liver YaYa ligandin radioimmunoassay) was present in peak 3 (elution volume 130-145ml). SDS-PAGE analysis of the immunoreactive peak revealed 2 bands consisting of equal amounts of Ya and Yc subunits. SDS-PAGE of peak 5 revealed a band in the Yc region.

Chromatofocusing of Testis Hexylglutathione Material

Re-chromatography of testis hexylglutathione material (Fig. 2.11, peak 2) on a chromatofocusing column resulted in the elution of several peaks exhibiting CDNB-GSH conjugating activity as shown in Fig. 2.20. The first peak (eluting between fractions 15 and 22) had an isoelectric point of approximately 8.5 and consisted of both Ya and Yc subunits on SDS-PAGE, with Yc present in greater quantities (Fig. 2.21). This peak ehibited GSH peroxidase activity with cumene.OOH as substrate. The Ya and Yc subunits on SDS-PAGE diminished as the isoelectric point of the fractions decreased. A band with molecular weight corresponding to that of Yb was present in these fractions.

The chromatofocusing polybuffer used here stains with Coommassie Blue on the SDS-polyacrylamide gels. Several washes with 10% TCA before staining will remove most of the polybuffer, except for areas at the front of the gel where the acrylamide concentration is between 20 and 25% (Fig. 2.21).

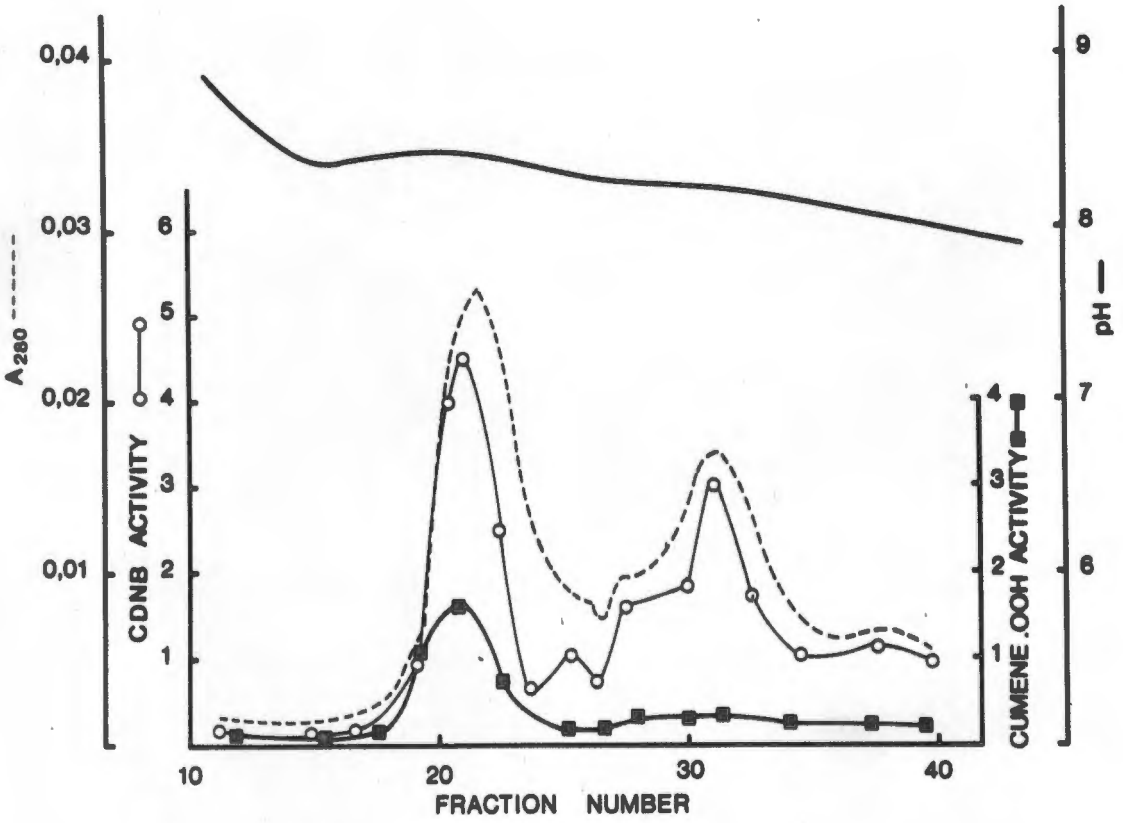


Fig. 2.20 **Separation of the basic testis GSH S-transferases by chromatofocusing.** Hexylglutathione-bound material from testis cytosol was eluted with a pH gradient, pH 9.4-8.0. GSH S-transferase activity was assayed with CDNB as substrate (O) and was expressed as umol/min/ml. GSH peroxidase II activity was assayed with cumene.OOH as substrate (■), with the results expressed as umol/min.

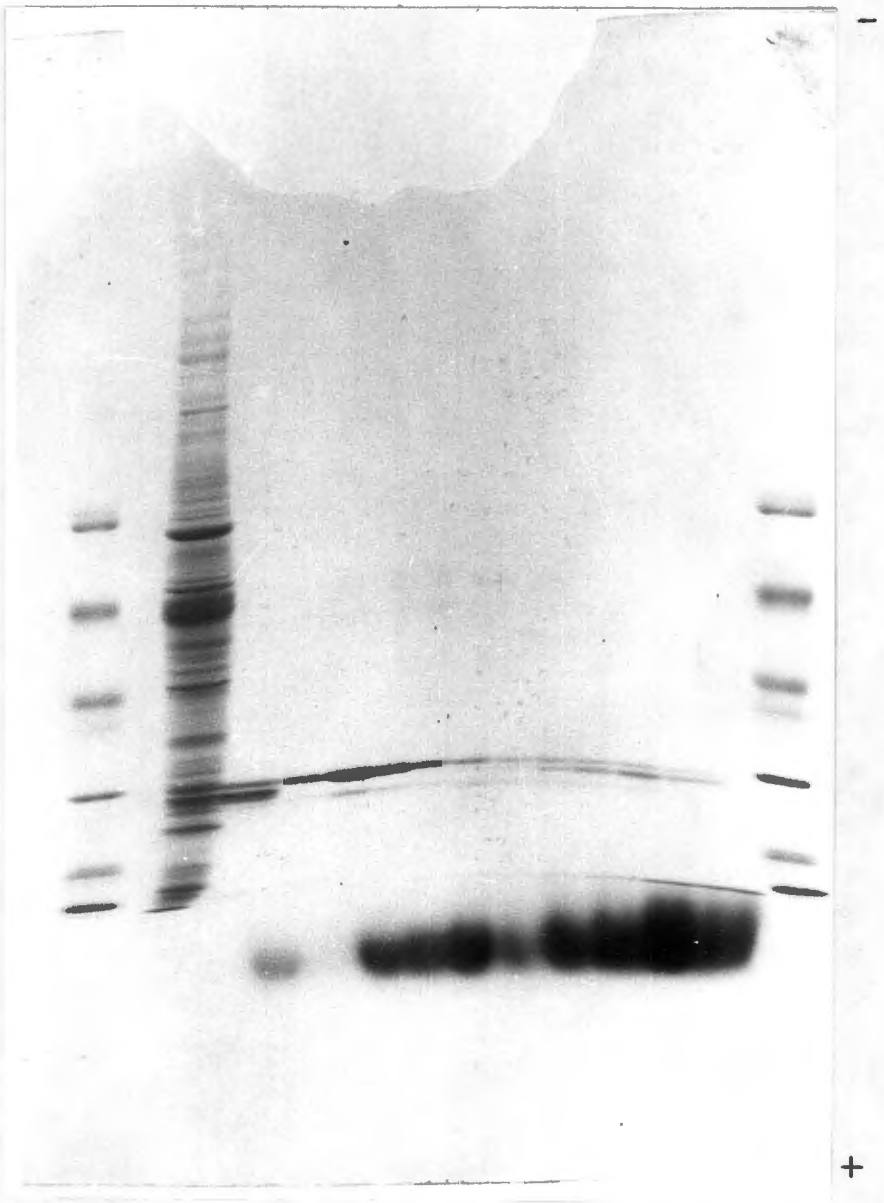


Fig. 2.21 **SDS-PAGE of the basic testis GSH S-transferases isolated by means of chromatofocusing.** Lanes; (2), testis cytosol before hexylglutathione affinity chromatography (Fig 2.11, peak 2), (4) to (14), fractions 19, 21, 23, 25, 28, 30, 32, 34 and 38 eluting from the chromatofocusing column (Fig. 2.20). (1) and (15) are the molecular weight markers.

(e) Discussion

Liver ligandin, as defined by Bass **et al** (1977a), consists of equal amounts of Ya and Yc. In contrast the Yc subunit accounts for as much as 90% of testis ligandin (Bhargava **et al** 1980a, Bass **et al** 1977a). Using the standard purification procedure, a protein with subunit structure, amino acid composition, binding characteristics and GSH S-transferase activity similar to that described by Bhargava **et al** (1980a) was purified. These workers found testis ligandin to have a lower affinity for bilirubin and BSP than liver. Circular dichroism spectra revealed absence of the primary binding site for bilirubin (Bhargava **et al** 1980a).

When testis ligandin was prepared using affinity chromatography with anti-liver YaYa antiserum, it consisted of equal amounts of Ya and Yc. This was clearly different to testis preparations obtained by the standard purification procedure. The testis starting material contained more Yc than Ya (Fig. 2.8, lane 1). A considerable amount of Yc was eluted in the wash (lanes 2 and 3), suggesting that this may not have been recognised by the antibody. The immunoreactive material eluted using glycine buffer pH 3.0 consisted of equal amounts of Yc and Ya (lane 4) resembling the proportions of these subunits found in liver ligandin. In addition antibodies raised against testis ligandin prepared by standard methods did not meet immunodiffusion and immunoelectrophoretic criteria for purity when tested against liver cytosol. Furthermore, SDS-PAGE of covalently cross-linked testis ligandin showed more than one band in the 50000 dalton region.

This finding differed from that reported by Bhargava **et al** (1978a) who noted only one band on SDS-PAGE for DMS cross-linked liver ligandin. Therefore another subunit combination in DMS cross-linked testis ligandin preparations exists, differing from that obtained with liver ligandin. This may be due to the presence of an additional protein in standard testis preparations.

The view that standard preparations of testis ligandin could be contaminated by a closely related protein was strengthened by the findings of Hayes **et al** (1980) and Scully and Mantle (1980) who studied the subunit composition of liver GSH S-transferases. The GSH S-transferases of rat liver (AA, A, B, C, D and E) have been classified according to their reverse order of elution from CM-cellulose (Habig **et al** 1976). Transferases D and E elute before the salt gradient is applied after which transferase C, B, A, and AA elute sequentially. Whilst there is overlap of substrate specificity the various tranferases may be identified by their order of elution from CM-cellulose and their ability to conjugate GSH to various substrates, they all conjugate CDNB. Only A and C conjugate GSH to DCNB (Habig **et al** 1974b), while GSH S-transferase B, A and AA have GSH peroxidase activity (Prohaska and Ganther 1977b, Lawrence **et al** 1978, Irwin **et al** 1980). On SDS-PAGE liver GSH S-transferase AA migrated as Yc, liver transferase B migrated as YaYc, while transferases A and C, which show immunological identity (Habig **et al** 1974b), migrated as Yb. This finding led Hayes **et al** (1979) to differentiate between GSH S-transferase B (YaYc) and ligandin (YaYa). Since this study has been aimed at determining the subunit composition of a protein in testis which reacts immunologically with anti-liver ligandin, the term ligandin has been used throughout, although in

the light of these findings the term GSH S-transferase B may be more appropriate.

Purified ligandin (following the standard procedure, Fig.1.6) from rat ovaries and adrenals consisted of more Yc than Ya on SDS-PAGE, similar to testis ligandin preparations. To determine whether testis and ovary GSH S-transferase AA migrated as Yc, the testis and ovary GSH S-transferases were separated by CM-cellulose chromatography and the order of elution, SDS-PAGE, radioimmunoassay and catalytic activity with different substrates were used to characterise the various fractions. CDNB and DCNB activity resembled that seen in liver. GSH peroxidase activity was found in peaks corresponding to liver GSH S-transferases B, A and AA. Of interest is the fact that transferase AA acounts for most of the GSH peroxidase II activity in testis cytosol. SDS-PAGE of the testis and ovary CM-cellulose peak with the elution characteristics of transferase AA revealed a major band in the Yc region, similar to that reported by Scully and Mantle (1981) for testis preparations. This peak was not recognised by antibody raised against liver YaYa ligandin. Immunoreactivity was confined to fractions eluting in the GSH S-transferase B region of the CM-cellulose profile. On SDS-PAGE this peak consisted of equal amounts of Ya and Yc. GSH S-transferases from adrenal cytosol exhibited very low activities with all the substrates tested. Thus similar CM-cellulose profiles from this tissues could not be obtained.

It was apparent that if GSH S-transferase AA was present in standard preparations of ligandin it would not be detected on SDS-PAGE. CM-cellulose chromatography was therefore used to study

"pure" testis ligandin and it was found to consist of GSH S-transferase B (equal amounts of Ya and Yc, recognised by antibody raised against liver YaYa) and GSH S-transferase AA (which resembles Yc on SDS-PAGE). The finding that standard preparations of testis ligandin are contaminated by GSH S-transferase AA offers a new explanation for studies previously reported and interpreted differently.

Difference spectroscopy data showed bilirubin binding with standard testis preparations. This may be due to the Ya subunit. High affinity binding for bilirubin (Bhargava **et al** 1978b) and for cholic acid (Hayes **et al** 1981) has been associated with Ya. GSH S-transferase AA (YcYc) has a very low affinity for bilirubin (Ketley **et al** 1975) and its presence in these testis preparations would not contribute to binding.

S-hexylglutathione affinity chromatography, a technique described by Guthenberg and Mannervik (1979) for preparing GSH S-transferases of rat liver and lung, was used to investigate the different enzyme forms of the testis GSH S-transferases. Material from testis cytosol which bound to this column was shown to consist of several bands on SDS-PAGE migrating between 25000 and 22000 daltons molecular weight. As very little enzyme activity eluted with the unbound material, most of the testis GSH S-transferase forms should be present in the bound peak (Fig. 2.11). Separation of these enzyme forms can be achieved by hydroxyapatite chromatography, or by isoelectric focusing. These methods do not provide satisfactory separation of the different enzyme forms. Application of a newly described technique, chromatofocusing (a combination of ion-exchange chromatography and isoelectric focusing) (Sluyterman and Elgersma 1978), resulted in several peaks of CDNB-GSH conjugating

activity. The first, most basic peak eluting from the chromatofocusing column displayed GSH peroxidase activity with cumene hydroperoxide as substrate. SDS-PAGE analysis of the peaks eluting from the chromatofocusing column showed a combination of the 3 subunits Ya, Yb and Yc in differing amounts. The Yc subunit predominated in fractions which displayed the highest isoelectric point, with Yb present at lower isoelectric points. It is interesting to note that the YaYc composition of the first peak resembled that obtained by standard purification procedures (Yc greater than Ya) previously defined as 'testis ligandin'.

Further characterisation of these peaks would be required in order to establish identity with respect to Habig's classification of the GSH S-transferases eluting from CM-cellulose (Habig **et al** 1976). Hayes **et al** (1979) used CM-Sephadex to separate the liver GSH S-transferase forms and found that order of elution differed. This new approach of chromatofocusing may reveal the presence of other previously undescribed forms of GSH S-transferases.

Thiol-Sepharose affinity chromatography achieved separation of the YaYa liver ligandin homodimer from the YaYc heterodimer. These species are separable by means of the cysteine residues which are present on the Yc subunit. Bhargava **et al** (1980b) reported similar findings. Testis ligandin (more Yc than Ya) could not be separated in this way as all the material bound to the column. There does not appear to be any evidence of a YaYa species in testis preparations.

During the course of this study other workers in the field have

made contributions which may have altered original ideas and concepts. It is now possible that homodimeric ligandin is another GSH S-transferase present in the liver in addition to heterodimeric GSH S-transferase B. The presence of this YaYa species in other tissues has not yet been demonstrated. Although Hayes **et al** (1981) using tryptic-digest peptide 'maps', have suggested that the Ya subunits of the homodimer (YaYa) and the heterodimer (YaYc) in liver are identical, it is possible that differences between the Ya subunits of other tissues exist. A similar argument can be used for the Yc subunits of GSH S-transferases AA and B. Differences occurring during preparation of liver ligandin using the standard purification procedure may well result in the co-purification of GSH S-transferase AA, as is the case in the testis.

Very recently Kitahara and Sato (1981) have shown that an immunological relationship exists between GSH S-transferase AA (YcYc) and GSH S-transferase B (YaYc). The anti-YaYc antiserum used by these workers recognised antigenic determinants on both YaYa and YcYc homodimers, while the anti-YaYa antiserum showed identity with only YaYa and YaYc. This finding would explain why immunoaffinity experiments (Chapter 2, Section A) using anti-YaYa ligandin only reacted with the YaYc species while YcYc washed through the column, and why the GSH S-transferase AA peak, eluting from CM-cellulose, showed no significant reaction with anti-YaYa ligandin antiserum used in the radioimmunoassay.

The question arose as to whether liver and testis ligandin from human tissues differed on SDS-PAGE following their purification according to the standard procedure. SDS-PAGE of material eluting from the final QAE-Sephadex chromatography step resulted in a

single band of molecular weight 23500, identical to that obtained with human liver ligandin (Fig. 2.15). Even though the amino acid composition, immunological characteristics and subunit molecular weights of the 5 cationic human liver ligandin species have been shown to be identical (Kamisaka **et al** 1975), they each exhibit differing substrate specificities (Table 1.7). Human liver and testis ligandin may differ in this way and it would be necessary to carry out further studies in order to completely characterise and compare these two preparations from differing sources.

Chromatography of testis cytosol on Sephadex G-75 results in an additional second large peak of GSH peroxidase II activity (Fig. 1.10). Although Prohaska and Ganther (1977b) suggested that the GSH S-transferases exhibit peroxidase activity, they did not identify which testis GSH S-transferases might be responsible. The results presented in Chapter 2, Section A indicate that GSH S-transferases AA, A and B have GSH peroxidase II activity (cumene hydroperoxide as substrate) in the testis with transferase AA contributing to the majority of this activity.

Although GSH peroxidase II activity is relatively high in both the ovaries and testis, the functional significance of these proteins in the gonads is unknown. It has been suggested that the GSH S-transferases play an important protective role in preventing damage to germ cells by xenobiotic compounds (Mukhtar **et al** 1978c), alternatively the enzyme may prevent damage caused by high concentrations of endogenous steroids (Bannikov and Tchipyseva 1978). The rat epididymis, site of spermatozoal maturation, as well as the vas deferens, display GSH S-transferase activity towards CDNB (Hales **et al** 1980) (see Table 1.5). The relative

activity of GSH S-transferase B was highest at the site of the epididymis-vas deferens where most spermatozoa gain their fertilizing capacity. Significant GSH S-transferase activity with CDNB, BPO and SO was also found in rat, mouse and human semen (Mukhtar **et al** 1978c). The hormonal regulation of these enzymes may thus be of key significance in understanding the process of spermatozoal maturation and fertilization as well as steroidogenesis.

SECTION B

DEVELOPMENTAL AND INDUCTION STUDIES OF LIGANDIN IN THE STEROIDOGENIC TISSUES OF THE RAT

(a) Introduction

Ligandin is present in high concentrations in the steroid synthesising tissues of the rat where it has been claimed to influence intracellular accumulation and efflux of steroid hormones and their metabolites (Smith and Litwack 1980). The developmental pattern of ligandin in these steroid producing tissues is therefore of interest, particularly during the neonatal and pubertal period.

In this study the emphasis has been placed on ligandin rather than the other GSH-transferases because of the availability of a sensitive and specific radioimmunoassay developed in our unit, and because we believed that a comparative study of the ontogeny of ligandin in steroidogenically active and non-steroidogenically active tissues might shed light on the role of this multifunctional protein in these tissues.

