STUDIES ON INTERACTIONS BETWEEN BILE ACIDS AND RAT LIVER

by

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DECLARATION OF ORIGINALITY

I declare that the work presented herein

and the composition of this thesis is my own.

John D. Hayes

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ABSTRACT

The intrahepatic transport of a number of anions is thought to be mediated by the cytosolic anion-binding protein ligandin (Arias et al., in Glutathione: Metabolism and Function, eds. I. M. Arias & W. B. Jakoby, Raven Press, New York, 1976, pp. 175-188). Ligandin also possesses enzyme activity and has been reported to be identical to glutathione S-transferase B (Habig et al., Proc. Natl. Acad. Sci U.S.A. 71, 3879 3882), one of at least 7 proteins in rat liver cytosol with transferase activity. Each of these enzymes comprise two of three possible monomers, Ya, Yb or Yc which have mol. wts. 22 000, 23 500 or 25 000 respectively. In this thesis bile acid-binding to rat liver cytosol proteins has been investigated.

In hepatic cytosol the major bile acid-binding activity was associated with glutathione S-transferase activity. Transferase activity was resolved into 7 peaks by cation exchange chromatography; at least 4 of these peaks bound cholic acid and at least 3 of the transferases bound lithocholic acid. Examination of the subunit compositions of different glutathione S-transferases indicated that cholic acid-binding, lithocholic acid-binding and GSH: 1, 2-dichloro-4-nitrobenzene conjugating activity may be ascribed

to different subunits.

The two major lithocholic acid-binding transferases were purified and were designated LBa and LBb; they comprised YaYa and YaYc subunits respectively. These two proteins were identified by comparing their elution volumes from CM-Sephadex with those of purified ligandin and glutathione S-transferase B. Although ligandin and glutathione S-transferase B have been reported to be identical they eluted separately, as single peaks of enzyme activity at volumes equivalent to LBa and LBb respectively. This suggests that ligandin and glutathione S-transferase B are not identical but separate proteins. LBa and LBb had similar amino acid compositions and were both induced by phenobarbitone. They may be coded by a single gene. Peptide "mapping" suggested that a product-precursor relationship may exist between LBa and LBb. The removal of 26 terminal amino acids from the Yc monomer of LBb (YaYc) may yield LBa (YaYa).

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Abbreviations

The abbreviations in this thesis are those used by the Biochemical Journal (Biochem.J. (1978) 169, 1-27). The following abbreviations have also been used:

GSH, glutathione

Bromosulphophthalein, disodium phenoltetrabromophthalein sulphonate

SDS, sodium dodecyl sulphate

Cholic acid, 3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid

Chenodeoxycholic acid, 3α, 7α-dihydroxy-5β-cholan-24-oic acid

Deoxycholic acid, 3α, 12α-dihydroxy-5β-cholan-24-oic acid

Lithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid

V_{max}, hepatic uptake capacity

T_m, maximal hepatic secretion rate .

INTRODUCTION

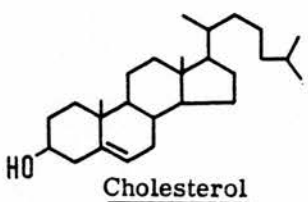
Bile acid structure

The bile acids are a group of acidic sterols with a four ringed cyclopentanophenanthrene structure (Fig. 1.1). Bile acids are based on cholanic acid and the individual acids differ in the number of hydroxyl groups on the rings; for example, cholic acid has hydroxyl groups at the 3, 7 and 12 positions, chenodeoxycholic acid and deoxycholic acid have hydroxyl groups at the 3 and 7 and 3 and 12 positions respectively, and lithocholic acid has one hydroxyl group at the 3 position. Neither the methyl, hydroxyl nor carboxyl groups are situated in the same steric plane as the steroid ring: the methyl groups are β-oriented while the hydroxyl groups and the carboxyl side chain are α-oriented. Bile acid molecules can be divided into two distinct regions; first, the polar hydroxyl and carboxyl groups and secondly, the non-polar steroid ring and methyl groups.

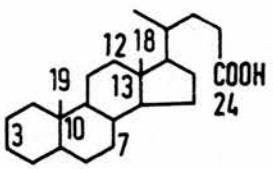
Physiological functions of the bile acids

Bile acids have three major physiological functions.