The maturation of the GSH S-transferases in the liver and kidney has previously been investigated by several workers and GSH S-transferase activity towards CDNB, immunoprecipitable by anti-GSH S-transferase B antiserum, has been examined by Hales and Neims (1976a). The developmental pattern of epoxide metabolising enzymes in rat testis, ovary and adrenal have been described (Mukhtar **et al** 1978a,b) but these measurements represent the

total activity of several GSH S-transferases and thus may not have bearing on the development of ligandin in the newborn and pubertal rat.

This study provides the specific developmental pattern of immunoreactive ligandin in the steroidogenic tissues of the rat, in correlation with the corresponding circulating levels of serum steroid hormones (testosterone and progesterone, Fig 2.22) in these animals. Attempts to stimulate gonadal and adrenal ligandin concentrations with phenobarbital and testis ligandin with various hormones were carried out.

The radioimmunoassay which was used for quantitating ligandin levels in these tissues is described in this section.

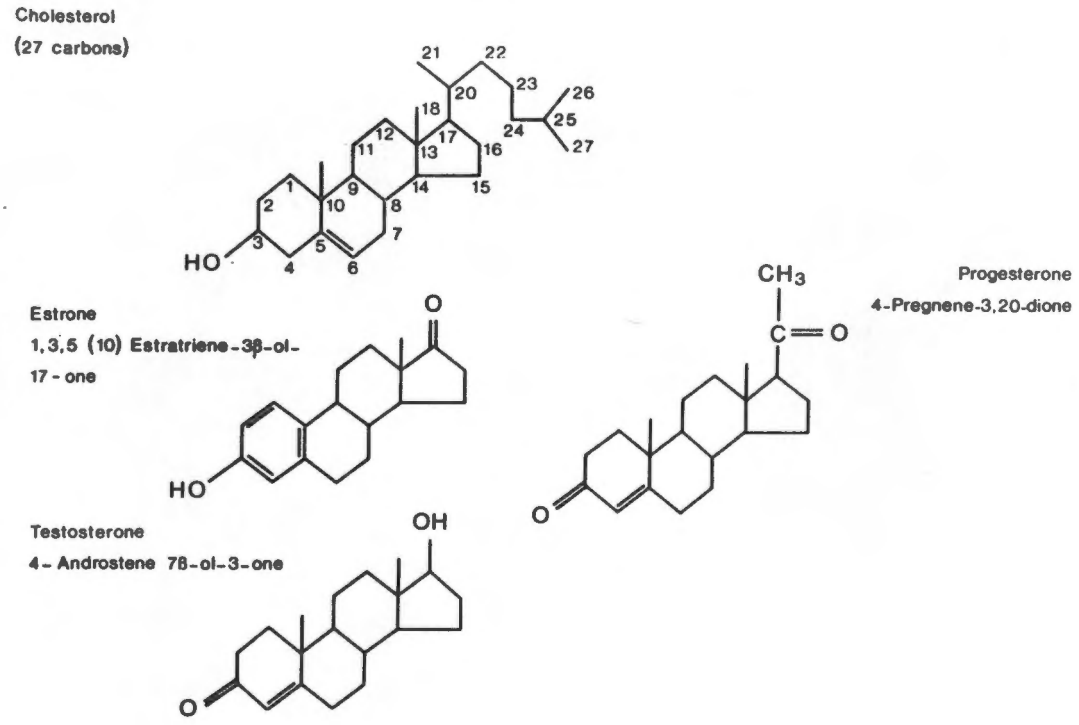


Fig. 2.22 Structural formulae of cholesterol, estrone, testosterone and progesterone.

(b) **Materials**

The reagents were purchased as follows:

^{125}-Iodine and 1,2,6,7,[N]-^{3}H Testosterone, used without further purification, were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England.

Donkey anti-rabbit serum from Wellcome Reagents, Ltd., Beckenham, England.

Testosterone, progesterone, Norit A Charcoal (purified) and Dextran T-70 were obtained from Sigma.

Gelatin and Chloramine T was supplied by BDH.

Toyo 514 Chromatography paper was from Toyo Kagaka Sangyo Co. Ltd., Tokyo, Japan.

Rabbit anti-progesterone, 11-alpha-bovine serum albumin was obtained from Miles Laboratories (Pty) Ltd., Goodwood, South Africa.

Falcon Plastic Tubes No. 2052 was from Div. Bec ton, Dickenson and Co. Cockeysville, MD, 21030, USA.

Human chorionic gonadotrophin (HCG), Human menopausal gonadotrophin (HMG) were from Propan Ethicals (Pty) Ltd., Goldbree, 334 Bree St. Johannesburg.

Dexamethasone sodium phospate from Merck Sharp and Dohme Park, Old Pretoria Main Rd, Halfway House 1685, South Africa.

Phenobarbitone sodium from Maybaker (S.A.) (Pty) Ltd., 21 McHardy Ave, Port Elizabeth, South Africa.

(c) Methods

Animals and Treatments For the developmental study Long-Evans rats aged 4, 9, 21, 36, 48 and 60 days were used. Liver, kidney, testis and adrenals were removed from male rats and ovaries from female rats. Serum was obtained from male and female rats aged 4, 9, 21, 24, 37, 44, 49, 57 and 75 days. Animals aged 21 days and over were bled by means of cardiac puncture, while younger animals were bled by decapitation. All serum specimens were stored at -20°C, until assayed for steroid hormones.

Ovaries, placenta and serum of pregnant female rats were collected at 4, 12, 16 and 20 days gestation.

Preparation of Cytosol The procedure for preparation of cytosol has been detailed in Section A of this chapter. The adrenals, ovaries and tissues from newborn animals were homogenised in 2 ml of buffer. Details of the dilutions used are found in legends to the figures.

Phenobarbital Induction Rats aged 4, 20 and 60 days were injected with 8mg/100g of phenobarbitone subcutaneously at approximately 0900hrs each day for 5 days. Control rats were injected with saline.

Hormone Induction Immature male rats (26 days) were injected with 0,4 mg Dexamethasone sodium phosphate, 100IU HCG and 10IU human menopausal gonadotrophin (HCG, 5IU FSH and 5IU LH) in 50ul saline (0.15M) per testis with a 26 gauge, short bevel needle. Control animals were injected with 50ul saline (0.15M). Injections were carried out intratesticularly as described previously (Reddy and Villee 1975).

Radioimmunoassay Procedures

(i) Preparation of Radio-labelled Ligandin

Rat liver ligandin was purified as described in Section A of Chapter 2. High specific activity ^{125}I-ligandin was prepared according to the method of Greenwood **et al** (1963) as modified by Bass **et al** (1977b). Using a small glass tube (2 x 0.5cm) the following reactants were mixed at room temperature in the following order using E-mil constriction pipettes:

10ul (1mCi) carrier free ^{125}I,

25ul 0.5M sodium phosphate buffer pH 7.4.

The following reactants were then added (in the same phosphate buffer, diluted 1:10) with mixing:

25ul (10-15ug) of ligandin,

25ul (25ug) of chloramine-T and after reaction had proceeded for 30 seconds,

100ul (50ug) sodium metabisulphite was added to terminate the reaction.

After addition of 200ul (2mg) of cold carrier iodine in the form of potassium iodide, the entire contents of the reaction vessel were mixed with 500ul of 0.3M Tris HCl buffer, pH 8.67 / 0.1% bovine serum albumin and applied to a combined gel filtration ion exchange chromatography column. The column (0.5cm diameter) was packed to a height of 4-5cm with TEAE-cellulose and overlaid with 25cm of Sephadex G-25, equilibrated with 0.03M Tris HCl buffer pH 6.67 and adjusted to a flow-rate of 18-20ml/hour. Fractions of 0.6ml were collected.

Assessment of the iodination was necessary in order to establish the extent of the dama

In order to accomplish this the following two procedures were carried out:

(1) Trichloroacetic acid (TCA) precipitation:

5ul of the reaction mixture, or 20ul of purification column fractions (peak fractions 10, 19 and 35, from Fig. 2.23) were added to 1ml of 0.05M sodium phosphate buffer pH 7.4 / 0.1% BSA, followed by addition of 1ml of 10% TCA with mixing. The mixture was incubated for 30 minutes at 4°C and was centrifuged. Radioactivity of the supernatant (500ul) and the precipitates were separately counted. The results of the procedure were expressed as the percentage of total counts present in the precipitate.

(2) Chromatoelectrophoresis (Berson **et al** 1956).

The various purification fractions were subjected to chromato-electrophoresis performed on 3,5 x 50cm strips of Toyo 514 paper using 5ul bovine serum albumin as carrier and Bromophenol Blue as a marker of damaged protein (Bass **et al** 1977b). Intact or undamaged ^{125}I-ligandin remained at the origin, while the damaged labelled protein migrated with the bromophenol blue and free ^{125}I migrated ahead of the damaged protein. Sections of the chromatoelectrophoresis strips were cut, placed in Falcon tubes and counted. The intact protein was expressed as a percentage of the total radioactivity present.

The ^{125}I-ligandin was diluted with diluent buffer to give approximately 20000 cpm / 200ul before use in the assay. The labelled protein was stored for not longer than 2 weeks at 4°C.

(ii) Ligandin Radioimmunoassay Procedure

Radioimmunoassay of ligandin from rat tissues was performed according to the method of Bass **et al** (1977b) using mono-specific rat liver ligandin antisera.

Preparation of antisera was described in Section A of this chapter. The radioimmunoassay procedure was carried out using a double antibody method (Morgan and Lazarow 1963) which separated the antibody-bound from free labelled antigen. High specific activity ^{125}I rat liver ligandin was prepared as previously described. Falcon plastic tubes (12 x 75mm) were used in the assay.

In order to determine the optimal anti-ligandin dilutions for the assay, incubation of serial dilutions of rabbit anti-liver ligandin antiserum with constant amounts of ^{125}I liver ligandin according to standard assay protocol were carried out. Bleeds 10 days after each booster were tested. The dilution which demonstrated 50% of maximum binding of the traces in the absence of unlabelled ligandin, ie the antibody titre, was used in the assay. In addition, optimisation of the non-immune rabbit serum and donkey anti-rabbit globulin dilutions was carried out by using constant concentrations of one reagent and varying the other. This was determined for each new batch of donkey anti-rabbit serum, as inter-batch variations were noted. Non-immune rabbit serum was used in a dilution of 1:400 and donkey anti-rabbit serum in a 1:20 dilution for all assays carried out.

Immunochemical displacement curves were constructed by means of serial dilutions of liver and testis ligandin assayed using

labelled liver ligandin and anti-liver ligandin antiserum. These dose response curves were linearised by logit/log transform using the relationship:

logit $(Y)=a + b \log_e(X)$, [where $Y=B/Bo$, the ratio of bound counts (B) to counts bound in the absence of unlabelled antigen (B_o), with both B and B_o corrected for non-specifically bound (NSB) counts],

logit $(Y)=\log_e (Y/1-Y)$

a is the intercept and b is the slope of the linearised curve, while X is the dose of unlabelled antigen.

All determinations were performed in duplicate. Dilutions were made using a diluent buffer comprising 0.5M sodium phosphate buffer pH 7.4 / 0.15M NaCl / 0.01M EDTA / 0.5% bovine serum albumin / 0.1% sodium azide.

The assay procedure consisted of the consecutive addition of the following:

(1) 200ul diluent buffer
(2) 200ul sample or standard
(3) 200ul ligandin antisera
(4) 100ul non-immune rabbit sera
(5) 200ul labelled ligandin

The reagents were mixed and incubated for 24 hours at 4°C after which 100ul donkey anti-rabbit serum was added with mixing. Following a further 24 hour incubation period at 4°C, the tubes were centrifuged at 2000 g for 30 minutes at 4°C in a Sorvall RC-3B refrigerated centrifuge. Supernatants were decanted and tubes drained for 10 minutes care being taken to ensure that the opaque

pellet was not dislodged. Counting was performed as previously described and the data recorded on paper tape for transfer to a computer file.

After determination of radioactivity the data were processed on a Univac series 1100 system computer using the Rodbard-Faden RIA-RUN programme (Rodbard 1974). This programme fits the standard curve of the assay to a linear function from which "potency estimates" of the unknowns can be derived. Statistical analysis of several criteria of assay performance, provided by this programme, was carried out. Assays with criteria falling more than two standard deviations from the mean were discarded and repeated.

Each assay incorporated the following protocol:

Total counts - 4 tubes containing labelled protein only.

Bo - (maximum binding) 4 tubes with no standards or unknowns added.

NSB - (non specific binding) 4 tubes with no anti-ligandin antibody added.

Standards - 22 tubes (11 duplicates) containing known concentrations of unlabelled pure ligandin prepared as follows: the protein concentration was estimated to be 1.5mg/ml, a 3000ng/ml stock solution was prepared (600ng/200ul) and diluted accordingly to provide standards of 600, 300, 150, 75, 37.5, 18.75, 9.38, 4.69, 2.34, 1.17 and 0.59ng/200ul.

Samples - Up to 200 tubes containing unknown samples, in differing dilutions

Internal Standards - 1:5000 and 1:1000 dilutions of known concentrations of ligandin, used in every assay for inter-assay comparisons.

(iii) Extraction of Sera for Testosterone Radioimmunoassay (Millar and Kewley 1976)

Extraction of sera was carried out as follows; serum aliquots (100ul) were pipetted into 10ml glass stoppered tubes. ^{3}H testosterone (approximately 2000cpm in 100ul) was added to each tube for internal recovery estimations, and left overnight to allow equilibrium binding of ^{3}H testosterone to serum proteins. After addition of 100ul of concentrated ammonium hydroxide, the serum samples were extracted by shaking for 5 min with 6ml diethyl ether. The aqueous phase was rapidly frozen in acetone / solid CO_2 mixture and the ether layer poured off and evaporated to dryness at 45°C under nitrogen. 400ul of 0.01M sodium phosphate buffer pH 7.0 / 0.15M NaCl / 0.1% sodium azide / 1% methanol solution (PBS / 1% methanol) was added to the tubes and mixed. 100ul was taken for scintillation counting to estimate the recovery, and the remainder used for assay.

(iv) Testosterone Radioimmunoassay Procedure

Duplicate 100ul aliquots of standard testosterone (0.0156 0.03139 0.625 0.125 0.25 0.50 and 1.0 pmoles/100ul) and serum extracts in the methanol phosphate buffer were pipetted into 12x75mm glass test tubes. 100ul of specific antiserum to testosterone (produced by immunising rabbits with testosterone-3-carboxymethyl oxime-

bovine serum albumin conjugate intradermally) diluted in PBS / 0.1% gelatin was added, the tubes vortexed and left for 30 min at room temperature. After addition of 100ul of ^{3}H testosterone (approximately 10000cpm) the tubes were again vortexed and incubated overnight at 4°C. The tubes were then placed in an ice bath and 500ul of dextran-coated charcoal, removed from a continuously stirring beaker, pipetted into the tubes. After standing for 10 min the tubes were centrifuged at 2000 g for 10 min in a Sorval RC-3B centrifuge, and the supernatant poured into counting vials with scintillant and the radioactivity counted.

All calculations were carried out using the computer data processing system for radioimmunoassays of Rodbard (1974).

$$\% \frac{B}{B_0} = \text{sample mean} \times \frac{100}{B_0}$$

S=reading interpolated from standard curve.

$$\text{nanomoles/litre} = \frac{(S)(\text{cpm total recovery})(10^3)}{(\text{sample vol})(\text{cpm sample recovery})}$$

(iv) Extraction of Sera and Procedure for Progesterone Radioimmunoassay (Abraham **et al** 1971).

A similar extraction procedure was followed to that previously described for the testosterone radioimmunoassay, excepting that 100ul of ^{3}H-progesterone (approximately 1000cpm) was added to all tubes, and 6ml of petroleum ether (between 40°C-60°C) was added to each tube. The tubes were then stoppered and extracted by shaking for 5 min. After freezing in acetone / solid CO_2, the

supernatant organic phase was decanted off into clean 10ml tubes and evaporated to dryness at 40°C under nitrogen. 800ul of benzene was added to the tubes and thoroughly mixed. 100ul was taken for recovery estimation of the organic solvent extraction efficiency into scintillation vials and 100ul pipetted in duplicate into Wasserman tubes. The contents of the Wasserman tubes were evaporated to dryness at 40°C under nitrogen and 100ul of PBS / 1% methanol pipetted into each tube, and the tubes vortexed.

Duplicate 100ul of progesterone standard (0.2, 0.5, 1, 2, 5, 10 nanomoles / litre) in PBS / 1% methanol was pipetted out into Wasserman tubes. 100ul of progesterone antiserum (rabbit anti-progesterone, 11-alpha-bovine serum albumin) diluted in PBS, 0.1% gelatin was added, and the tubes vortexed and incubated for 30 min at room temperature. After addition of 100ul of ^{3}H-progesterone (approximately 10000 cpm) the tubes were again vortexed and incubated overnight at 4°C. The assay procedure then followed the same steps for charcoal treatment, centrifugation, radioactive counting and calculation of data as described for the testosterone radioimmunoassay.

(d) Results

RAT LIVER YaYa LIGANDIN RADIOIMMUNOASSAY

Rat Liver Ligandin Iodination The elution profile of the iodination reaction mixture chromatographed on Sephadex G-25/TEAE-cellulose is shown in Fig. 2.23. Three peaks of radioactivity were apparent and an aliquot from each peak tube was assessed by TCA precipitation and chromatoelectrophoresis. Table 2.4 lists the results obtained from a typical iodination of rat liver ligandin. The first peak consists almost entirely of TCA precipitable protein and was shown by means of chromatoelectrophoresis to remain at the origin. These results suggest that peak 1 contains mainly intact protein. The percentage obtained of TCA precipitable protein as well as the results obtained from chromatoelectrophoresis suggests that peak 2 and 3 consists largely of damaged protein and free iodine respectively.

Mean data obtained from chromatoelectrophoresis and TCA precipitation (15 procedures) demonstrated incorporation of ^{125}I into liver ligandin to be 42%. The specific activity of ^{125}I-liver ligandin was estimated to be 35uCi/ug.

FRACTION	TCA%	CHROMATOELECTROPHORESIS%		
		Intact	Damaged	Free
Reaction mixture	32.8	25	38	37
9	99.8	99.4	0.1	0.5
19	8.2	5	80.4	14.6
35	1.6	0.4	2.6	97

Table 2.4 - Purification of Iodination Reaction Mixture by Gel-filtration/Ion Exchange Chromatography

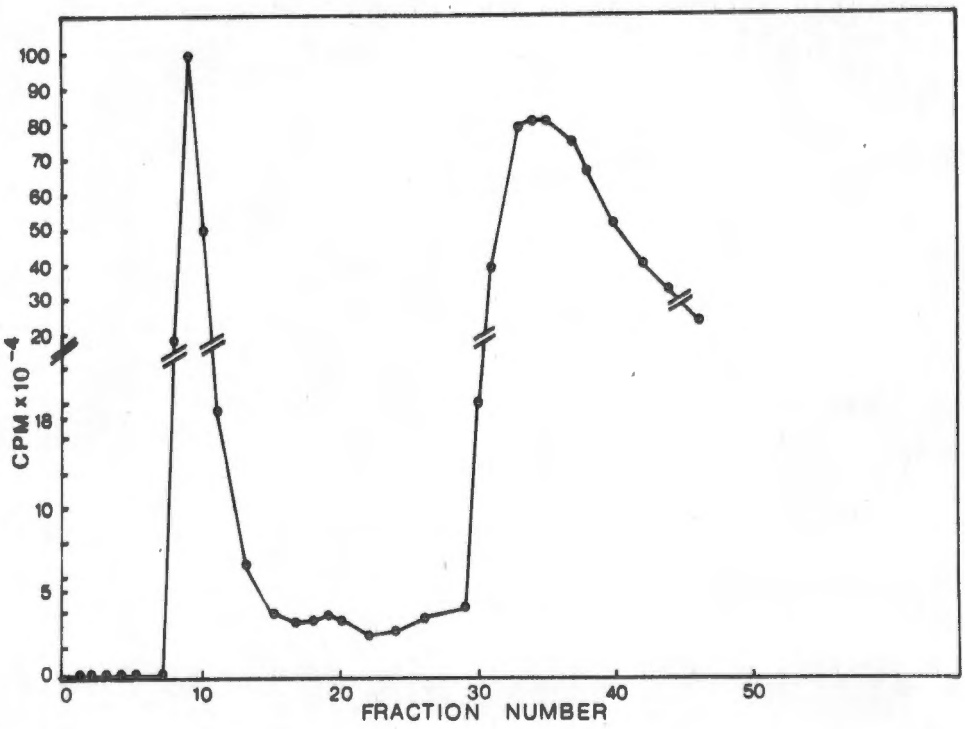


Fig. 2.23 Purification of ^{125}I ligandin by Sephadex G-25/ TEAE cellulose chromatography. The iodination mixture was purified according to details described in Chapter 2, Section B, Methods.

Determination of First Antibody Titre for Radioimmunoassay Serial dilutions of rabbit anti-liver YaYa ligandin antiserum bound by a constant amount of ^{125}I-liver ligandin are shown in Fig. 2.24. Antiserum 1234/10/04 exhibited 50% of maximum binding at an initial dilution of 1:40000, while following a booster inoculation, antiserum 1234/29/04 exhibited 50% of maximum binding at a dilution of 1:80000, demonstrating a rise in titre (the "titre" of antiserum is that demonstrating binding of 50% of tracer).

At an initial dilution of 1:800 antiserum 1817/10/04 bound 95% of freshly prepared ^{125}I-liver YaYa ligandin, while antisera 1234/10/04 and 1234/29/04 only bound 70% of label at this dilution. Thus antiserum 1817/10/04 was used for all subsequent assays. 50% of the tracer was bound at an initial dilution of 1:12000.

Parallelism of the Standard Curves The standard curve obtained by addition of increasing amounts of unlabelled liver YaYa ligandin to tubes containing constant amounts of anti-liver YaYa ligandin and ^{125}I-labelled liver YaYa ligandin, was shown to be identical to a curve obtained when unlabelled testis ligandin replaced liver ligandin (Fig. 2.25).

Characteristics of the Assay The characteristics of the assay were similar to those obtained by Bass **et al** (1977b). The percentage of total ^{125}I-ligandin bound in the absence of anti-liver ligandin antiserum averaged 2.8 $\pm$ 0.2% (mean $\pm$ SEM) in 10 consecutive assays. Interassay variation over 10 consecutive assays for testis ligandin samples were 50.4 $\pm$ 2.3 ng/tube (1:1000 dilution) and 15.1 $\pm$ 0.6 ng/tube (1:3000 dilution) both values, mean $\pm$ SEM, with coefficients of variation 9.3% and 8.8% respectively. The minimal detectable dose averaged 1.54 $\pm$ 0.16 ng/ml (mean $\pm$ SEM) and the slope of the standard curve averaged -0.78 $\pm$ 0.03 (mean $\pm$ SEM) over 10 consecutive assays.

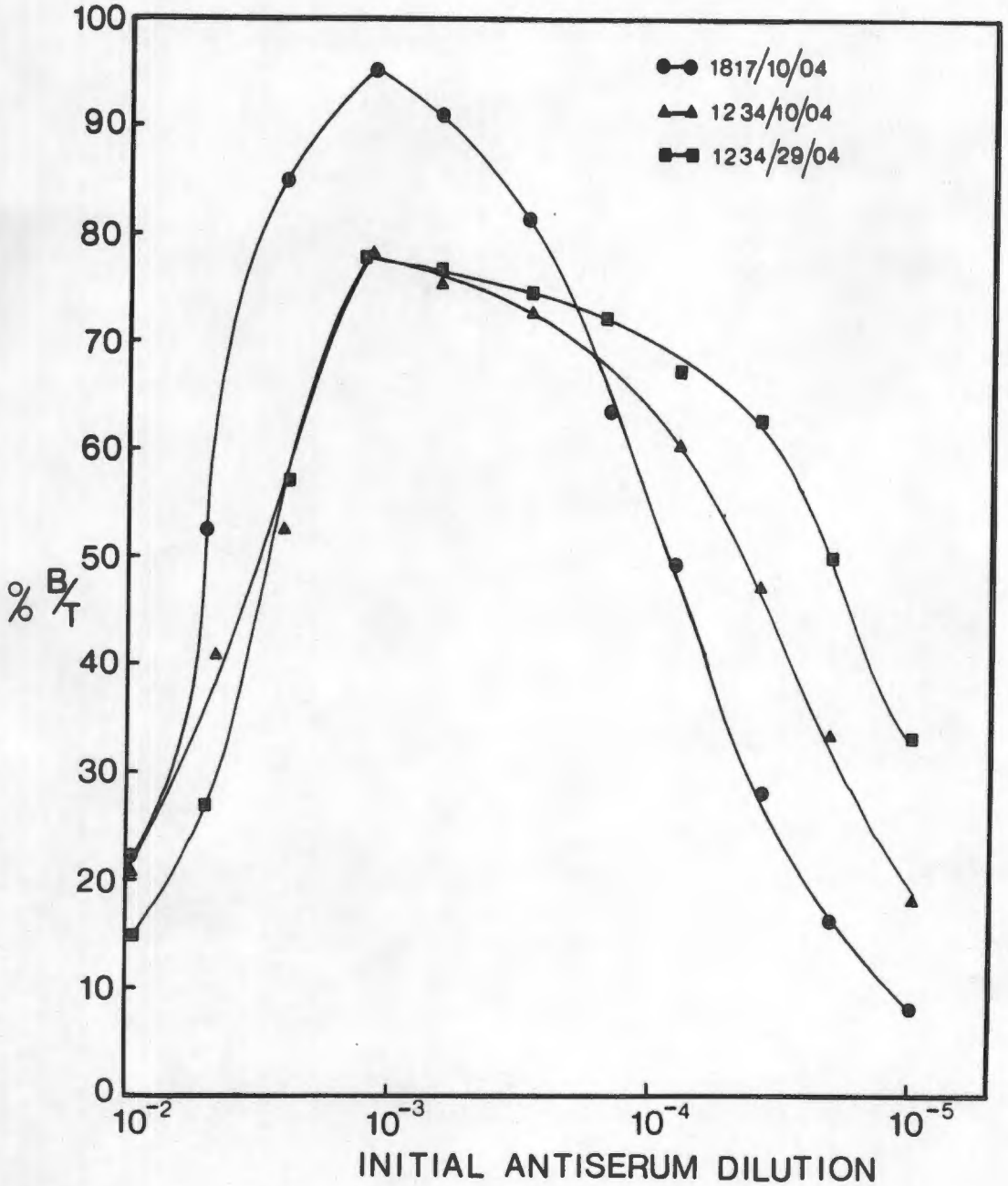


Fig. 2.24 Antiserum dilution curves of rabbit anti-YaYa ligandin antiserum. Serial dilutions of antisera harvested from rabbit 1234 following the first and second booster inoculations, and antiserum from rabbit 1817 following second booster inoculations, were incubated with constant amounts of ^{125}I-labelled liver ligandin. The standard radioimmunoassay procedure described in Chapter 2, Section B, Methods, was followed.

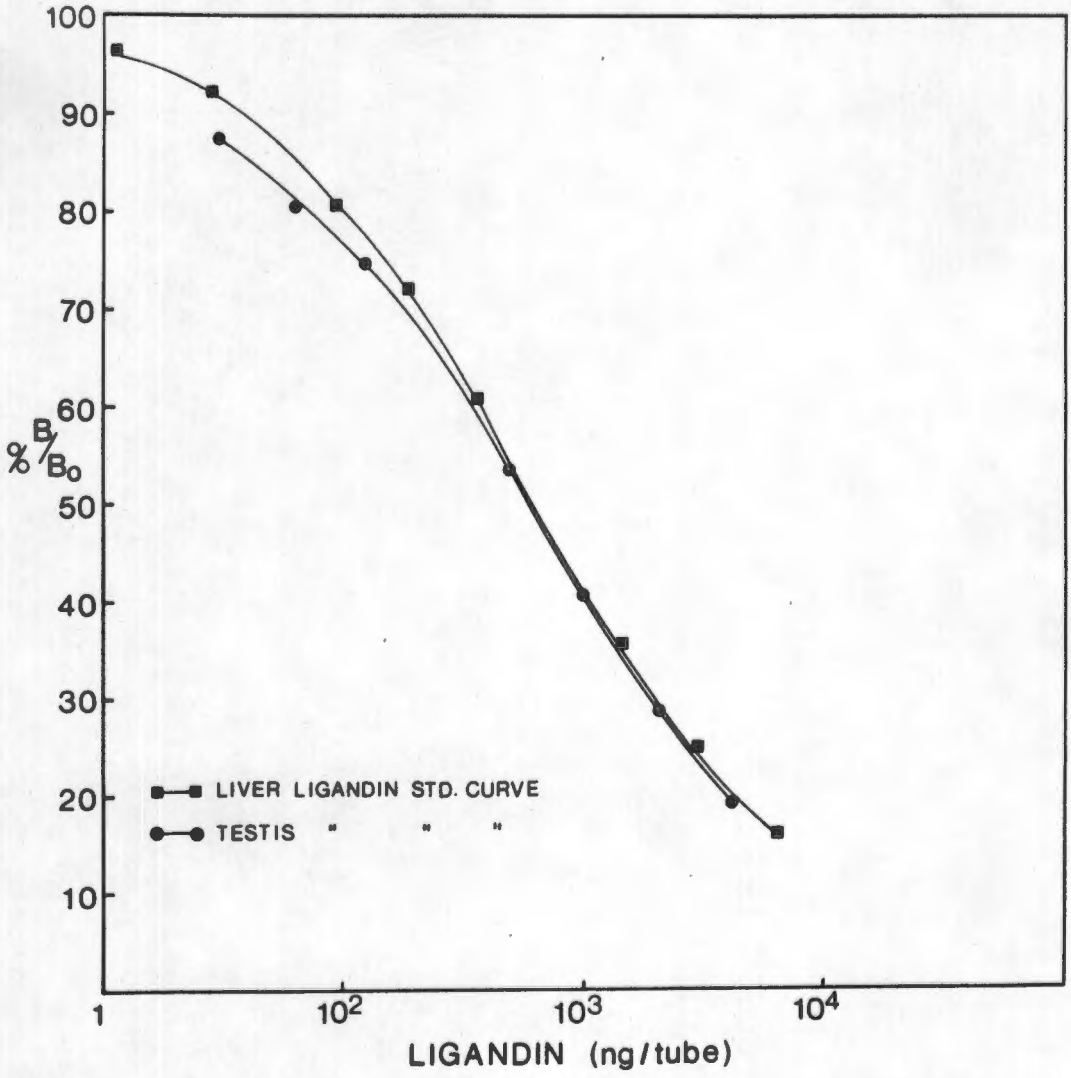


Fig. 2.25 **Ligandin radioimmunoassay standard curves.** These were obtained when liver YaYa ligandin and testis ligandin was added to the liver ligandin radioimmunoassay according to the procedure described in Chapter 2, Section B. On the ordinate is the percentage ratio of antibody-bound radioactivity to radioactivity bound in the absence of unlabelled ligandin (% B/Bo) and on the abscissa (log scale), the amount of unlabelled ligandin.

LIGANDIN AND SERUM HORMONE LEVELS IN RELATION TO DEVELOPMENT OF THE RAT

Ligandin levels were measured in rat tissue using the anti-liver YaYa ligandin radioimmunoassay in steroid-producing tissues of rats at 4, 9, 20, 31, 46 and 60 days postpartum.

Testicular Ligandin Concentration During Development The developmental pattern of testicular ligandin is shown in Fig. 2.26. Relatively high concentrations of ligandin expressed as micrograms/milligram of supernatant protein were present at 4 days. By 9 days the relative concentration of ligandin had decreased to low levels. These levels then rose sharply during puberty (30-40 days), and then increased gradually until 60 days of age. Serum testosterone levels (expressed as nanomoles/litre) paralleled those of ligandin (Fig. 2.26).

Ligandin concentration expressed as micrograms per wet weight of tissue exhibited a similar pattern of change (Fig. 2.30).

Adrenal Ligandin Concentration During Development Ligandin concentration at 4 days was shown to be relatively high when expressed as micrograms/milligram of supernatant protein (Fig. 2.27). These values decreased by 9 days and thereafter increased sharply during puberty reaching a plateau between 46 and 60 days.

Measurements of adrenal ligandin expressed per wet weight of tissue are shown in Fig. 2.30. Ligandin levels decreased between day 4 and day 9 and then increased progressively to reach adult levels by 46 days.

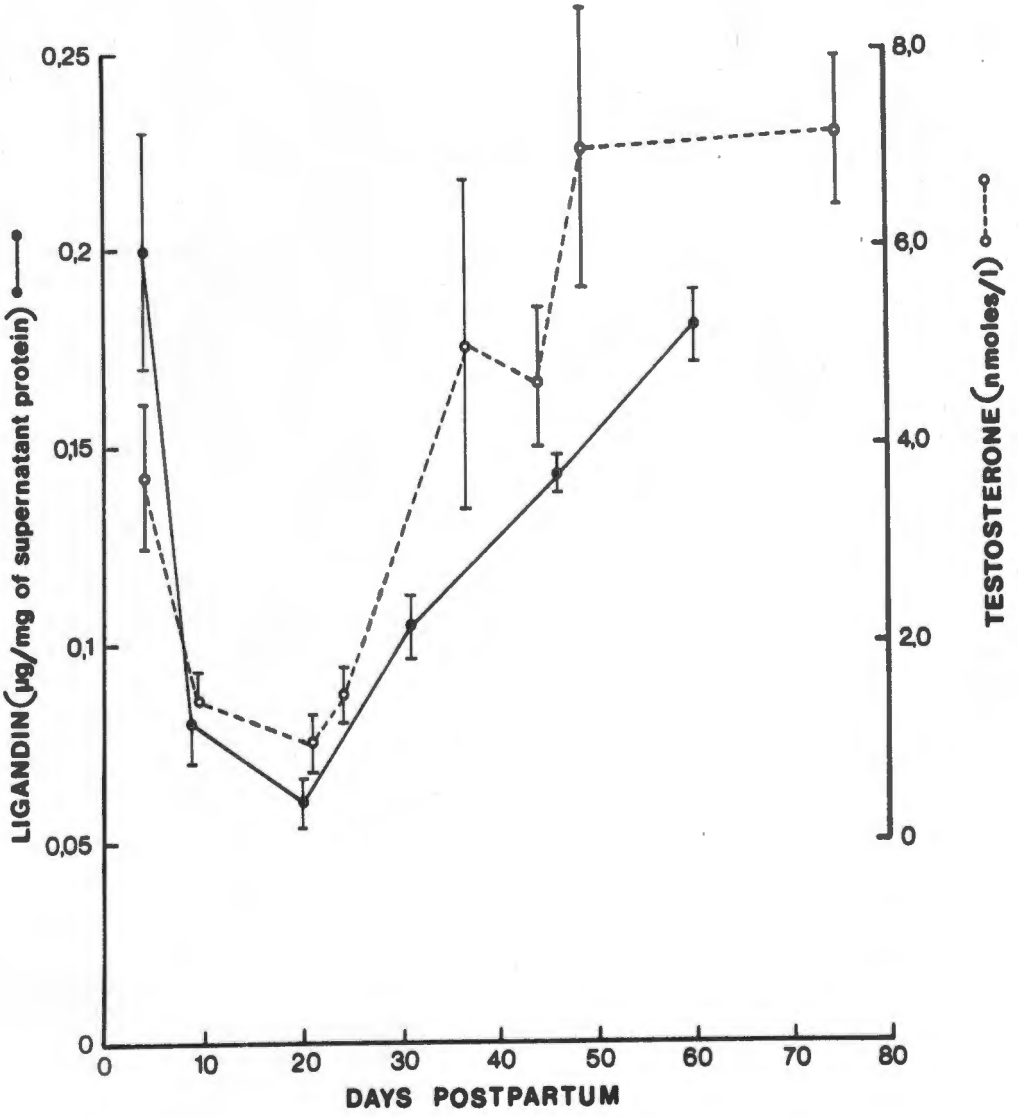


Fig. 2.26 Postnatal development of rat testis ligandin and serum testosterone levels. Ligandin is expressed as micrograms-/milligram supernatant protein in 100000 g testis supernatant. For 4-day-old animals 6 testes were pooled, for 9-day-old animals 9 testes were poooled, for 20- and 31-day-old animals 6 testes were pooled and for 46- and 60-day-old 2 testes from the same animal were pooled. Each point represents the mean + SEM of six individual determinations at each time interval studied. The serum testosterone is expressed in nanomoles/litre, and was taken from male animals at 4, 9, 21, 24, 37, 44, 49 and 75 days postpartum. Each point represents the mean ± SEM of six individual determinations.

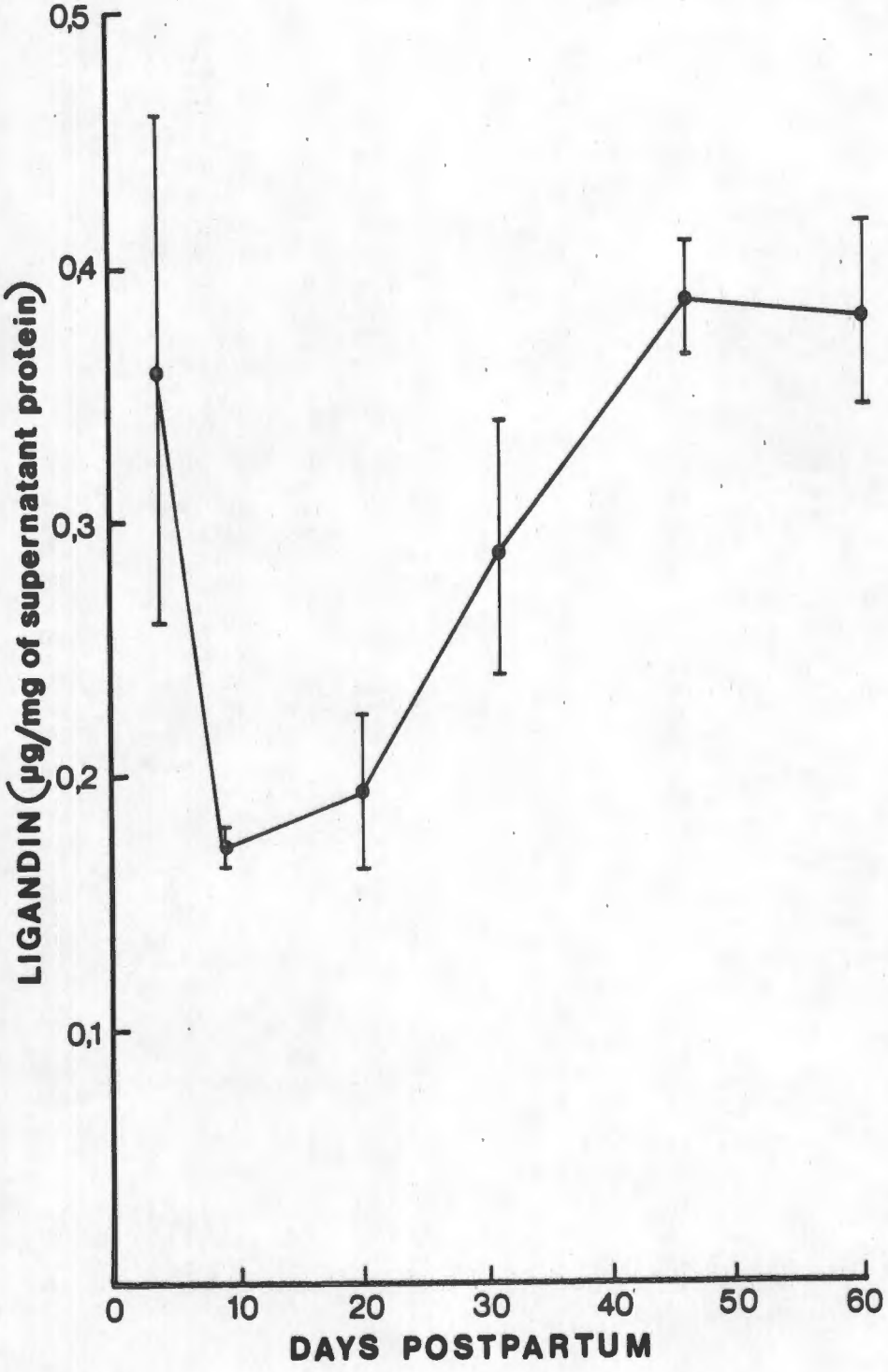


Fig. 2.27 Postnatal development of rat adrenal ligandin. Ligandin is expressed as micrograms/milligram of supernatant protein, in 100000 g adrenal supernatant. For 4-, 20- and 30-day-old animals 6 adrenals were pooled, for 9-day-old animals 9 adrenals were pooled and for 46- and 60-day-old animals 2 adrenals from the same animal were pooled. Each point represents the mean ± SEM of six individual determinations at each time interval studied. Only adrenals from male animals were used.