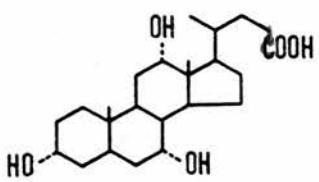
First they solubilize biliary cholesterol (Admirand & Small, 1968; Neiderhiser & Roth, 1968; Dam & Hegardt, 1971) and emulsify the digestion products of dietary lipid in the gut (Hofmann & Borgström,



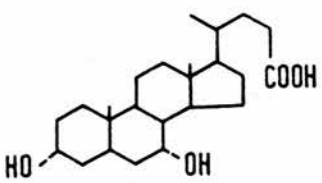
Cholesterol



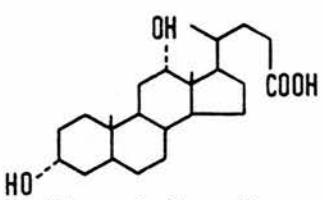
Cholanic acid



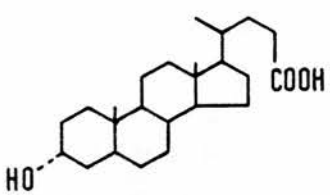
Cholic acid
(3α, 7α, 12α-trihydroxy
cholanic acid)



Chenodeoxycholic acid
(3α, 7α-dihydroxy
cholanic acid)



Deoxycholic acid
(3α, 12α-dihydroxy
cholanic acid)



Lithocholic acid
(3α-monohydroxy
cholanic acid)

Figure 1.1 Structural formulae of cholesterol, cholanic acid, cholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid.

1962; Hofmann, 1963). Bile acids also appear to enhance the process of lipolysis in vitro (Borgström, 1954) and in vivo (Borgström et al., 1957). Although the mechanism of this process is not clear it has been discussed in a review on the roles of bile acids in fat and cholesterol absorption (Holt, 1972). Whilst it is possible that bile acids may exert their effect on lipolysis by interacting directly with pancreatic lipase it is more probable that bile acids increase lipolysis in the gut through emulsifying the substrate. Sarda & Desnuelle (1958) demonstrated that lipolysis occurs at an oil-water interface and therefore emulsification of large triglyceride droplets into a small stable droplet emulsion would increase the interface available for the reaction. Holt (1972) also suggested that the emulsification properties of bile acids may help remove the products of lipolysis from the interface reducing product inhibition of the reaction. The emulsification activity of bile acids depends on their amphipathic properties. They are surface-active in water and at low concentrations are most densely distributed at the air:water interface. At a certain critical concentration, when bile acid molecules are densely crowded together at the interface and the surface activity has reached a maximum, the excess bile acid molecules, which must enter the bulk phase, spontaneously aggregrate to form small clusters of molecules called micelles. This critical bile acid concentration is called the critical micellar

concentration(C. M. C.) and it has been calculated to be 0.75-2.0 mM (Holt, 1972). The micelle is about 10 nm in diameter and is discoid in shape. It comprises 4-20 bile acid molecules, oriented with non-polar areas in the centre of the aggregate and the polar groups exposed to the aqueous phase (Hofmann & Small, 1967; Carey & Small, 1972). The solubilization of biliary cholesterol as well as the digestion products of dietary lipid occurs by formation of mixed micelles in which lipid molecules interdigitate between detergent molecules (Small, 1970). In the rat, the concentration of bile salts in the liver tissue is 0.14-0.28 mM (Greim & Popper, 1971). Bile acids therefore cross the hepatocyte as single molecules, not as micelles.

The second physiological role which has been ascribed to bile acids is the stimulation of bile flow. A linear relationship has been demonstrated between the rate of bile acid secretion into bile and bile flow (Wheeler, 1972; Javitt, 1976a, b). Because of their choleretic activity bile acids are determinants of bile flow and they therefore facilitate the removal of many endogenous and exogenous substances from the body by excretion into bile. Sperber (1959) proposed that the choleretic effect of bile acids results from their active transport across the canalicular membrane.

The osmotic gradient thereby established allows water to pass across the canalicular membrane into bile. Active transport of any solute should therefore promote bile flow.

The third physiological role of bile acids may be to regulate the size of the body cholesterol pool. Since bile acids are quantitatively the major products of cholesterol catabolism, the formation of bile acids and their ultimate elimination in the faeces represents a major mechanism of cholesterol degradation and elimination from the body (Siperstein & Chaikoff, 1952; Elliott & Hyde, 1972; Danielsson & Sjövall, 1975). For an animal to be in cholesterol balance the rates of cholesterol supply and removal must be equal. Cholesterol supply involves both dietary intake and cholesterol acquired from endogenous synthesis; cholesterol removal involves both the catabolism of cholesterol to bile acids and the secretion of cholesterol into the gut with their ultimate excretion into the faeces. When the rates of supply and removal are equal the cholesterol pool remains constant. There is evidence which suggests that hepatic cholesterol synthesis is in part regulated by bile acids in the enterohepatic circulation (the enterohepatic circulation is described on pp.14-17). However, the mechanism of this effect is not well understood. First, the external diversion of bile flow

leads to a striking increase in the rate of cholesterol synthesis in the liver (Eriksson, 1957; Myant & Eder, 1961). Second, the fact that feeding the bile acid binding resin cholestyramine also results in increased hepatic cholesterol synthesis suggests that the bile acid component in bile is responsible for the increase in synthesis (Huff et al., 1963). Third, interference with the enterophepatic circulation of bile acids as a result of ileal bypass operations also results in a striking increase in cholesterol synthesis in the liver (Borgström, 1960; Moutafis & Myant, 1968). The bile acids are an integral part of cholesterol catabolism and anabolism; it has been suggested that the net effect of the bile acids on cholesterol metabolism is to maintain a balance in which synthesis and absorption tend to equal excretion and degradation under diverse physiological conditions (Wilson, 1972).