Ovarian Ligandin Concentration During Development There was an initial decline of ovarian ligandin (ug/mg protein) between 4 and 9 days of age, followed by a rapid increase between 20 and 40 days which gradually levelled off at about 50 days (Fig. 2.28). This pubertal rise, was closely paralleled by an increase in serum progesterone levels (Fig. 2.28). Once again, when the ligandin levels were expressed as micrograms per wet weight of tissue (Fig. 2.30) the pattern was similar to that shown in Fig. 2.28.

Liver and Kidney Ligandin Concentration During Development In order to compare the developmental pattern of ligandin in steroidogenic and non-steroidogenic tissues measurements were made using the same system in liver and kidney. Hepatic and renal ligandin development differed from that of testis, ovary and adrenal. At birth hepatic ligandin was low, increasing to approximately 50% of adult values by 20 days, and thereafter showing a progressive rise to 60 days (Fig. 2.29, ug hepatic ligandin/mg protein; Fig. 2.30, ug hepatic ligandin/wet weight of tissue).

Renal ligandin levels were also low at birth, but increased rapidly at 21 days and levelled off at 31 days, after which they increased gradually to 60 days of age (Fig. 2.29).

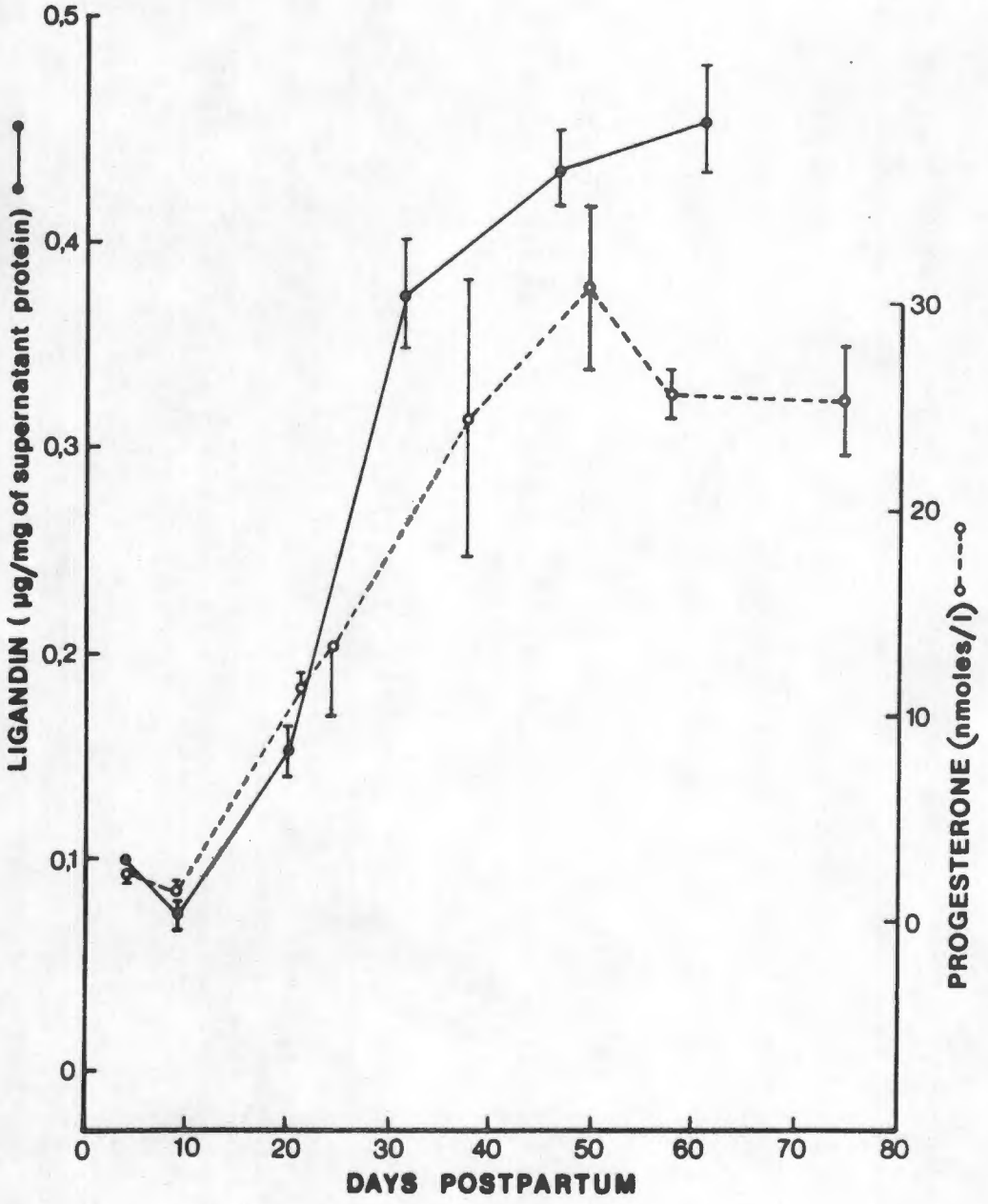


Fig. 2.28 Postnatal development of rat ovary ligandin and serum progesterone levels. Ligandin is expressed as micrograms/ milligram supernatant protein, in 100000 g ovary supernatant. Four-day-old values are from a single pool of ovaries from 10 rats. For 9-day-old animals 8 ovaries were pooled, for 20- and 31-day-old animals 6 ovaries were pooled, and for 46- and 60-day-old animals 2 ovaries from the same animal were pooled. Each point represents the mean + SEM of six individual determinations at each time interval studied. The serum progesterone is expressed in nanomoles/litre and was taken from female animals at 4, 9, 21, 24, 37, 49, 57 and 75 days postpartum. Each point represents the mean + SEM of six individual determinations.

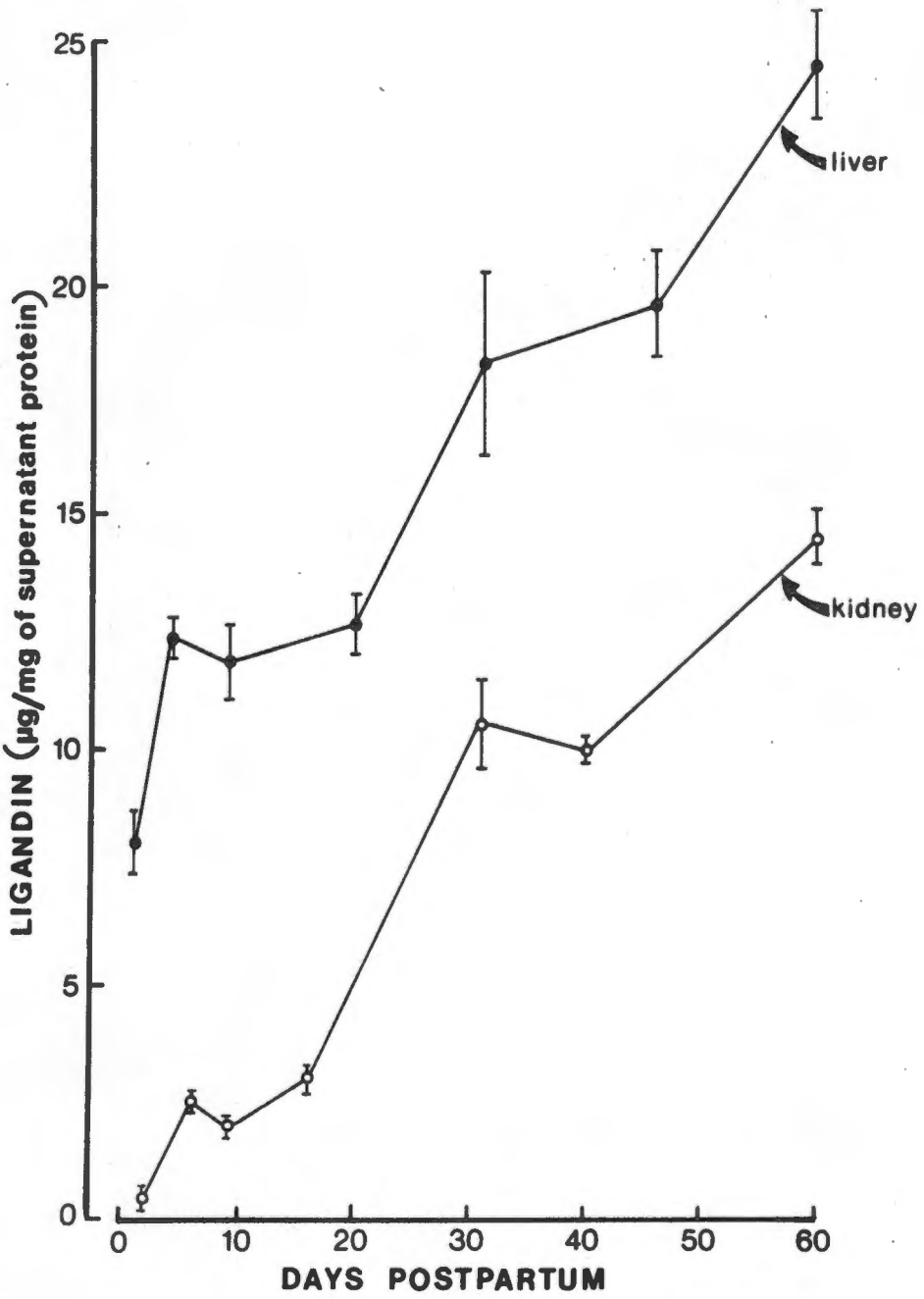


Fig. 2.29 **Postnatal development of rat liver and kidney ligandin.** Ligandin is expressed as micrograms/milligram supernatant protein. For 4- and 9-day-old animals 2 livers were pooled. Livers of older animals were assayed individually. Values are means of six individual determinations ± SEM. Renal ligandin values were determined in the same manner.

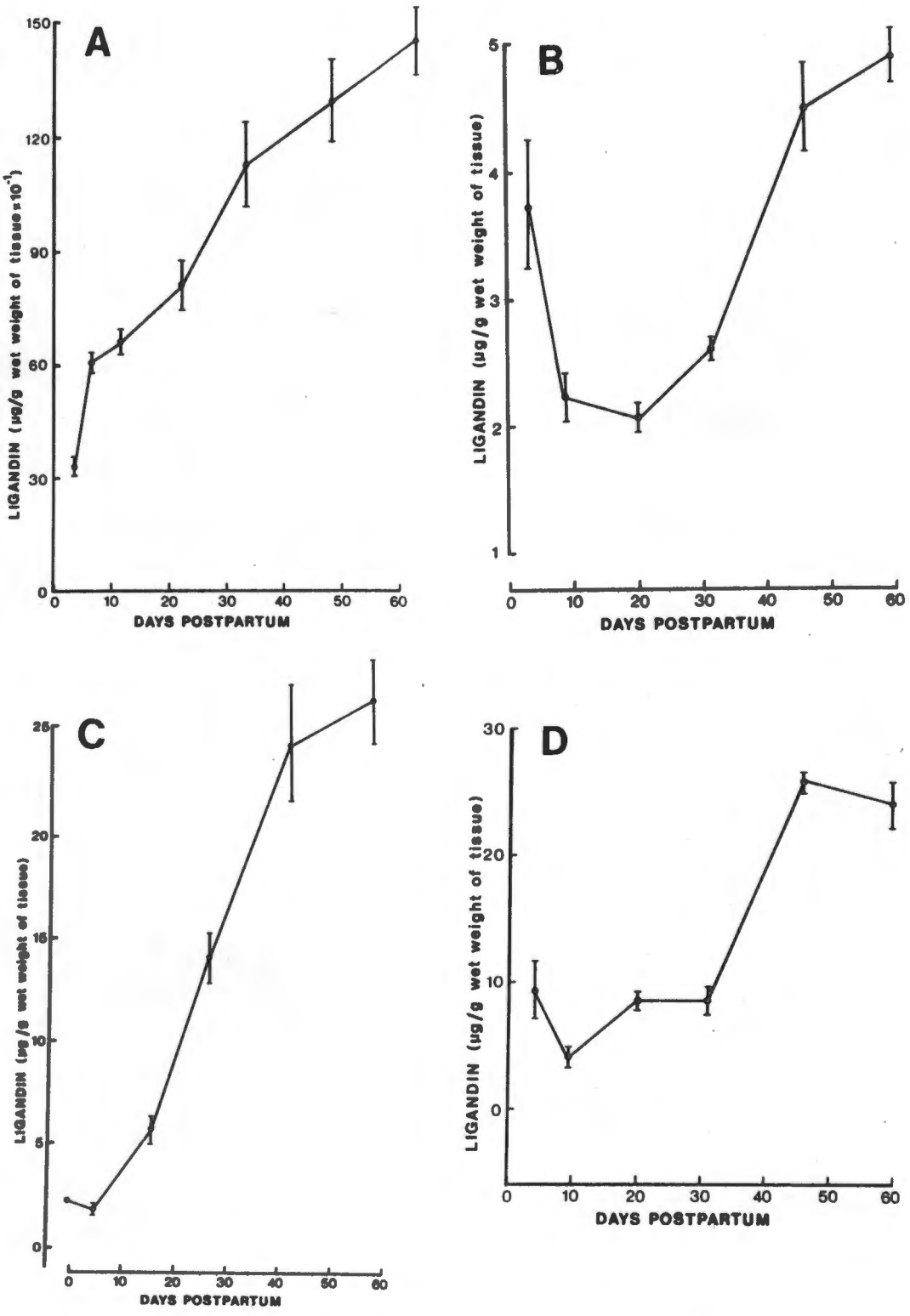


Fig. 2.30 Postnatal development of rat liver (A), testis (B), ovary (C) and adrenal (D) ligandin expressed as micrograms of ligandin/gram wet weight of tissue.

POSTNATAL DEVELOPMENT OF RAT BODY AND TISSUE WEIGHT

Other parameters of maturation, including total body weight and the weight of the various tissues studied, are all summarised in Figs 2.31 to 2.32.

OVARIAN LIGANDIN CONCENTRATION AND ENZYMATIC ACTIVITY DURING PREGNANCY

Table 2.5 shows the results of assays performed on pregnant ovaries at different stages of gestation. Ligandin levels in the early stages of pregnancy showed no significant changes compared to non-pregnant controls. By 16 and 21 days ligandin concentration had decreased. This was contrasted by a significant rise in GSH S-transferase specific activity (approximately 70%) towards CDNB by mid-pregnancy (12 days), relative to non-pregnant rats. GSH peroxidase specific activity (cumene hydroperoxide as substrate) showed a 91% increase at 12 days compared to controls with enzyme specific activity declining by 21 days gestation. Serum progesterone levels doubled by 4 days gestation, increased during the course of pregnancy and decreased slightly just before birth.

EFFECT OF PHENOBARBITAL ON LIGANDIN CONCENTRATION DURING DEVELOPMENT

The effect of phenobarbital treatment on liver, testis, ovary and adrenal ligandin concentration in developing rats is shown in Table 2.6. A significant increase in hepatic ligandin for each time interval studied was associated with phenobarbital treatment, while testicular, ovarian and adrenal ligandin concentration showed no change.

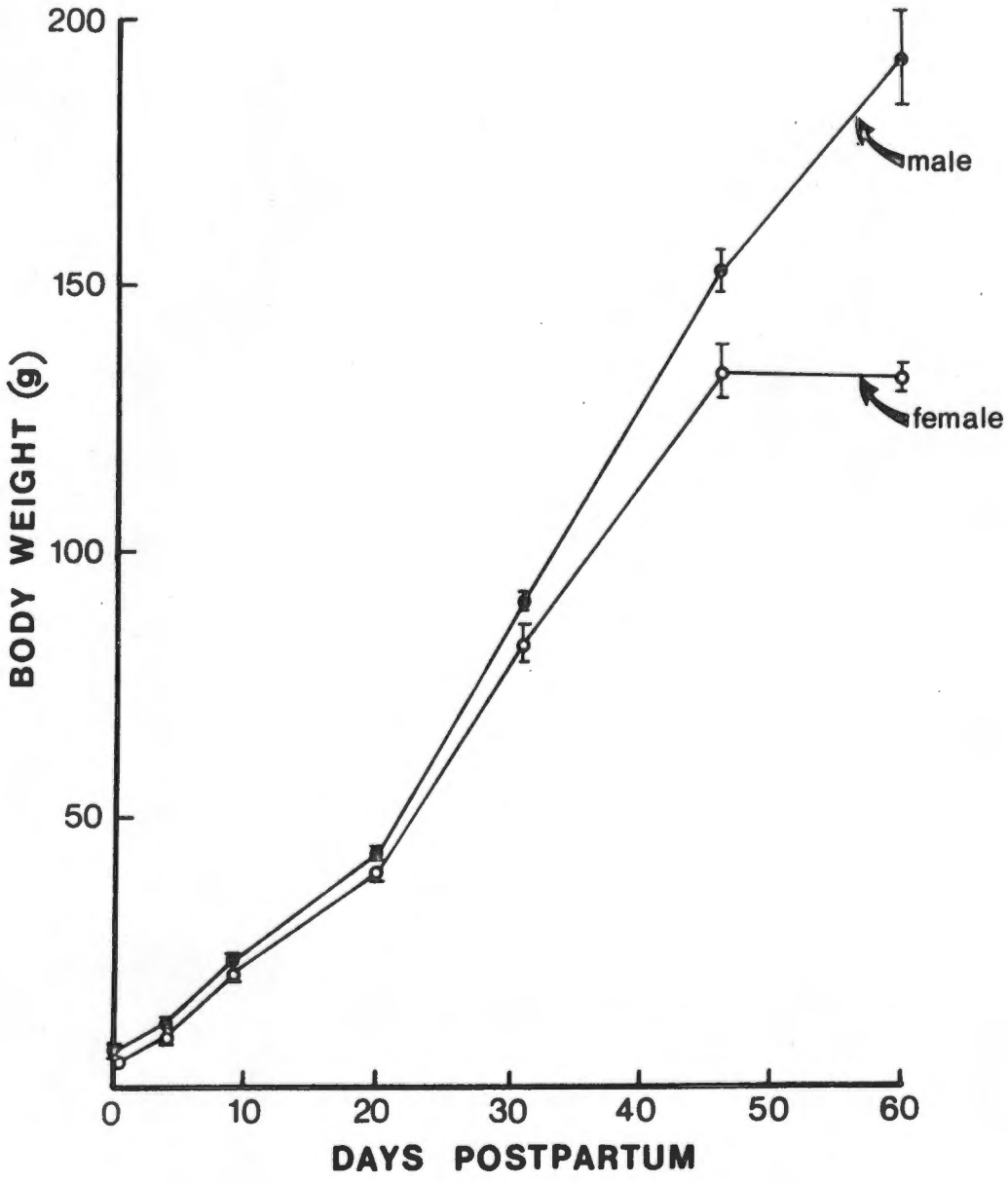


Fig. 2.31 **Postnatal development of male and female rat body weight.** For day 0 and 4, 14 animals were used. Thereafter 6 animals were used for each tested age.