Bile acid synthesis

Bile acids are synthesised from cholesterol in the liver. Synthesis involves five separate modifications of the cholesterol molecule, (a) introduction of α-hydroxyl groups at positions 12 and/or 7; (b) alteration of the orientation of the 3-hydroxyl group from β to α ; (c) saturation of the double bond between C5 and 6;

(d) cleavage of the side chain between the carbon atoms 24 and 25 and (e) oxidation of the terminal carbon atom of the side chain to form a carboxylic acid group.

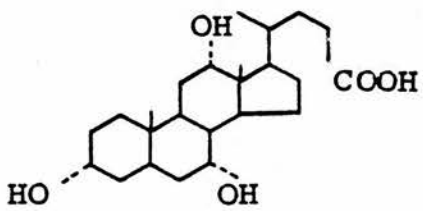
In rat liver the two major synthetic pathways result in the formation of cholic acid and chenodeoxycholic acid. These bile acids are formed by pathways which are initiated by modifications to the steroid ring structure of the cholesterol molecule and are followed by modifications to the side chain. The first two reactions in both pathways are common and therefore a branch point exists before cholesterol degradation is committed to either cholic acid synthesis or chenodeoxycholic acid synthesis.

Cholic acid is the major bile acid in rat liver (70 % of total) (Okishio & Nair, 1966). The first step in the conversion of cholesterol into either cholic acid or chenodeoxycholic acid is the formation of 7α-hydroxycholesterol. This reaction is catalysed by cholesterol 7α-hydroxylase, a cytochrome P-450 dependent enzyme system present in the microsomal fraction of the liver, which requires oxygen, reduced nicotinamide adenine dinucleotide phosphate (NADPH) and a thiol cofactor (Scholan & Boyd, 1968; Grimwade et al., 1973). This reaction is rate-limiting; procedures, such as biliary drainage, which result in an

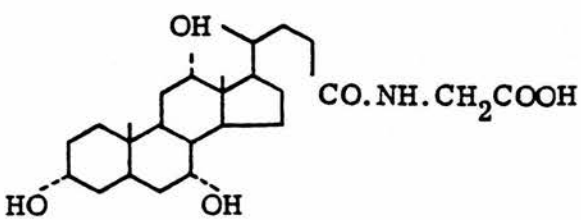
increase in the rate of bile acid synthesis also result in an increase in the activity of the cholesterol 7α-hydroxylase enzyme system (Danielsson et al., 1967; Boyd et al., 1969). The regulation of bile acid synthesis has been reviewed by Boyd & Percy-Robb (1971) and Danielsson & Sjövall (1975); it is widely accepted that bile acids control the rate of their own synthesis through a feedback inhibition mechanism which alters the activity of the cholesterol 7α-hydroxylase enzyme system, possibly by inducing the synthesis of a component of this enzyme system. The mechanism of such an effect is unknown.

Bile acid metabolism and degradation

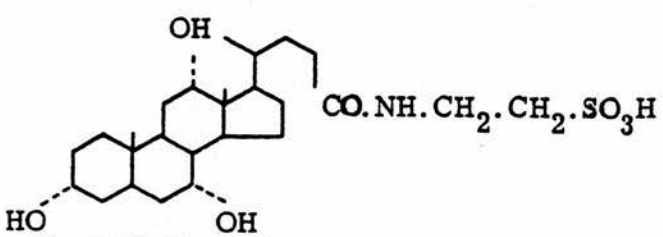
After synthesis the bile acids are conjugated with either taurine or glycine through an amide bond at carbon atom 24. Vessey & Zakim (1977) showed that in the guinea-pig the enzymes responsible for conjugation are located in the microsomal fraction of the liver. The two conjugates of cholic acid, glycocholic acid and taurocholic acid, are shown in Fig. 1.2. The ratio of taurine-conjugated to glycine-conjugated bile acids in normal human bile is approximately 1:3 and in rat bile 20:1. These ratios are species specific but can be changed by nutritional or hormonal factors (Mosbach, 1972).



Cholic acid



Glycocholic acid
(cholyl glycine)



Taurocholic acid
(cholyl taurine)

Figure 1.2 Structural formulae of cholic acid, cholyl glycine and cholyl taurine.