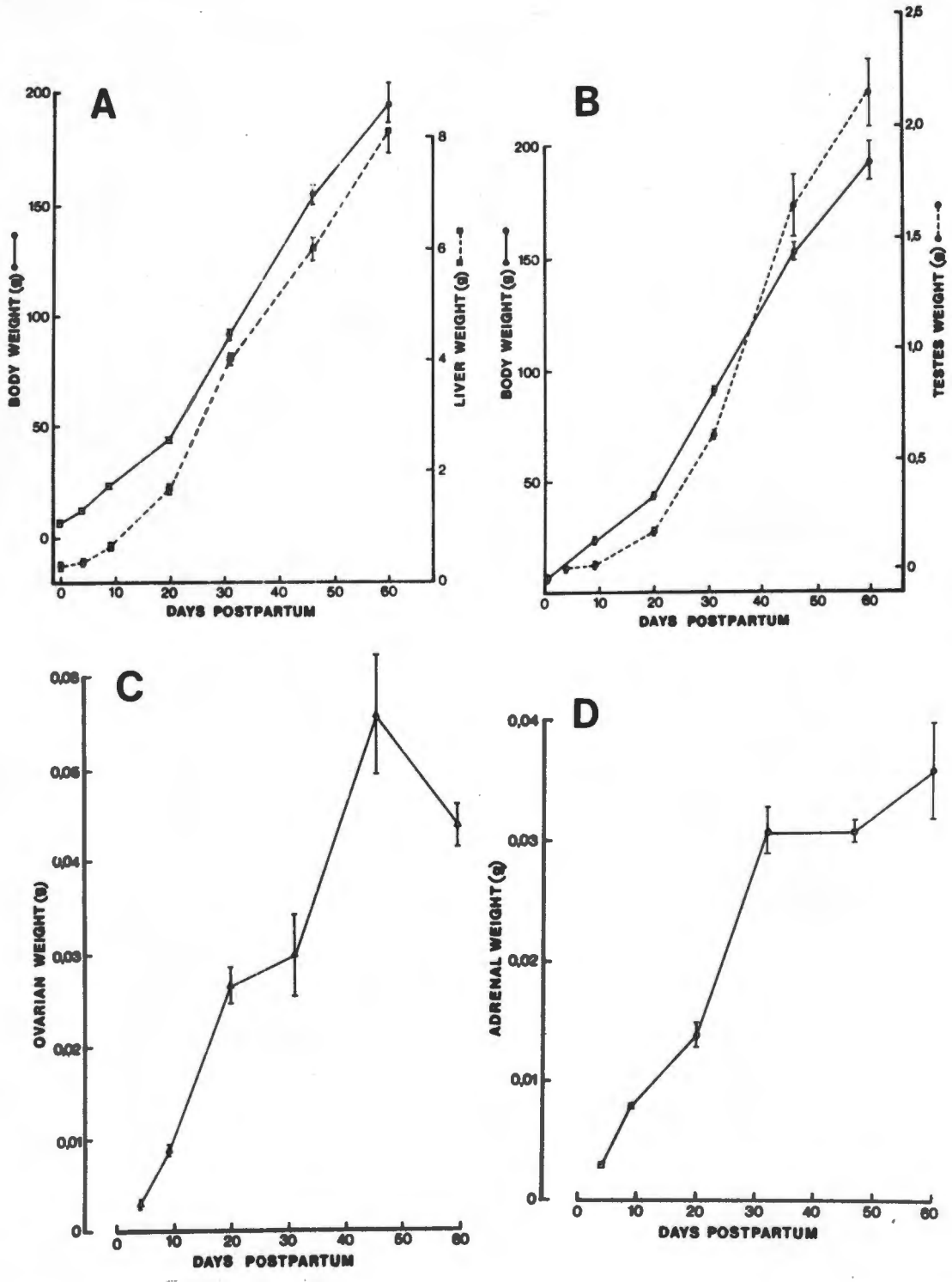


Fig. 2.32 Postnatal development of male rat liver weight and male rat body weight (A) (details of male rat liver weight data were as in Fig. 2.31). Postnatal development of rat testes weight and male rat body weight (B). Testes from 14 animals at 4 days were used. Thereafter testes from 6 individual animals were used at each time point studied. Postnatal development of rat ovary weight (C) and male rat adrenal weight (D).

		DAYS PREGNANT				
	CONTROL	4	9	12	16	21
CDNB umol/min/mg protein	0.041±0.003 (n=4)	0.045± 0.002 (n=4)	0.06 ±0.004 (n=4)*	0.07 ± 0.008 (n=4)**	0.075±0.014 (n=4)***	0.05 ± 0.004 (n=6)
Cumene.OOH umol/min/mg protein	0.033±0.007 (n=5)	0.046± 0.005 (n=6)	0.051±0.007 (n=4)	0.063± 0.013 (n=4)***	0.034±0.002 (n=6)	0.034± 0.003 (n=6)
Ligandin ug/mg supernatant protein	0.301±0.034 (n=4)	0.34 ± 0.05 (n=5)	0.23 ±0.014 (n=4)	0.225± 0.037 (n=4)	0.158±0.008 (n=8)*	0.122± 0.006 (n=6)*
Progesterone umoles/litre	37.84 ±7.38 (n=5)	70.36 ±14.65 (n=4)*	80.0 ±3.1 (n=4)*	114.75 ±22.25 (n=4)*	- -	97.6 ±12.32 (n=4)*

Table 2.5 **Effect of pregnancy on rat ovary ligandin, serum progesterone concentration, GSH S-transferase and GSH peroxidase activities.** Tissues from the same animal were pooled for each determination. The number of determinations are in parentheses. All values represent the mean ± SEM. Significant differences from controls shown by *P < 0.005 **P < 0.01 ***P < 0.025

TISSUE	DAY	CONTROL*	PHENOBARBITAL-* TREATED
Liver	4	12.29±0.41	20.03±1.78
	20	12.62±0.64	26.68±2.37
	60	24.66±1.3	33.96±3.35
Testis	4	0.2 ±0.03	0.21±0.04
	20	0.06±0.01	0.07±0.00
	60	0.18±0.01	0.16±0.01
Ovary	4	0.17±0.01	0.17±0.01
	20	0.15±0.01	0.11±0.02
	60	0.45±0.03	0.44±0.05
Adrenal	4	0.36±0.11	0.36±0.09
	20	0.19±0.03	0.20±0.04
	60	0.38±0.04	0.38±0.05

Table 2.6 **Effect of phenobarbital on immunoreactive ligandin concentration during development.** Tissues from 3 animals were pooled for each 4-day-old and 20-day-old determination and tissue from the same animal were pooled for the 60-day-old animals. *Ligandin expressed as micrograms/milligram supernatant protein, all values represent the mean ± SEM of six individual determinations at each time interval studied.

EFFECT OF VARIOUS HORMONES ON LIGANDIN CONCENTRATION AND ENZYME ACTIVITY OF IMMATURE RAT TESTIS.

The effect of the gonadotrophins, HCG, HMG and dexamethasone on testis ligandin concentration and GSH S-transferase activity in 26 day-old rats, 36 hours after hormonal injection is shown in Table 2.7. GSH S-transferase activity with CDNB as substrate was significantly increased by administration of HCG and HMG at a dose of 100IU and 10IU per testis respectively, although ligandin concentration when expressed per mg of supernatant protein was not significantly increased.

TREATMENT	CDNB umol/min/mg protein	LIGANDIN ug/mg protein
CONTROL	0.56 ± 0.05	1.54 ± 0.084
DEXAMETHASONE	0.67 ± 0.04	1.31 ± 0.21
HMG	0.89 ± 0.04**	1.60 ± 0.14
HCG	1.01 ± 0.17*	1.70 ± 0.12

Table 2.7 **Effect of various hormones on ligandin concentration, and GSH S-transferase activity in immature rat testis.** The values represent mean ± SEM of 5 individual determinations. Significant differences from controls shown by *$P < 0.005$ and **$P < 0.01$. Testes from 2 rats were used for each determination.

Discussion

Developmental studies, using radioimmunoassay, provided data which may elucidate the role of ligandin in steroidogenically active tissue. Previous ontogenetic studies of ligandin in these tissues are limited to qualitative immunocytochemistry (Bannikov **et al** 1979) using immunofluorescent techniques and to studies of enzyme activity with substrates known to react with several species of GSH S-transferases (Mukhtar **et al** 1979a,b). Consequently, previously available information regarding ligandin maturation in the steroid-producing tissues of the rat is insufficient to allow for any conclusions.

The characteristics of the rat ligandin radioimmunoassay used here were similar to those obtained by Bass **et al** (1977b). Non-specific binding of ^{125}I-ligandin was minimal, as was cross-reactivity with other intracellular proteins. The sensitivity of the binding assay (minimal detectable concentration) was capable of measuring less than 1 ng/tube. The anti-liver YaYa ligandin antiserum used in the assay (1817/10/04) had the highest affinity of all the antisera tested. Thus the assay was considered to be sufficiently sensitive and specific for the studies undertaken.

Several points of interest were noted with development of adrenal and gonadal ligandin in the rat. Firstly, relatively high levels of ligandin (ug ligandin per mg soluble protein) were present soon after birth, dropped during early life and rose with the onset of puberty. This pattern of development was similar when values obtained were expressed per wet weight of tissue. Secondly, a

close relationship was shown to exist between testicular ligandin and serum testosterone concentration and between ovarian ligandin and serum progesterone concentration during the developmental period.

A different pattern was observed when liver and kidney ligandin maturation was compared to that of the steroidogenic tissues, ie concentrations were low soon after birth, rose to 50% of adult values by approximately 25 days and then increased progressively to reach adult values by 60 days. These results for liver and kidney ligandin agree with the findings of Bass (1977). Baars **et al** (1980) demonstrated that rat liver GSH S-transferase activities towards CDNB and SO are low at birth and develop regularly during the first 5 postnatal weeks, thus exhibiting a pattern similar to that of rat liver ligandin maturation. The development of hepatic and renal ligandin has been claimed to be related to the maturation of organic anion transport mechanisms (Kirsch **et al** 1975). Phenobarbital pre-treatment of neonatal, prepubertal and adult rats showed an increase in hepatic ligandin concentrations at each stage of development, while adrenal and gonadal ligandin concentrations were not affected.

Ligandin in the testis is present mainly in the interstitial cells (Chapter 2, Section C), which is the principal site of steroidogenic activity and also considered to be the major source of androgen production. The possibility exists that testicular interstitial or Leydig cell development may bear a relationship to the maturation of ligandin in this tissue. Two distinct phases of Leydig cell activity have been described during development. The first consists of foetal cells which peak in number shortly after

birth and then regress and disappear. The second phase is seen during puberty and persists into adult life (Christensen 1975). Histological and physiological studies have confirmed the existence of a quiescent interval between the neonatal and pubertal period during which Leydig cell activity is markedly diminished (Niemi and Ikonen 1963, Knorr **et al** 1970). These phases of Leydig cell activity parallel the changes in ligandin concentration and testosterone secretion during development.

In humans serum testosterone levels parallel the decline of foetal Leydig cells (Forest **et al** 1973). Similarly in rats, serum testosterone levels are high at the end of foetal life and for the first few days of neonatal life. Thereafter testosterone levels drop and remain low until puberty (approximately 4 weeks) at which stage they increase abruptly to reach adult levels (Moger and Murphy 1977, Steinberger **et al** 1979). The results presented in this chapter are in accordance with these observations. The loss of foetal Leydig cells mirrors the loss of the foetal zone of human adrenal cortex. Recently Paz **et al** (1980) have shown that, although there is a quiescent period of steroidogenic activity in the rat testis during the 3rd to 5th week of life, the Leydig cells are still capable of responding to HCG from foetal life through to adulthood. The cells of the seminiferous tubules may also be capable of significant steroid synthesis, although the products and their functions may differ from those of Leydig cells (Lacy 1973).

In the data presented here, the effect of the gonadotrophins, HCG, and HMG (FSH and LH) on immature rat testis, showed that these hormones are able to significantly increase GSH S-transferase activity with CDNB as substrate. LH and HCG act on the Leydig

cells of the testis causing an increase in steroidogenesis and intratesticular injection of HCG has been shown to stimulate mRNA synthesis in the Leydig cells (Reddy and Villee 1975). FSH and pregnant mare serum gonadotrophin (PMSG) which mimics the actions of FSH, act on the seminiferous tubules causing an increase in general protein synthesis (Means and Hall 1968). Activity of the enzyme glucosamine 6-phosphate synthase in immature rat testis was increased following intratesticular injection of PMSG, while HCG and LH had no effect, suggesting that this enzyme is present only in the seminiferous tubules (Rukmini and Reddy 1981). As ligandin has been localised immunohistochemically to the interstitial cells of the testis (Chapter 2, Section C), stimulation of enzyme specific activity by HCG provides additional evidence regarding the distribution of this protein.

The results of the pregnant rat ovary study indicated that the GSH S-transferase and GSH peroxidase specific activities increase until mid-pregnancy (9-12 days) was reached. GSH S-transferase activity towards CDNB remained high until the 16 day measurement, decreasing by 21 days, while GSH peroxidase activity decreased earlier. This increase in ovarian enzyme activity during pregnancy did not correlate with relative ligandin concentration measured in the same specimens. Another GSH S-transferase form may be responsible for the increased enzyme activity. Immunocytochemical studies presented in Chapter 2, Section C, although not quantitative, suggest that GSH S-transferase AA may be induced during pregnancy and be responsible at least in part for the increase in enzyme activity with both CDNB and cumene hydroperoxide.

GSH S-transferase activities towards CDNB in the placenta at mid-pregnancy is found to be much higher (0.09 umoles/min/mg protein) than in the full-term placenta (0.047 umoles/min/mg protein) (Polidoro **et al** 1981b). The decline in GSH S-transferase and GSH peroxidase activity in the later stages of gestation suggest a progressive decrease in importance of this enzyme system in the placenta (Polidoro **et al** 1981b) and, as was shown in these studies, in the ovary.

The present findings show a close association between steroid activity and ligandin concentration in testis, ovary and adrenal during development. Although the specific role of ligandin and the GSH S-transferases in steroidogenically active tissue is not known, this correlation indicates that these proteins may be involved in several related functions which will be mentioned in the Concluding Discussion.

SECTION C

IMMUNOCYTOCHEMICAL STUDIES LOCALISING LIGANDIN IN THE STEROID-PRODUCING TISSUES OF THE DEVELOPING RAT

(a) Introduction

The presence of ligandin in appreciable amounts in testes, ovaries and adrenals at different stages of development has been demonstrated by means of radioimmunoassay (Chapter 2, Section B). Although several workers using the immunofluorescent technique have confirmed the presence of ligandin in the liver, kidney, small intestinal mucosa (Fleischner **et al** 1977), and more recently in the rat gonad cells (Bannikov and Tchipyseva 1979), others working in the field have failed to demonstrate the presence of ligandin in the rat steroid-producing tissues (Arias 1981). Localisation of ligandin in the rat adrenal or placenta has not been achieved.

The purpose of this immunocytochemical study was to localise ligandin in the cells of the endocrine tissues of the maturing rat using an indirect immunoperoxidase sandwich method, a technique which has the potential for avoiding some of the shortcomings of the immunofluorescence method (Taylor 1978). This method which can be used on formalin fixed, paraffin-embedded tissues has a sensitivity estimated to be 100 to 1000 times that of immunofluorescence. The antibody selectively localises the tissue antigen under investigation (Fig. 2.33 - Stage (1)). Instead of visualising this reaction by labelling the antibody directly (as in the immunofluorescence technique), visualisation is achieved by

using a second specific antibody (swine, antirabbit IgG) (Fig. 2.33 - Stage (2)), and utilising the bivalence of IgG which can then bind to the rabbit IgG of the PAP-immune complex (consisting of rabbit antibody to horseradish peroxidase and horseradish peroxidase antigen) (Fig. 2.33 - Stage (3)). Thereafter bound peroxidase is reacted with its substrates, hydrogen peroxide and diaminobenzidine. Enzyme action forms the insoluble polymeric oxidation product of diaminobenzidine which yields a brown colour in light microscopy.

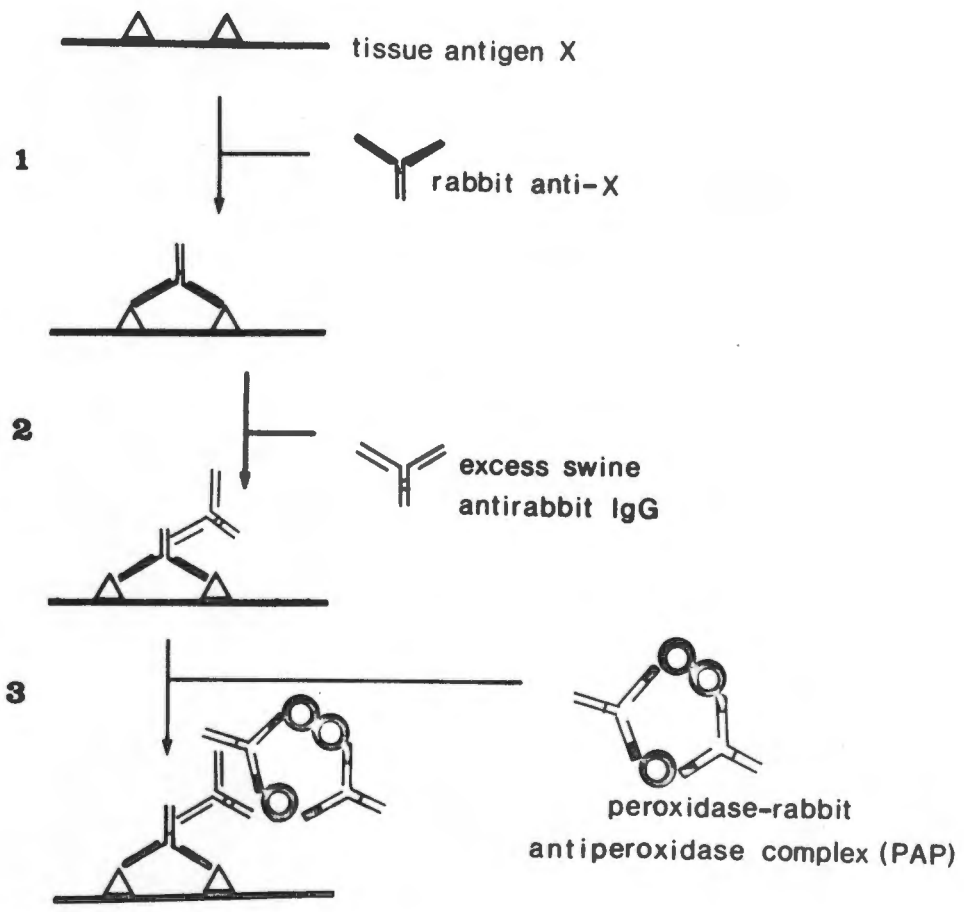


Fig. 2.33 **Peroxidase antiperoxidase (PAP) immune complex method.** Stage 1: diluted antiserum from rabbit species specific to tissue constituent X. Stage 2: excess of antiserum to IgG of rabbit, produced in swine species. Stage 3: PAP-complex. Stage 4 (not shown): Localisation of peroxidase site demonstrated by means of chromogenic substrate, diaminobenzidine tetrahydrochloride-hydrogen peroxide. Adapted from Sternberger 1974.

(b) Materials

Normal swine serum, swine anti-rabbit IgG and PAP-complex were purchased from Dako Immunoglobulins Ltd., 22, Guldborgvej, 2000 Copenhagen F, Denmark. H_2O_2 and 3,4',3', tetraminobiphenyl-hydrochloride (3-3 diaminobenzidine) were obtained from BDH.