Conjugated bile acids possess several physiological advantages over their unconjugated counterparts. They are more polar and are therefore secreted, by the liver, into bile more rapidly than unconjugated bile acids (O'Maille et al., 1965). Although free bile acids precipitate from solution, as the insoluble protonated acids, at pH's which lie close to neutrality (Dowling & Small, 1968) conjugated bile acids are resistant to precipitation, as protonated acids, at the pH of the upper gut (pH about 6.5); conjugated bile acids are therefore more effective promoters of absorption within the intestine than their unconjugated counterparts (Schiff et al., 1972). Conjugated bile acids also appear to be less toxic to the intestinal mucosa. For example, cholic acid (8 mM) caused a 99% inhibition of glucose transport by segments of jejunum, whereas equivalent concentrations of glycocholic acid or taurocholic acid produced no inhibition (Pope et al., 1966). Further, Teem & Phillips (1972) reported that deoxycholic acid, but not conjugated deoxycholic acid, produced gross morphological damage to the jejunum.

The conjugated bile acid is commonly called a bile salt whilst the unconjugated form is referred to as a bile acid. However, this terminology is not used rigorously. The terms bile salts and bile acids to describe conjugated and unconjugated bile acids are commonly interchanged. In this thesis the unconjugated bile acids will be

referred to as bile acids and the conjugated bile acids as bile salts. In dilute solutions (below their C.M.C.'s), the free bile acids cholic, chenodeoxycholic and deoxycholic have pKa values about 5, their glycine conjugates about 3.7 and their taurine conjugates about 1.5 (Heaton, 1972). At physiological pH's, therefore the majority of both conjugated and unconjugated bile acids will be present largely as sodium salts.

Apart from conjugation with the amino acids taurine or glycine, bile acids can react with sulphuric acid. This occurs in the liver and the kidney and results in the formation of a sulphate ester with the 3α-hydroxyl group (Palmer, 1971; Cronholm et al., 1972). The intracellular location of enzymes responsible for this reaction is not known. The ratio of sulphated to non-sulphated bile acid varies with the bile acid; lithocholic acid is the most completely sulphated bile acid (Makino et al., 1974; Stiehl, 1974). The sulphation process does not appear to be affected by conjugation since both bile salts and bile acids are sulphated (Stiehl et al., 1977). Bile acid sulphates differ in excretion and toxicity from non-sulphated bile acids (Stiehl et al., 1977). The introduction of an SO_2H group into the bile acid molecule improves its water solubility. Once excreted into bile the polar sulphated bile acids are poorly absorbed from the small intestine. They pass into the colon and the majority of the sulphated bile acids are lost in the faeces (Cowen et al., 1975). Sulphation

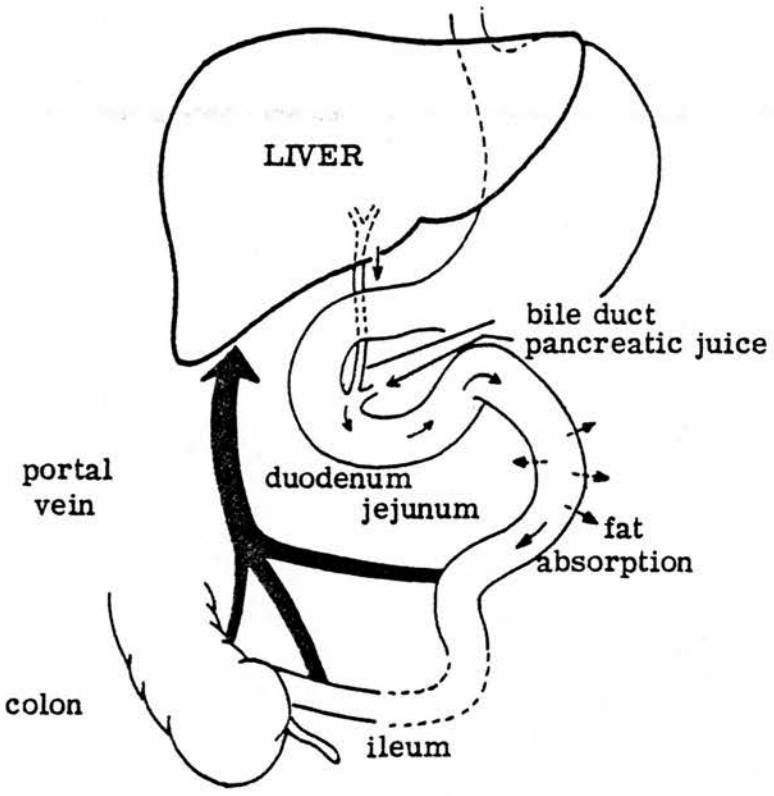
is therefore of special importance in helping to eliminate the potentially toxic bile acid, lithocholic acid, from the body. In patients with cholestasis, which results in increased serum concentrations of bile salts, elimination of bile salts in the urine may be considerable (Back, 1973; Norman & Strandvik, 1974; Stiehl et al., 1975; Van Berge Henegouwen & Brandt, 1976). A major part of such bile salts are present in the urine as sulphate esters (Makino et al., 1974; Stiehl, 1974).