(c) Methods

The indirect peroxidase-antiperoxidase (PAP) method was used to locate ligandin in buffered formalin fixed, paraffin-embedded rat tissues. The method (Fig. 2.33), described in detail by Taylor (1978), consists of the following steps. Sections were dewaxed and taken into absolute alcohol, followed by blocking in both 0,3% H_2O_2 in methanol for 30 minutes, and exposure to 1:20 dilution of normal swine serum for 15 minutes. Sections were then incubated for 30 minutes at room temperature (24°C) with antiligandin at a 1:200 dilution which gave no background staining. Various dilution and incubation times were tested, the above giving the most consistent and comparable results. This was followed by incubation in 1:20 swine antirabbit IgG for 30 minutes at room temperature and then PAP complex for 30 minutes at room temperature. Sections were washed for 30 minutes in buffer A between each step on a magnetic stirrer.

Buffer A (0,05M phosphate / 0.14M NaCl pH 7.4), was used for all dilutions and washes. The colour reaction was developed for 5 minutes in a solution of 5mg 3-3 di-aminobenzidine in 10ml Buffer A to which 0,1ml of 1% H_2O_2 was added. After washing for 15 minutes, sections were counter-stained with Mayers haematoxylin, dehydrated in alcohol, cleared in Xylol and mounted with Canada Balsam.

Uninoculated rabbits from the colony provided non-immune control sera which were diluted in the same way.

Besides using a non-immune control serum, certain agents were omitted eg antisera or PAP from the staining schedule to see if a non-specific reaction occurred between any of the reagents and the tissue. All proved to be negative. As a final control the anti-ligandin was absorbed with purified ligandin. The absorbed serum showed no specific staining.

Two antisera, anti-liver ligandin (YaYa) antiserum (rabbit 1234) and anti-testis "ligandin" antiserum (rabbit 255) were used at similar dilutions (1:200).

(d) Results

PAP - STAINING WITH ANTI-LIVER LIGANDIN ANTISERUM

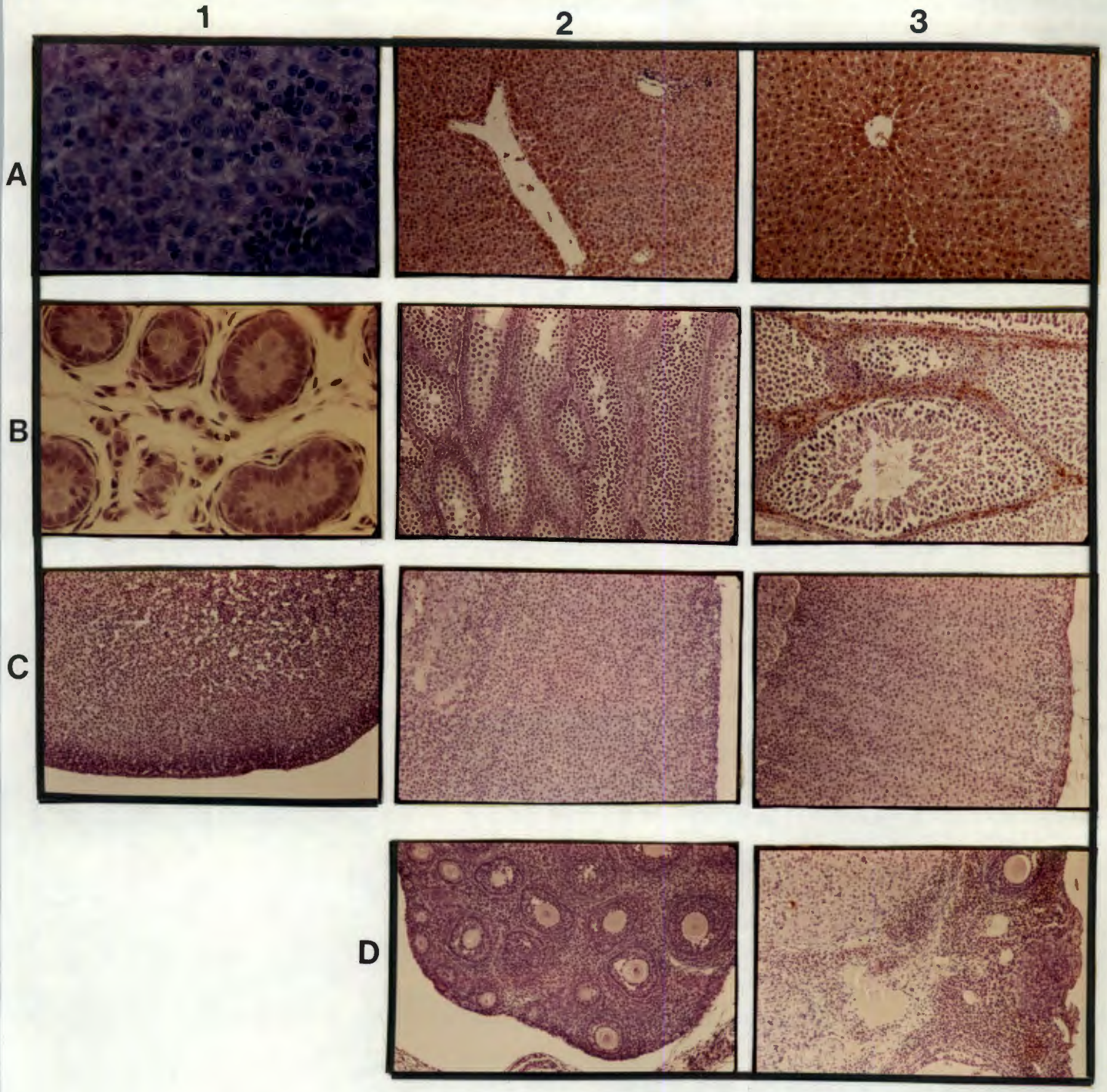
Immunocytochemical preparations using a specific antiserum to liver ligandin and the peroxidase-antiperoxidase (PAP) technique (Fig. 2.34) showed sparse staining of hepatocytes from 4-day-old rat livers, the rest of the tissue remaining negative. By 20 days postpartum a stronger, more diffuse staining was evident, particularly at the edge of the portal tract. At 60 days the adult liver tissue showed a strongly positive, brown reaction product, the intensity of staining for ligandin increasing from the central vein to the portal tract.

All the tissue sections in which the anti-liver ligandin antiserum was replaced by non-immune control serum showed no brown colour at all (Fig. 2.36).

The seminiferous tubules of neonatal testis appeared solid with the epithelium composed principally of Sertoli cells, while the surrounding interstitium was more cellular than that seen in adult sections (Fig. 2.34, 1B). Specific staining was not evident at this stage of development. Sections of early pubertal testis (20 days) contained larger seminiferous tubules, separated by a slight amount of interstitial, connective tissue containing groups of Leydig cells (Fig. 2.34, 2B). No reaction product was seen. By 60 days positive staining was present, but was limited to the interstitial cells (3B).

Early neonatal adrenal tissue did not show any specific staining in the glomerular, fascicular and reticular zones of the adrenal

Fig. 2.34 PAP-staining with anti-liver (YaYa) ligandin antiserum, of liver **(A)**, testis **(B)**, adrenal **(C)** and ovary **(D)** in neonatal **(1)**, pubertal **(2)** and adult **(3)** rats. Magnifications of 1A and 1B x 600; 1C, 2A, 2B, 2C, 2D, 3C and 3D x 60; 3A and 3B x 150. See Chapter 2, Section C for details.


1
2
3
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D

cortex or in the medulla (1C). Pubertal and adult adrenal cortex show equivocal staining only (2C and 3C).

No staining was evident in the prepubertal ovary (1D). Weakly positive staining of the stromal cells surrounding the follicles and corpus luteum was present in mature adult ovaries (3D). Pregnant ovary showed a similar response with this antibody, while placental tissue (not shown) was negative.

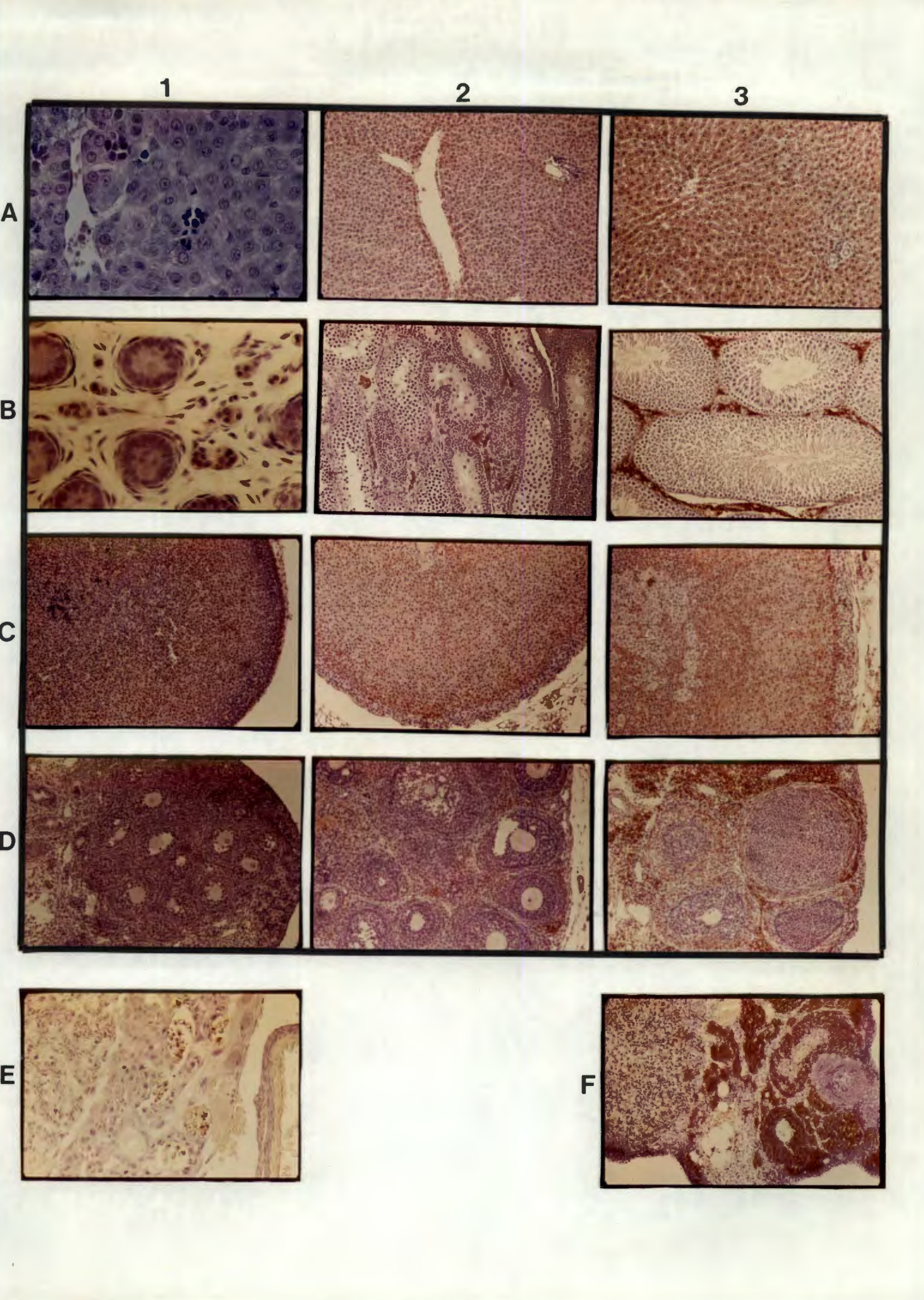
PAP - STAINING WITH ANTI-TESTIS LIGANDIN ANTISERUM

Antiserum raised against standard preparations of testis "ligandin", described in Chapter 2, Section A, was also used for immunocytochemical studies.

The intensity of staining was studied in various tissues using the PAP technique and the anti-testis ligandin antiserum (Fig. 2.35). The liver showed faint non-specific staining at 4 days of age (1A). By 20 days, however, a uniform light-brown stain of the hepatocytes was noted, with slightly darker staining at the portal tract edge (2A). At 60 days of age, the liver showed a strongly positive stain which was slightly darker in the cells surrounding the central vein (3A).

4-day-old testis showed slight staining of the interstitial cells (1B). At 20 days scattered staining of these cells could be seen (2B), and by 60 days very dense staining of the interstitial cells was observed (3B). The seminiferous tubules remained negative.

Fig. 2.35 PAP-staining with anti-testis ligandin antiserum, of liver (A), testis (B), adrenal (C) and ovary (D) in neonatal (1), pubertal (2) and adult (3) rats. (E), placenta and (F), ovary of a pregnant rat at 12 days gestation. Magnifications of 1A, 1B and E x 600; 1C, 1D, 2A, 2B, 2C, 2D, 3D and F x 60; 3A, 3B, and 3C x 150.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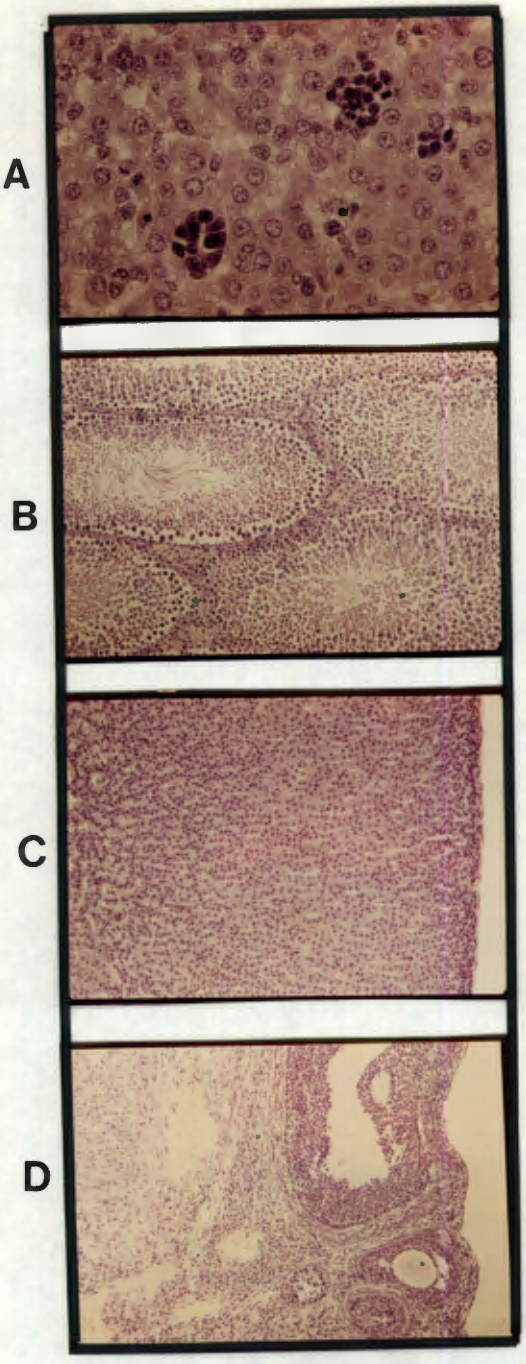
F

4-day-old adrenal glands showed specific staining of the capsule and diffuse staining of the reticular zone of the cortex (1C). By 30 days distinct staining was seen extending from an area immediately beneath the glomerular zone, through the fascicular zone to the reticular cells of the cortex (2C). The medulla did not stain. In the adult adrenal gland there was dense staining of the cortex but not of the medulla (3C).

Staining of the stromal cells of the ovary was just discernable by 9 days. The immature follicles were negative (1D). At 20 days, reaction product was present in the stromal cells surrounding the developing follicles (2D). A similar pattern with increased intensity was found in the adult ovary (3D). In the pregnant ovary the stromal cells stained with an even greater intensity, and for the first time a faint, diffuse staining was seen in the corpus luteum (F).

The clear trophoblast cells of the placenta showed slight staining with the anti-testis ligandin antiserum (E).

Fig. 2.36 PAP-staining with non-immune control serum of neonatal liver (A) (x 600), adult testis (B) (x 60), adrenal (C) and ovary (D).


A
B
C
D

FOOTNOTE TO FIGS 2.34 AND 2.35

Note: The histology slides were filed and stored in the dark. After storage for one year, it was noted that the brown reaction product had faded considerably. The mounting solution, Canada Balsam, which is an acidic, natural product, may have contributed to this occurrence. Use of a synthetic, neutral mounting medium would be more advisable.

(e) Discussion

Karavanova **et al** (1980), using immunofluorescent techniques found ligandin to be uniformly distributed in the liver of foetal and newborn rats. By 4 days postpartum the number of ligandin positive cells had decreased sharply. Their data suggests adult liver lobule formation is complete by the 14th day postpartum, at which stage synthesis of ligandin abruptly increases. Campbell **et al** (1980) showed positive staining of isolated hepatocytes in a 16mm-long human foetus, while heavier staining of numerous hepatocytes was observed in a larger 50 mm foetus.

Livers of 1-day-old rats were examined (results not shown) using anti-liver YaYa ligandin and the PAP technique. Staining for ligandin was similar to that seen in 4-day-old livers. The immunocytochemical studies of ligandin in rat liver at different stages of development (Figs 2.34 and 2.35), correlates with the radioimmunoassay measurements of liver ligandin (Chapter 2, Section A) during maturation. These findings support the studies done by Fleischner **et al** (1976), Hales **et al** (1976a) and Bass **et al** (1977b), which show ligandin concentration to be considerably lower in the neonatal rat than in the adult animal. This may be related to the functional capacity of the liver to handle organic anions which only develops after the first week of life. A gradient of ligandin-positive cells increasing from the periportal to the centrilobular areas of the liver is seen in Fig. 2.34, 2A, a finding similar to that of Karavanova **et al** (1980).

It is not intended here to compare the PAP reaction product of anti-liver ligandin antiserum with that of anti-testis ligandin antiserum, but rather to compare the reaction which takes place in the different tissues with the individual antiserum. The two antisera, although used at the same dilution and antibody titre, have different affinities and specificities. Even antisera raised against the same antigen, in different rabbits, may have different properties. Therefore the only comparisons which are valid are those in pattern and intensity of staining of different tissues with the same antiserum. The reaction of anti-liver ligandin antiserum in adult hepatic tissue is strongly positive, while its reaction in the steroid-producing tissues is weak. Normal adult kidney and intestine show strong staining with this antiserum (not shown). Thus antiserum raised against liver ligandin appears to react strongly with tissues involved in storage and transport (liver, kidney and gut) while steroid-producing tissues evoke a weak response.

A different pattern is observed when antiserum raised against testis "ligandin" is used. The substance which reacts with this antiserum is distributed diffusely in the hepatic tissue, while a selective, intense reaction is observed in the steroid-producing tissues of testis, ovary, adrenal and placenta. The reaction is seen in developing tissues soon after birth, increasing progressively in intensity during maturation. Examination of the reactions of this antiserum with kidney and gut sections revealed relatively little staining (not shown). Therefore, the anti-testis "ligandin" antiserum behaves differently to the anti-liver ligandin antiserum in that the steroid-producing tissues are strongly positive, while a weaker response is seen in the liver, kidney and gut.

These immunocytochemical studies were carried out before it was established that standard preparations of testis ligandin contain another GSH S-transferase (Chapter 2, Section A). It can, therefore, be assumed that anti-testis ligandin antiserum has been raised against both ligandin (YaYc) and GSH S-transferase AA (YcYc), and this must be considered when attempts are made to interpret these findings.

A radioimmunoassay for GSH S-transferase AA has not yet been established. No published studies are available which describe the tissue distribution of this protein. Results obtained in Chapter 2, Section B show that GSH S-transferase AA is a major basic transferase of testis and and ovary, and may thus account for the intense staining seen in the testis, ovary, pregnant ovary and perhaps adrenal sections.