Bile salts can be degraded by aerobic and anaerobic bacteria in the small and large intestine. Two types of bacterial action are important in bile acid metabolism (Lewis & Gorbach, 1972). First, the main initial reaction is the deconjugation of bile salts to produce the respective free acid. Several strains of bacteria , including Streptococcus faecalis, Bacteroides fragilis, Clostridium perfringens and Bifidobacterium adolescentis, produce a cholanyl glycine hydrolase which catalyzes the deconjugation of bile salts (Shimada et al., 1969; Aries & Hill, 1970; Lewis & Gorbach, 1972). The specificity of this reaction is not absolute and most organisms found to deconjugate a specific bile salt will, under suitable conditions, deconjugate others presented to them (Drasar et al., 1966; Dickenson et al., 1971). Secondly, the two primary bile acids, cholic acid and chenodeoxycholic acid, may subsequently be dehydroxylated at the 7 position to form the

secondary bile acids deoxycholic acid and lithocholic acid (Lewis & Gorbach, 1972). As the rat eats its own faeces it normally possesses bacterial colonisation of the small gut. Bacterial modification of bile acids is therefore quantitatively important in the rat. In the rat half the cholic acid pool is dehydroxylated each day and up to one third of the bile salt pool is deconjugated each day (Lindstedt & Samuelsson, 1959; Heaton, 1972).

Bile acid enterohepatic circulation

After conjugation in the liver, the bile salts are secreted into bile and pass down the bile duct into the third part of the duodenum. During passage down the small intestine the bile salts promote the digestion of dietary fat and in the rat a proportion of the bile salts will be deconjugated by intestinal bacteria to form free bile acids. The bile salts and bile acids are reabsorbed largely at the terminal ileum (Dietschy, 1968; Lack & Weiner, 1971) and return to the liver in the portal blood bound to albumin (Rudman & Kendall, 1957; Burke et al., 1971). The bile salts and acids are extracted from the blood by the hepatic parenchymal cells (O'Máille et al., 1967), transported across the hepatocyte and resecreted against a concentration gradient into the bile canaliculi (O'Máille et al., 1965).



The enterohepatic circulation

Figure 1.3 Diagramatic representation of the enterohepatic circulation of bile acids.

This recycling system, in which bile salts are circulated between the liver and the intestine, is called the enterohepatic circulation. It effectively conserves bile salts and bile acids and enables the body to make maximum use of this group of substances. A diagramatic scheme of the enterohepatic circulation in the rat is shown in Fig. 1.3. This differs from the enterohepatic circulation in man in that the rat does not possess a gallbladder. The human gallbladder has a capacity of about 40 ml and can concentrate bile acids to about 0.2 M (Lindstedt, 1957; Abvarre et al., 1969; Vlahcevic et al., 1970). It is theoretically capable of storing 8 mmol of bile acid, which is equivalent to the total bile acid pool. The possession of a gallbladder, with its large storage capacity, therefore enables man to secrete bile in response to the ingestion of food more effectively than the rat.

The bile acid pool is continually depleted by faecal excretion and repleted by hepatic synthesis. In normal man the bile acid pool is 2 - 4 g and it loses, and is replaced with 500 - 700 mg (15 - 20%) of the total) per day. Since the bile acid pool circulates 5 - 15 times a day, approximately 30g of bile acid are transported across the liver and are reabsorbed from the gut. This demonstrates that about 95% of the bile salts and bile acids are reabsorbed during each cycle of the pool (Brunner et al., 1972;

Heaton, 1972). In the rat the pool is about 20mg and about 200mg of bile salt and bile acid are transported across the liver each day (Cronholm & Sjövall, 1967). Maintenance of an adequate bile acid pool is dependent upon efficient absorption by the ileum, return to the liver in portal blood, removal from the blood by the liver and secretion into bile. These transport processes are very efficient and except for a small quantity of bile acid in the systemic circulation the bile acid pool is restricted to the enterohepatic circulation (Ng & Hofmann, 1977).

Hepatic transport of bile acids

This thesis is principally concerned with the intrahepatic transport phase of the enterohepatic circulation. The hepatic transport of bile acids includes three steps: uptake across the sinusoidal membranes of the parenchymal cell, intracellular transport and secretion across the canalicular membrane into bile. Little is known about the molecular mechanisms of these steps.