Immunocytochemical studies do not provide quantitative results and can only show the distribution of the protein in question and its concentration relative to other tissues, provided sections were prepared under identical conditions. This technique is probably not as sensitive as radioimmunoassay which is able to detect very low concentrations of protein. It is therefore not surprising that the radioimmunoassay using anti-liver ligandin antiserum was able to detect ligandin in neonatal steroidogenic tissues (Chapter 2, Section B) while immunocytochemical techniques using this antiserum were probably not sensitive enough to detect the low concentrations found in these tissues. These immunocytochemical studies did demonstrate that the increased concentration of ligandin, measured by radioimmunoassay, in cytosol prepared from whole-organ homogenates at different stages of development, is due to increased amounts of ligandin in specific cell types.

Chapter 3

CONCLUDING DISCUSSION

AND DIRECTION FOR FUTURE STUDIES

CONCLUDING DISCUSSION

The use of the term Ligandin to describe several, distinct proteins has resulted in considerable confusion. It would appear that different tissues and different methods of isolation do not neccessarily give rise to the same protein with the same subunit composition. This study attempted to provide insight into these anomalies by making use of various purification techniques and by studying other tissues where ligandin is present in appreciable quantities. Initially, comparisons between liver preparations and those obtained from steroidogenic tissue (testis, ovary and adrenal) isolated by the standard procedure, indicated that there were differences in subunit composition. Further studies however, showed that identity betweeen liver ligandin and testis ligandin existed, indicating that previously described differences (Bhargava **et al** 1980a) were not valid since an extra chromatographic step produced a protein with equal amounts of Ya and Yc which reacted with anti-liver ligandin YaYa.

In the course of this study the different enzyme forms of the GSH S-transferases were investigated by affinity chromatographic techniques including S-hexylglutathione bound to epoxy-activated Sepharose. In addition the GSH S-transferases were separated using a chromatofocusing technique which, when refined, may reveal the presence of additional, previously undescribed forms of these enzymes.

Different combinations of the Ya, Yb and Yc subunits give rise to the multiple forms of the GSH S-transferases which differ in isoelectric point and substrate specificity. The overlapping specificities displayed by the different transferases may be accounted for by the presence of certain of the subunits in several peaks. Structural characterisation of these proteins has contributed to the understanding of the many catalytic and binding functions attributed to them.

All organs and tissues are potential targets for environmental toxins. Such exposure could be particularly critical in the testis and ovary since it may result in increased frequency of germ cell mutations and result in genetically-linked disease. Rat testis contains high levels of ligandin and the GSH S-transferases.

Although there is no reported evidence linking these proteins to androgen metabolism or activity, there are several possible roles for ligandin in the steroidogenic tissues. Firstly it may act as a low affinity, high capacity binding protein for steroids. Purified rat liver ligandin has been shown to bind non-covalently to a wide variety of steroids and their metabolites **in vitro**, including estrone, estradiol, cortisol, testosterone, pregnenolone sulphate and dihydroepiandrosterone sulphate with association constants ranging from 10^5 to 6×10^5 M^{-1} (Ketterer **et al** 1976). Ligandin may be capable of binding more steroids **in vivo** than these results indicate. As such, the high levels of ligandin contained in the testis may be involved in the transport of steroids or the intermediates formed during steroidogenesis. Although it would seem plausible that hormone receptors with much higher affinities and capacities would be responsible for the

binding of steroids, the interstitial tissue of the testis does not appear to have specific receptors for these products, unlike target tissue of the body. The presence of high affinity binders in the steroid-synthesising tissue would retard the passage of hormones from their site of production to their site of action. Ligandin may be also be involved in the uptake of steroids from the plasma to the cell interior. Steroids have affinity constants for human serum albumin in the range 10^4 - 10^5 M^{-1} which is lower than that reported for ligandin. This may be a passive process where ligandin influences the efflux of steroids from the cell.

Secondly, ligandin and the GSH S-transferases may be involved in either synthesis or metabolism of steroids and other compounds. Ligandin has been shown to have delta^5-3-ketosteroid isomerase activity (Benson **et al** 1977). In the liver ligandin also has been shown to effect the catalysis of prostaglandin endoperoxides to prostaglandins F_2 and E_2 (Christ-Hazelhof **et al** 1976).

A third role for these enzymes in the steroid-producing tissue may be to detoxify xenobiotics. The GSH S-transferases are able to detoxify reactive electrophiles, thus allowing for excretion of these foreign compounds. Another possible enzymatic function would be to inhibit lipid peroxidative damage which can be initiated by the reactive products of metabolism and oxidation (see Chapter 1, Section C). Lipid hydroperoxides can be described as the endogenous substrates for the GSH S-transferases which exhibit GSH peroxidase II activity. The data presented here have shown that gonadal tissue of the rat displays this activity. These proteins could therefore play a vitally important protective role in the steroidogenically active tissues of the rat.

AREAS FOR FUTURE RESEARCH

It is hoped that the results of this study will stimulate further investigations of the function(s) of ligandin in the steroid-producing tissues. The correlation between steroid hormone synthesis and gonadal and adrenal ligandin development could be studied further by means of modifying physiological occurences, eg by delaying or inducing puberty. The former may be achieved by means of hypophysectomy in prepubertal animals, while the latter could result from the administration of prolactin or other hormones. Hypophysectomies are difficult to perform and non-specific changes may complicate interpretation of results.

Another approach would be to examine the ontogeny of other GSH S-transferases in the steroidogenically active tissues and their relationship with the development of ligandin. Measurements using specific radioimmunoassays for these proteins would provide much information. This would require production of monospecific antibodies to the individual GSH S-transferases. However, immunological cross-reactivity which occurs between GSH S-transferases A and C and possibly between B and AA, would prevent specific quantitation of these transferases in the different tissues. The exact nature and distribution of the other GSH S-transferases in steroid-synthesising tissues requires further study and will depend on their purification and successful antisera production.

In terms of enzyme function, the relative importance of GSH S-transferases B and AA as GSH peroxidases requires further study. This additional information may elucidate their role in the prevention of lipid peroxidation which could have serious effects in reproductive organs.

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Identity of ligandin in rat testis and liver

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1. One of the main problems in the field of multifunctional proteins such as ligandin is the possibility that multiple forms and isoproteins may exist. Because liver ligandin [GSH (reduced glutathione) *S*-transferase B] consists of equal amounts of Ya (22000 Da) and Yc (25000 Da) subunits, and testis ligandin, prepared by the standard technique of anion-exchange and molecular-exclusion chromatography, contains more Yc subunit than Ya, it has been claimed that testis and liver ligandin are different entities. 2. We purified testis ligandin by immunoaffinity chromatography and have obtained a product identical with liver ligandin (Yc = Ya). This suggests that the differences previously described may be due to contamination of testis ligandin by a closely related species. In fact sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic analysis of testis GSH *S*-transferases separated by CM-cellulose chromatography showed that GSH *S*-transferase AA, present in large amounts, migrated in the same region as Yc subunit. 3. Testis ligandin prepared by the standard technique was similar to that reported [Bhargava, Ohmi, Listowsky & Arias (1980) *J. Biol. Chem.* **255**, 724–727] and contained more Yc subunit than Ya. CM-cellulose chromatography of this 'pure' preparation revealed significant amounts of GSH *S*-transferase AA migrating as Yc subunit, in addition to ligandin consisting of equal amounts of Ya and Yc subunits. 4. Our studies show that testis ligandin is identical with liver ligandin. Previously described differences are due to a contaminant identified as GSH *S*-transferase AA.

Ligandin is a basic protein (pI 8.9–9.3) present in abundance in the livers of all air-breathing vertebrates studied (Litwack *et al.*, 1971; Arias *et al.*, 1976). It has also been described in intestinal mucosa, proximal renal tubules and steroid-producing tissues of the rat (Fleischner *et al.*, 1972; Kirsch *et al.*, 1975; Bass *et al.*, 1977*a*). Rat liver ligandin has the chemical structure, enzymic activity, ability to bind non-substrate ligands and immunological properties of GSH *S*-transferase B (EC 2.5.1.18) (Habig *et al.*, 1974). SDS/polyacrylamide-gel electrophoresis of rat liver ligandin in a discontinuous system revealed two non-identical subunits, Ya (22000 Da) and Yc (25000 Da) (Bass *et al.*, 1977*a*). By using non-denaturing systems YaYa and YaYc dimers have been purified (Bass *et al.*, 1977*a*; Hayes *et al.*, 1979). It has been suggested that ligandin exists as a YaYc heterodimer (Listowsky *et al.*, 1976; Bhargava *et al.*, 1978) or as a mixture of a YaYa homodimer and a YaYc heterodimer (Carne *et al.*, 1979). Indeed, Hayes *et al.* (1979) distinguish between ligandin (YaYa) and GSH *S*-transferase B (YaYc). However, this does not explain the finding by Bass *et al.* (1977*a*) and by Bhargava *et al.* (1980) that testis ligandin consists predominantly of a Yc fraction. We have investigated the possibility that a substance other than ligandin may contribute to this electrophoretic finding and to the differences in function attributed to testis ligandin. Our data suggest that GSH *S*-transferase AA accounts for part of the predominant Yc band seen on SDS/polyacrylamide-gel electrophoresis of testis preparations, that this protein is a major contaminant of testis ligandin purified by standard techniques, and that testis ligandin is made up of equal amounts of Ya and Yc subunits and is not different from liver ligandin.

Abbreviations used: GSH, reduced glutathione; SDS, sodium dodecyl sulphate.

Materials and methods

Chemicals

NADPH and bovine serum albumin were purchased from Miles Laboratories (Pty.) Ltd.,

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Goodwood, South Africa. Glutathione reductase (Type IV) prepared from yeast was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Cumene hydroperoxide (70% in cumene) was from Fluka A.G., Buchs, Switzerland. Complete Freund's Adjuvant and Agar Noble were from Difco Laboratories, Detroit, MI, U.S.A. Acrylamide, *NN'*-methylenebisacrylamide and SDS were from BDH Chemicals, Poole, Dorset, U.K. CNBr-activated Sepharose 4B was from Pharmacia, Uppsala, Sweden. Donkey anti-rabbit globulin was from Wellcome Reagents, Beckenham, Kent, U.K. ^{125}I was from The Radiochemical Centre, Amersham, Bucks., U.K. Dimethyl suberimidate, 2HCl was from Pierce Chemical Co., Rockford, IL, U.S.A. All reagents used were of analytical grade.

Purification of ligandin

Ligandin was purified from the liver and testis of Long–Evans rats (250–300g) as described by Kirsch *et al.* (1975) and Bhargava *et al.* (1980). In brief, cytosol prepared by centrifugation at 100000*g* for 120min was obtained from 100g of rat tissue, was dialysed against 0.01M-Tris/HCl, pH8.8 (buffer A), and chromatographed on a column of TEAE-cellulose (triethylaminoethyl-cellulose) in buffer A. The single protein peak eluted was pooled, concentrated (Amicon cell, PM-10 membrane) and chromatographed on a column of Sephadex G-100 in 0.01M-sodium phosphate buffer (pH7.4)/0.1M-NaCl. Fractions exhibiting enzyme activity with 1-chloro-2,4-dinitrobenzene were pooled, concentrated, dialysed against buffer A and chromatographed on a column of QAE-Sephadex (quaternary aminoethyl-Sephadex) A-50 in buffer A. The single protein peak eluted was pooled and stored at 4°C.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in 0.1% SDS was performed on vertical slab gels (Laemmli, 1970). The system comprised a 3% (w/v) stacking gel (3cm × 14cm × 0.2cm) in 0.14M-Tris/HCl buffer, pH6.8, and a 5–25% (w/v) gradient gel (20cm × 14cm × 0.2cm) in 0.56M-Tris/HCl buffer, pH8.8. Samples were prepared as described by Maizel (1971). Gels were fixed and stained for 2.5h in 10% (v/v) trichloroacetic acid/1.0% Coomassie Brilliant Blue R in methanol/water (1:2, v/v) and destained in 5% methanol/7% acetic acid (both v/v).

Molecular weights were determined by comparison with standards of known molecular size.

Immunological studies

Antisera to liver and testis ligandin were raised by injecting 50μg of purified proteins in Freund's complete adjuvant into popliteal lymph nodes of male albino rabbits. Thereafter, 50μg of ligandin in adjuvant was injected into multiple subcutaneous sites at 3-week intervals. Animals were bled 10 days after booster inoculations. The separated sera were tested for specific antibody production by immunodiffusion and immunoelectrophoresis (Bass *et al.*, 1977*a*). Radial immunodiffusion used 1.2% agar gels (Ouchterlony, 1958) and immunoelectrophoresis used 1% agar gels (Bass *et al.*, 1977*a*). Gels were thoroughly washed with iso-osmotic saline (0.9% NaCl) before staining with Amido Black. Affinity reagents, consisting of the globulin fraction either of anti-(rat liver ligandin) serum or anti-(rat testis ligandin) serum prepared by $(NH_4)_2SO_4$ precipitation (Hebert *et al.*, 1973), were coupled overnight at 4°C to CNBr-activated Sepharose 4B. The remaining active groups were treated with 1M-ethanolamine at pH8 for 2h, after which several washing cycles (0.1M-acetate buffer/1M-NaCl, pH8.0, and 0.1M-borate buffer/1M-NaCl, pH4.0) were carried out to remove non-covalently adsorbed protein. Thus 98.4% of liver antibody and 94.2% of testis antibody were coupled. Partially purified rat tissue ligandin (TEAE-cellulose eluate, Fig. 3) was applied to a column (10cm × 0.9cm) of affinity material. After unbound protein had been washed from the column, immunoreactive material was eluted with 0.1M-glycine, pH3.0. The pH of all fractions was immediately adjusted to 7.4 with 1M-Tris buffer.

Radioimmunoassay of ligandin from rat tissues was performed by the double antibody method of Morgan & Lazarow (1963), with anti-(rat liver ligandin) serum as the first antibody. The assay procedure and the antisera used in this study have been described in detail (Bass *et al.*, 1977*b*).

Separation of glutathione S-transferases

Rat liver and testis supernatants were chromatographed on a DEAE-cellulose column (2.6cm × 35cm) in 0.01M-Tris/HCl buffer, pH8.0. Fractions exhibiting absorbance at 280nm were pooled and concentrated to a volume of 10ml (Amicon cell, PM-10 membrane). After dialysis into 0.01M-potassium phosphate buffer, pH6.7 (buffer C), the preparation was applied to a CM-cellulose column (2.6cm × 30cm) previously equilibrated with buffer C (Habig *et al.*, 1974). A 600ml linear gradient of 0–120mM-HCl in buffer C was applied to the column, and 3ml fractions were collected and stored at 4°C before assay.

In a separate experiment, testis ligandin prepared as described by Bhargava *et al.* (1980) was chromatographed on a CM-cellulose column (1.6cm × 10cm) as described above.

Assays

Glutathione *S*-transferase activity was measured by following the conjugation of GSH with either 1-chloro-2,4-dinitrobenzene at 340nm or 1,2-dichloro-4-nitrobenzene at 345nm (Habig *et al.*,

1974). Glutathione peroxidase activity was assayed, as described by Prohaska & Ganther (1977), with 0.1mM-cumene hydroperoxide dissolved in 10% (v/v) ethanol. One enzyme unit is defined as 1μmol of NADPH oxidized/min. Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Covalent cross-linking of ligandin

Ligandin in 0.05M-triethanolamine buffer (pH8.0)/0.05M-KCl/0.4M-NaCl was mixed with dimethyl suberimidate at 4°C to give final concentrations of 0.3mg of protein/ml and 30mg of cross-linking reagent/ml. The reaction was terminated by addition of an equal volume of 0.3M-ethanolamine, before electrophoresis in SDS on polyacrylamide gradient gels (Davies & Stark, 1970).

Results

Purification of testis ligandin

Specific activity, with 1-chloro-2,4-dinitrobenzene and GSH as substrates, was determined at each stage of testis ligandin purification by the method of Kirsch *et al.* (1975) and Bhargava *et al.* (1980). The mean specific activity of the final product of four separate testis preparations was 15.6μmol/min per mg of protein. SDS/polyacrylamide-gel electrophoresis of each stage of purification is shown in Fig. 1. The final preparation of testis ligandin has more Yc subunit than Ya. These findings and those of difference spectroscopy (results not shown) are identical with those obtained by Bhargava *et al.* (1980), and suggest that we are dealing with the same preparation.

Antisera

Radial immunodiffusion and immunoelectrophoresis of anti-(liver YaYa ligandin) or anti-(liver YaYc ligandin) against liver and testis cytosol revealed single lines of identity. Antisera raised against testis 'ligandin' revealed a single line of identity when immunoelectrophoresed against testis cytosol. However, a faint second line was noticed on immunoelectrophoresis against liver cytosol (Fig. 2).

Affinity chromatography

The profile obtained when the testis protein peak (TEAE-cellulose eluate) was applied to an anti-(liver ligandin) affinity column is shown in Fig. 3. Fractions were analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 4). The TEAE-cellulose eluate had more Yc subunit than Ya. In contrast, the material recognized by the matrix-coupled liver ligandin antibody (elution volume 85ml) had equal quantities of the two subunits. Unbound fractions (elution volume 15ml) contained no Ya subunit, but

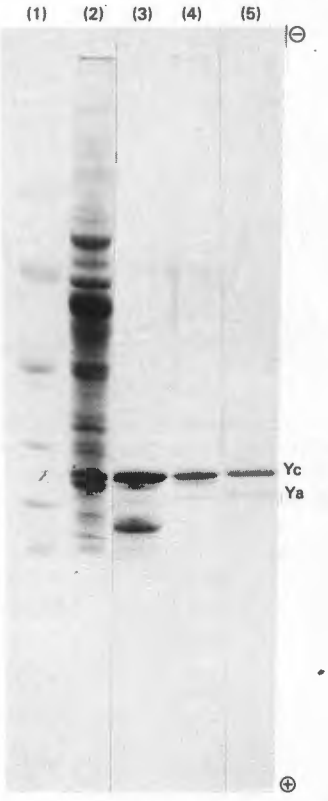

Fig. 1. *SDS/polyacrylamide (5–25%, w/v)-gradient-gel electrophoresis at each stage of testis ligandin purification*

(1) Molecular-weight markers (94000, 67000, 43000, 30000, 20100, 14400 daltons). (2) Testis cytosol (100000*g* supernatant). (3) Protein peak after TEAE-cellulose chromatography. (4) Peak exhibiting GSH–chlorodinitrobenzene-conjugating activity after Sephadex G-100 chromatography. (5) Pooled fractions from QAE-Sephadex A-50 eluate. Between 10 and 30μg of protein was loaded per sample. Samples were prepared as described by Maizel (1971).

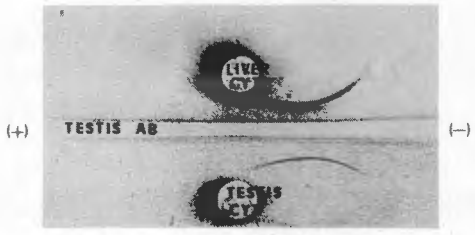

Fig. 2. *Immunoelectrophoresis of testis ligandin antibody against liver and testis cytosols in 1% agar gel*

The peripheral wells contain liver cytosol and testis cytosol (CYT; 1μg in 10μl). The central trough contains 10μl of rabbit antiserum (AB) to testis ligandin. Electrophoresis was performed in 0.07M-veronal buffer, pH8.7, at 12mA for 2h. The precipitates were stained with Amido Black.

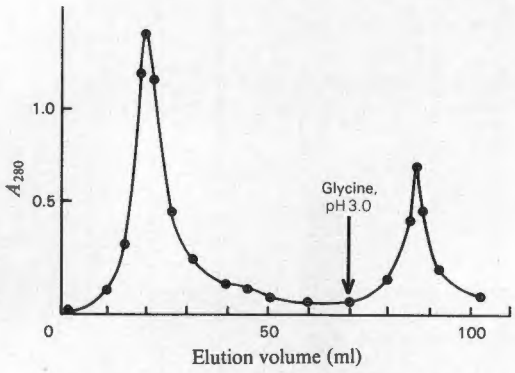

Fig. 3. *Chromatography of partially purified (TEAE-cellulose eluate) rat testis ligandin on anti-ligandin–Sepharose 4B affinity columns*

The elution profiles for anti-(liver ligandin) and anti-(testis ligandin) affinity columns are identical. Unbound material was eluted in the first peak (elution volume 10–40 ml). Bound material was eluted with 0.1 M-glycine, pH 3.0 (elution volume 80–95 ml).