Several studies have investigated the kinetics of bile acid uptake. O'Maille et al. (1967) have shown that in dogs both taurocholic acid and cholic acid are efficiently removed by the

liver after intravenous injection. By applying the Fick principle, to determine the total hepatic blood flow, they showed that about 90% of the taurocholate and 80% of the cholate was extracted from the blood during a single passage through the liver. Smallwood et al. (1974) injected a bolus of radiolabelled bile acids into the portal vein of rats and showed that radioactivity appeared in bile within 30 s and the maximal excretion rate was reached within 3 min. They showed that cholic acid was transported into bile more quickly than chenodeoxycholic acid. Similar experiments by Strange et al. (1979c) demonstrated that lithocholic acid excretion into bile was slower than that of the tri- or di-hydroxy bile acids.

Studies in dogs suggest that bile acid uptake from plasma is saturable and therefore carrier-mediated (Glasinovic et al., 1975). Bile acid uptake has also been studied in the isolated perfused rat liver (Reichen & Paumgartner, 1975) and isolated rat liver cells (Schwartz et al., 1975). These also indicate that a carrier-mediated transport mechanism, located in the sinusoidal membrane, is responsible for uptake (Reichen & Paumgartner, 1975). The process is specific for bile acids (Paumgartner & Reichen, 1975, 1976) and is Na^+ dependent (Schwartz et al., 1975; Reichen & Paumgartner, 1976). The demonstration of competitive

inhibition between the uptake of different bile acids suggests that they share a common pathway which is different from that by which organic anionic dyes enter the hepatocyte (Paumgartner & Reichen, 1975).

Uptake of bile acids and bile salts from the plasma is probably not rate limiting as their hepatic uptake capacity (Vmax) is 5 - 10 times greater than their maximal secretion rate (Tm) (Wheeler, 1972; Glasinovic et al., 1975; Reichen & Paumgartner, 1976). The difference between the capacity of the hepatic uptake and secretion processes has given rise to the concept of hepatic storage of bile acids and salts (Heaton, 1972; Wheeler, 1972; Reichen & Paumgartner, 1975).

Secretion of bile acids into bile is also probably carrier-mediated as it is saturable (O'Máille et al., 1967). However the Tm of bile acids exceeds the usual demands imposed by the enterohepatic circulation (O'Máille et al., 1965, 1967; Wheeler, 1972). This is greater than the Tm for other organic anions (Wheeler et al., 1960; O'Máille et al., 1966). Bile acids are excreted across the canalicular membrane into bile against a large concentration gradient. In the rat, the concentration of bile salts in the liver tissue is 0.14 - 0.28 mM (Greim & Popper,

1971) while in bile it is 16 mM (Klaasen, 1971). The excretion process is energy requiring and is postulated to be regulated by the membrane-bound enzyme (Na^+- K^+)ATPase (Erlinger et al., (1970).

The existence of bile acid carriers in the sinusoidal and canalicular membranes has been supported by the identification of bile acid-binding sites in liver surface membranes (Accatino & Simon, 1976). Characterisation of these binding species showed that their binding kinetics were similar to the uptake process in the intact liver. Further,Gonzalez et al., (1979) demonstrated that in rats administered cycloheximide, a drug which inhibits protein synthesis, both the number of bile acid receptors in liver surface membranes and the bile acid Tm were decreased. However, although the sinusoidal and canalicular membranes are morphologically distinct it is not clear whether these surface membranes receptors originate from the sinusoidal or canalicular membranes. Accatino & Simon (1976) suggested that they were present in both membranes but since the canalicular membrane represents only 13% of the total hepatic surface membranes the majority of the receptors were present in the sinusoidal membrane.

Comparatively little is known about how bile acids traverse

the hepatocyte. It is not clear whether they cross it in free solution or are transported from the sinusoidal membrane to the canalicular membrane bound to a carrier protein. Strange et al. (1976, 1977a) demonstrated the presence, in rat liver cytosol, of proteins which can bind cholic acid, chenodeoxycholic acid and lithocholic acid. Subsequent partial purification of two lithocholic acid-binding proteins showed that these both possessed glutathione S-transferase activity (Strange et al., 1977b). Although neither of the two proteins was identified it was suggested that one may be the well-described, non-specific, anion-binding protein, ligandin, which also possesses glutathione S-transferase activity (Kaplowitz et al., 1973). The physiological role of these binding proteins is not clear. However, the interaction of bile acids with subcellular organelles in the liver has recently been studied with a view to determining how bile acids cross the liver and how the cytosolic binding proteins effect bile acid transport (Strange et al., 1979a, b, c). These studies showed that the bile acids were non-specifically distributed (partitioned) between nuclei, microsomes, mitochondria or free solution and that only cytosol contained saturable bile acid-binding sites. Further these workers constructed a compartmental model for bile acids in the liver which indicated that 90% of the cholic acid conjugates and 99% of the lithocholic acid in the liver would be bound to nuclei, microsomes,

mitochondria or cytosolic proteins with 10% and 1%, respectively, in free solution. Strange et al. (1979b) considered ways in which bile acids may be passively transported across the liver cell. A comparison between the observed cholic acid transit time, across the liver into bile, and the calculated transit time it would have if it diffused across the hepatocyte (a) in free solution, (b) bound to protein or (c) bound to phospholipid, suggested that bile acids may diffuse across the liver in free solution. Strange et al. (1979c) suggested that the physiological role of the cytosolic binding proteins is to restrict the amount of bile acid available for partitioning into subcellular organelles.

Origin of the term ligandin

A large number of compounds, including bilirubin, bromosulphophthalein and indocyanine green, are transported across the hepatocyte and may be bound to cytosolic proteins. The hypothesis that cytosolic proteins are involved in the hepatic transport of various anions has largely originated from the work of Arias and his colleagues. They first described the presence of the anion-binding proteins, X, Y & Z by gel-exclusion chromatography of a mixture of hepatic cytosol and either bilirubin or bromosulphophthalein (Levi et al., 1969b). This resulted in the elution of 3 peaks of

protein-bound anion; these fractions were called X (eluted in the void volume), Y (mol. wt. approx. 45 000) and Z (mol. wt. approx. 12 000). The binding component in the Y fraction, initially called Y protein, bound a wide variety of organic anions including bilirubin, bromosulphophthalein and indocyanine green. It was subsequently reported that Y protein was identical to the Azodye carcinogen-binding protein (β-ABP) described by Ketterer et al. (1967) and the Corticosteroid Binder I described by Singer et al. (1970). The protein was therefore called ligandin because of its binding properties (Litwack et al., 1971).

Ligandin and the glutathione S-transferases

Habig et al. (1974a) reported that ligandin, prepared by the method of Arias and his colleagues, was identical to glutathione S-transferase B since both proteins had the same specificity for substrates and for ligands, both proteins had similar physicochemical properties, both proteins were induced by phenobarbitone treatment and both proteins reacted similarly to antibody produced against ligandin. There are at least 7 glutathione S-transferases in rat liver cytosol; they each catalyse the conjugation of glutathione to a number of electrophilic compounds. As examples, the reaction between glutathione and 1-chloro-2, 4-dinitrobenzene and between

(i)

(ii)

Figure 1.4 The reactions between (i) GSH and 1-chloro-2, 4-dinitrobenzene and (ii) GSH and 1,2-dichloro-4-nitrobenzene. Both reactions are catalysed by glutathione S-transferase enzymes. Reaction (i) results in the formation of HCl and of S-(2,4-dinitrophenyl)-glutathione. Reaction (ii) results in the formation of HCl and of S-(2-chloro-4-nitrophenyl)glutathione.

glutathione and 1, 2-dichloro-4-nitrobenzene are shown in Fig. 1.4. The conjugation of glutathione with various electrophilic compounds is the first step in the synthesis of the mercapturic acids. The thioesters formed by the reaction catalysed by the glutathione S-transferases are subsequently converted in separate stages by (a) removal of the γ-glutamyl moiety through the action of a γ-glutamyl transferase, (b) removal of the glycine moiety through the action of a dipeptidase and (c) N-acetylation of the cysteine conjugate by an acetyl-CoA linked acetylase to form a N-acetylcysteine thioether, which is the mercapturic acid (Fig. 1.5) (Barnes et al., 1959; Bray et al., 1959; Boyland & Chasseaud, 1969: Habig et al., 1974b).

Attempts to classify the glutathione S-transferases on the basis of their substrate specificity have resulted in the use of such terms as aryl transferase, alkyl transferase, aralkyl transferase, alkene transferase and epoxide transferase (Boyland & Chasseaud, 1969). However the overlapping catalytic abilities of individual members of this enzyme group has prevented their being definitively classified solely by substrate specificity. For example, transferases E, D, C, B, A and AA can all catalyse the conjugation of glutathione with 1-chloro-2, 4-dinitrobenzene. The Commission on Biochemical Nomenclature now recognises the glutathione S-transferases as