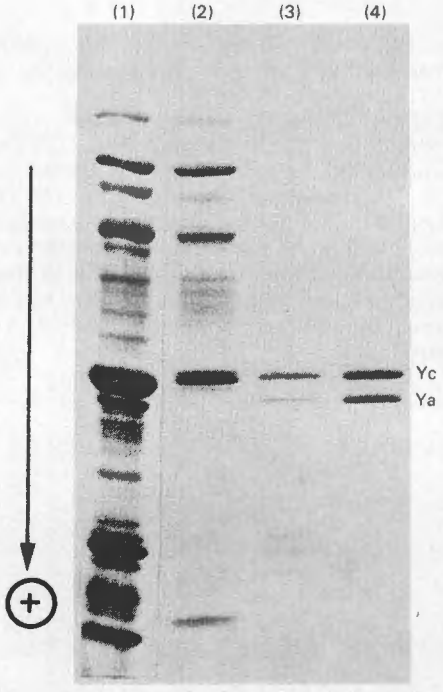

Fig. 4. *SDS/polyacrylamide-gel electrophoresis of peaks eluted after anti-(liver ligandin)-Sepharose 4B affinity chromatography*

(1) Testis TEAE-cellulose eluate (starting material); (2) material at elution volume 15 ml; (3) material at elution volume 30 ml; (4) material after elution with 0.1 M-glycine, pH 3.0. See Fig. 3 for details.

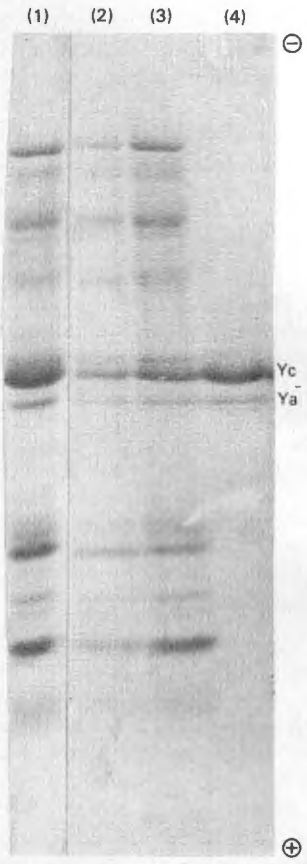

Fig. 5. *SDS/polyacrylamide-gel electrophoresis of eluate from anti-(testis ligandin)–Sepharose 4B affinity column*

(1) Testis TEAE-cellulose eluate (starting material), (2) material eluted at 15 ml, (3) material eluted at 25 ml, (4) material eluted after application of 0.1 M-glycine buffer, pH 3.0 (elution volume 80–95 ml). See Fig. 3 for details.

later fractions (elution volume 30 ml) contained trace amounts of this species.

When the same testis starting preparation was applied to a column of anti-(testis ligandin) affinity material, SDS/polyacrylamide-gel electrophoresis (Fig. 5) revealed more Yc subunit than Ya in the immunoreactive peak (elution volume 85 ml).

Elution of testis GSH S-transferases from CM-cellulose

CM-cellulose chromatography resolved liver DEAE-cellulose eluate into five peaks of GSH *S*-transferase activity (GSH–chloronitrobenzene conjugating activity) similar to those described by Habig *et al.* (1976). Fig. 6 shows the results of a similar experiment with testis DEAE-cellulose

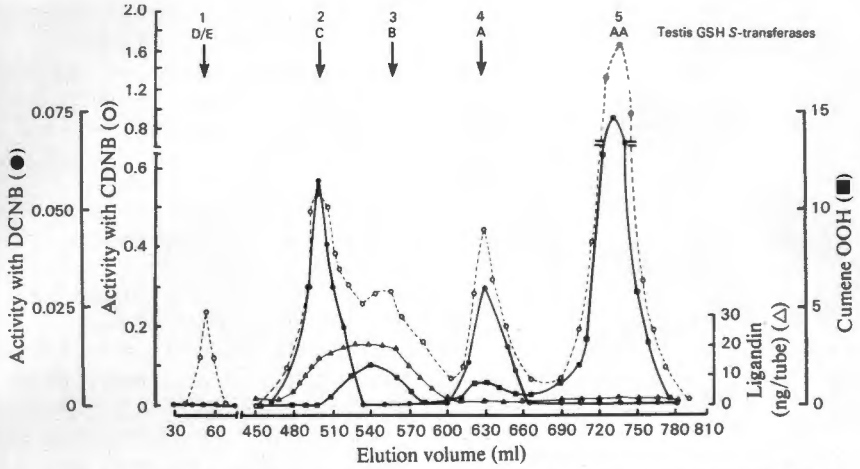

Fig. 6. *Elution pattern from CM-cellulose of testis DEAE-cellulose eluate at pH 6.7*
GSH *S*-transferase activity was assayed with 1-chloro-2,4-dinitrobenzene (CDNB, ○) and 1,2-dichloro-4-nitrobenzene (DCNB, ●). Enzyme activity is expressed as μmol/min per ml. GSH peroxidase II activity was assayed with cumene hydroperoxide (cumene HOOH) as substrate (■). Results were expressed as μmol of cumene hydroperoxide/min, by using the absorption coefficient (ε) for NADPH of 6×10^3 litre · mol^{-1} · cm^{-1}. Ligandin as measured by anti-(liver ligandin) radioimmunoassay is expressed as ng of ligandin/tube (△). The KCl gradient was started after peak 1 had been eluted (elution volume 0–180 ml).

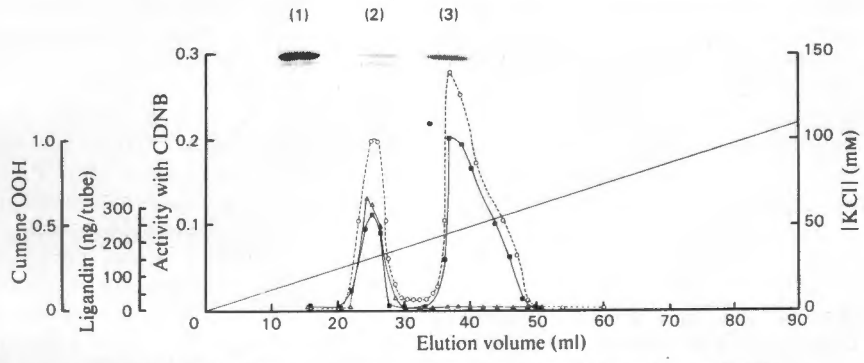

Fig. 7. *CM-cellulose chromatography of 'pure' testis ligandin at pH 6.7*
The KCl gradient (——, 0–120 mM) was started at the beginning of the column run. GSH *S*-transferase activity was assayed with chlorodinitrobenzene (CDNB, ○) and expressed as μmol/min per ml. GSH peroxidase II activity was assayed with cumene hydroperoxide (cumene HOOH) and expressed as μmol/min (■). Radioimmunoassay with anti-(liver ligandin) measured ng of ligandin per tube (△). Comparisons of the standard preparation of testis ligandin (1) with fractions from each peak (2 and 3) by SDS/polyacrylamide-gel electrophoresis are shown.

eluate. Peak 1 (elution volme 40–70 ml) represents material that did not bind to the column at pH 6.7. After initiation of the salt gradient, a further four peaks were obtained, peak 2 (elution volume 460–530 ml), peak 3 (530–600 ml) peak 4 (600–660 ml) and peak 5 (690–780 ml). Dichloronitrobenzene-conjugating activity was limited to peaks 2 and 4. GSH peroxidase II activity with cumene hydroperoxide as substrate was present in peak 5 and to a lesser extent in peaks 2 and 4. GSH peroxidase I activity was not measured in this experiment. Immunoreactivity determined by radioimmunoassay with antibody raised against liver ligandin was present in fractions eluted between 480 and 600 ml. No immunoreactive material was found in peak 5. SDS/polyacrylamide-gel electrophoresis of the immunoreactive peak revealed equal amounts of Ya and Yc subunits, but for peak 5 it revealed a major band in the Yc region but no Ya subunit.

Chromatography of 'purified' testis ligandin

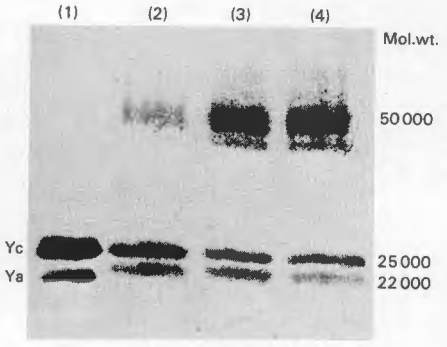

Fig. 8. *SDS/polyacrylamide-gel electrophoresis of 'pure' testis ligandin cross-linked with dimethyl suberimidate*
(1) Testis ligandin, purified by standard procedures, no dimethyl suberimidate added. (2) Testis ligandin, after addition of dimethyl suberimidate and cross-linking reaction allowed to proceed for 1.2h under conditions described in the Materials and methods section, (3) after cross-linking for 2.5h, and (4) after cross-linking for 3.5h.

yielded two peaks of GSH–chlorodinitrobenzene-conjugating activity. Both peaks had GSH peroxidase II activity. Only peak 1 contained immunoreactive material as determined by radioimmunoassay by using antiserum raised against liver ligandin. SDS/polyacrylamide-gel electrophoresis of peak-1 material revealed equal amounts of Ya and Yc subunits; peak 2 consisted entirely of Yc subunit (Fig. 7).

Covalent cross-linking of testis ligandin

SDS/polyacrylamide-gel-electrophoretic analysis of covalently cross-linked testis ligandin resulted in two bands in the region of approx. 50000 mol.wt. after cross-linking had been allowed to proceed for 2.5h under the conditions described in the Materials and methods section (Fig. 8).

Discussion

Liver ligandin, as defined by Bass *et al.* (1977*a*), consists of equal amounts of Ya and Yc subunits. In contrast, the Yc subunit accounts for as much as 90% of testis ligandin (Bhargava *et al.*, 1980; Bass *et al.*, 1977*a*). By the standard purification procedure we were able to purify a protein with subunit structure, amino acid composition, binding characteristics (results not shown) and GSH *S*-transferase activity similar to that described by Bhargava *et al.* (1980). They found testis ligandin to have a lower affinity than liver ligandin for bilirubin and for sulphobromophthalein. Circular-dichroism spectra revealed the absence of the primary binding site for bilirubin (Bhargava *et al.*, 1980).

When testis ligandin was prepared by affinity chromatography, with antibody raised against liver ligandin, it consisted of equal amounts of Ya and Yc subunits. This was clearly different from testis ligandin preparations obtained by the standard purification procedure. The testis starting material contained more Yc than Ya subunit (Fig. 5, lane 1). However, a considerable amount of Yc subunit was eluted in the wash (Fig. 5, lanes 2 and 3), suggesting that this may not have been recognized by the antibody. The immunoreactive material eluted with glycine buffer, pH 3.0, consisted of equal amounts of Yc and Ya subunits (Fig. 5, lane 4), resembling the proportions of these subunits found in liver ligandin. In addition, antibodies raised against testis ligandin prepared by standard methods consistently failed to meet immunoduffusion and immunoelectrophoretic criteria for purity when tested against liver cytosol. Furthermore, SDS/polyacrylamide-gel electrophoresis of covalently cross-linked testis ligandin revealed more than one band in the 50000Da region. This finding differed from that reported by Bhargava *et al.* (1978) for liver ligandin and thus may suggest the presence of another protein in our testis preparations. Our suspicion that standard preparations of testis ligandin could be contaminated by a closely related protein were strengthened by the findings of Hayes *et al.* (1980) and Scully & Mantle (1980), who studied the subunit composition of liver GSH *S*-transferases. The GSH *S*-transferases of rat liver (AA, A, B, C, D and E) have been classified according to their reverse order of elution from CM-cellulose (Habig *et al.*, 1976). Transferases D and E are eluted before the salt gradient is applied, after which transferases C, B, A and AA are eluted sequentially. Although there is overlap of substrate specificity, the various transferases may be identified by their order of elution from CM-cellulose and their ability to conjugate GSH to various substrates. All conjugate chlorodinitrobenzene. Only transferases A and C conjugate GSH to dichloronitrobenzene (Habig *et al.*, 1974), and transferases B, A and AA have GSH peroxidase activity (Prohaska & Ganther, 1977; Lawrence *et al.*, 1978; Irwin *et al.*, 1980). On SDS/polyacrylamide-gel electrophoresis, liver transferase AA migrates as subunit Yc, liver transferase B migrated as YaYc dimer, and transferases A and C [which show immunological identity (Habig *et al.*, 1974)] migrate as Yb subunit. This finding led Hayes *et al.* (1979) to differentiate between GSH *S*-transferase B (YaYc) and ligandin (YaYa). Since our study has been aimed at determining the subunit composition of a protein in testis which reacts immunologically with anti-(liver ligandin), the term ligandin has been used throughout, although in the

light of our findings the term GSH *S*-transferase B may be more appropriate.

To determine whether testis transferase AA migrated as Yc subunit, we separated the testis GSH *S*-transferases by CM-cellulose chromatography and used order of elution, SDS/polyacrylamide-gel electrophoresis, radioimmunoassay and catalytic activity with different substrates to characterize the various fractions. Activity with chlorodinitrobenzene and dichloronitrobenzene resembled that seen in liver. We found GSH peroxidase activity in peaks corresponding to liver GSH *S*-transferases B, A and AA. Of interest is the fact that transferase AA accounts for most of the GSH peroxidase II activity in testis cytosol. SDS/polyacrylamide-gel electrophoresis of the CM-cellulose peak with the elution characteristics of transferase AA revealed a major band in the Yc-subunit region, similar to findings reported by Scully & Mantle (1981). This peak was not recognized by antibody raised against liver ligandin. Immunoreactivity was confined to fractions eluted in the transferase-B region of the CM-cellulose profile. On SDS/polyacrylamide-gel electrophoresis, this peak consisted of equal amounts of Ya and Yc subunits.

It was apparent that if GSH *S*-transferase AA was present in standard preparations of ligandin it would not be detected on SDS/polyacrylamide-gel electrophoresis. We thus used CM-cellulose chromatography to study 'pure' testis ligandin and found it to consist of GSH *S*-transferase B (consisting of equal amounts of Ya and Yc subunits and recognized by antibody raised against the liver Ya subunit) and GSH *S*-transferase AA (which resembles Yc subunit on SDS/polyacrylamide-gel electrophoresis). Our finding that standard preparations of testis ligandin are contaminated by GSH *S*-transferase AA offers a new explanation for studies previously reported and interpreted differently.

We thank Professor Wieland Gevers, Dr. Morris Sherman, Dr. Langley Purves, Dr. George Lindsey, Dr. Paul Adams and Mr. Kenneth Jacobs. This study was supported by the South African Medical Research Council and the South African Atomic Energy Board.

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CAN ACTH ALONE MAINTAIN ADRENAL ANDROGEN SECRETION IN THE HYPOPHYSECTOMIZED CHIMPANZEE? B.D. Albertson, W.C. Hobson, and G.B. Cutler, Jr. Developmental Endocrinology Branch, NICHD, Bethesda, MD. 20205, and Primate Research Center (WCH), New Mexico State Univ., Holloman AFB, NM 88330.

The chimpanzee undergoes an adrenal maturation (adrenarche) that is qualitatively and quantitatively similar to that in man. We have used the chimpanzee to test the hypothesis that adrenal androgen production is supported by a pituitary factor distinct from ACTH. Six adult male chimpanzees who had completed both gonadal and adrenal maturation were castrated and either hypophysectomized (hypox) or sham-hypophysectomized. Completeness of hypophysectomy, evaluated by provocative tests one week after surgery, was 60, 80, and 91 percent in the 3 hypox animals. Hypox chimps received a daily I.M. injection of synthetic ACTH 1-24 and oral thyroxine to prevent adrenal insufficiency and hypothyroidism. Adrenal function was evaluated with a 3-h ACTH infusion before and at 7, 21, 40, 120, and 180 days after hypophysectomy. Plasma cortisol (F), dehydroepiandrosterone (DHA), DHA sulfate (DHAS), and androstenedione (Δ^4A) were measured at 6 time points during the ACTH infusions. The mean ratios of DHA/F, DHAS/F, and Δ^4A/F during ACTH infusion were calculated as indices of the relative activity of the androgen pathway compared to the cortisol pathway. The DHA/F and DHAS/F ratios decreased to 30 percent of pre-treatment levels at 40 days after hypophysectomy ($p<.01$), to 50 percent of pre-treatment levels at 120 days after hypophysectomy ($p<.05$ for DHAS/F), and to 60 percent at 180 days after hypophysectomy (p, NS). The Δ^4-A/F ratio did not change after hypophysectomy. None of the ratios changed in the sham-hypox controls. Metabolic clearance rates for cortisol and DHA were measured before and at 180 days after hypophysectomy or sham-hypophysectomy to assess the possible contribution of clearance changes to the changes in plasma DHA/F ratio. None of the clearance rates changed significantly, but cortisol clearance at 180 days was 57 ± 12 (mean ± SE) percent of the mean pre-treatment level. We conclude: (1) ACTH alone maintained a normal Δ^4-A/F ratio but failed to maintain normal DHA/F and DHAS/F ratios after hypophysectomy in the chimpanzee. (2) The data are consistent with the hypothesis that Δ^5 adrenal androgen production is regulated by a non-ACTH pituitary factor, but we cannot exclude the possibility that a decrease in cortisol clearance may explain the fall in DHA/F and DHAS/F ratios.

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WITHDRAWN

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ONTOGENY OF LIGANDIN AND STEROID HORMONES IN RAT TESTIS, OVARY AND ADRENAL: EVIDENCE FOR A RELATIONSHIP.
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Ligandin is present in high concentrations in steroid synthesising tissues and has been claimed to influence intracellular accumulation and efflux of steroid hormones and their metabolites. Using radioimmunoassays, we have studied the ontogeny of ligandin in various tissues of the rat in relation to serum testosterone and progesterone levels. Ligandin levels in testis, ovary and adrenal tissue are relatively high after birth (table), decrease by day 9 and increase during puberty to reach adult levels. These changes are closely paralleled with changes in circulating levels of testosterone and progesterone. In contrast, ligandin in non-endocrine tissues is low at birth and displays a progressive rise which is not correlated with changes in steroid-producing tissues.

DAY	ADRENAL*	TESTIS*	TESTOST.♂	OVARY*	PROGEST.♀
4	0.36±0.11	0.20±0.03	3.65±0.68	0.17±0.01	2.69±0.57
9	0.17±0.01	0.08±0.01	1.40±0.25	0.07±0.01	1.67±0.40
20	0.19±0.03	0.06±0.01	1.05±0.24	0.15±0.01	11.49±0.72
31	0.29±0.05	0.10±0.01		0.37±0.13	
37			5.05±1.64		24.35±6.80
46	0.39±0.02	0.14±0.01		0.43±0.02	
49			7.71±1.43		31.04±4.01
60	0.38±0.04	0.18±0.01	7.12±0.76	0.45±0.03	25.52±1.25

*ligandin expressed as ug/mg soluble protein.
♂testosterone and ♀progesterone expressed as nM/l, all values±SEM

The correlation between ligandin and sex hormone levels in developing rat steroid-producing tissues strongly suggests a functional relationship. Our immunohistochemical studies localise ligandin to the steroidogenic cells of testis, ovary, and adrenal cortex. Ligandin is immunologically indistinguishable from glutathione S-tranferase B and has delta-5,3-keto-steroid isomerase and glutathione peroxidase activity. It may thus be involved in steroid hormone biosynthesis and also play an important role in protecting steroid-producing tissues from toxic products of metabolism.

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WITHDRAWN