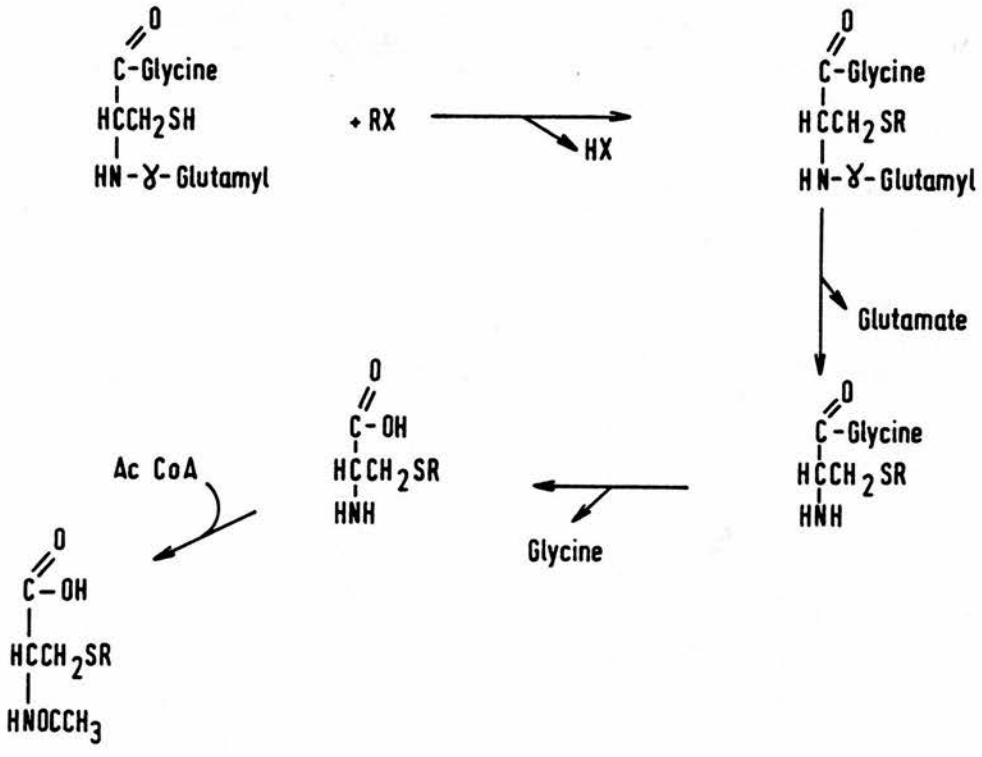


Figure 1.5 Synthetic pathway of the mercapturic acids. The initial step is catalysed by the glutathione S-transferases.

EC 2.5.1.18. The rat liver enzymes are basic proteins, which have pI values greater than 7.1. They can be separated by cation-exchange chromatography and they have been classified by their elution order. Habig et al. (1974b, 1976a) designated the transferases by their order of elution from CM-cellulose as E, D, C, B, A and AA and have described the abilities of these transferases to conjugate glutathione with a variety of substrates. This nomenclature is not entirely comprehensive as an additional cytosolic enzyme, glutathione S-transferase M, was omitted from the CM-cellulose chromatography as it was removed by preliminary purification steps (Jakoby et al., 1976b). Recently a separate glutathione S-transferase has been reported in the microsomal fraction of rat liver indicating that these enzymes are not restricted to the cytosol (Morgenstern et al., 1979).

The glutathione S-transferases are a group of enzymes which are functionally similar and, in addition to their overlapping substrate specificities, they also exhibit overlapping binding activities towards a large number of non-substrate ligands which possess hydrophobic moieties (Ketley et al., 1975). They have similar molecular weights (approx. 46 000) and each comprises two subunits (Jakoby et al., 1976b). Bass et al. (1977a) have examined the proteins

in the Y fraction, obtained by gel-exclusion chromatography of hepatic cytosol, using discontinuous SDS/polyacrylamide gel electrophoresis and found 3 major monomeric bands: Ya (mol. wt. approx. 22 000), Yb (mol. wt. approx. 23 500) and Yc (mol. wt. approx. 25 000). Since all the cytosolic transferases elute in the Y fraction this finding raised the possibility that certain glutathione S-transferases possess common subunits. Support is given to this hypothesis by the observation that antisera raised against transferase A cross-reacted with transferase C and vice versa (Habig et al., 1974b).

Ligandin: one protein or two?

The most widely studied glutathione S-transferase is ligandin from rat liver. Preparation of purified ligandin has been described by several groups using different purification techniques (Morey & Litwack, 1969; Habig et al., 1974a; Kamisaka et al., 1975; Tipping et al., 1976). Present evidence shows ligandin to be a basic protein (pI 8.7-9.0) which comprises 4.0-4.5% of the total protein in rat liver cytosol; it has a mol. wt. of 47 000 and consists of two subunits Ya (mol. wt. 22 000) and Yc (mol. wt. 25 000) (Bhargava et al., 1978; Ketterer et al., 1978). However, there is now doubt whether the different purified preparations of ligandin

contain only one protein or indeed if they comprise the same protein. These doubts arise partly because of the wide range of pI values (8.4-9.8) obtained by different groups (Litwack et al., 1971; Jakoby et al., 1976b) and also because these preparations exhibit properties compatible either, with their containing contaminating glutathione S-transferases or, with their modification during storage to a mixture of ligandin and other proteins (Habig et al., 1974b; Ketterer et al., 1976a; Listowsky et al., 1976). For example, Bass et al. (1977a) and Carne et al. (1979) have demonstrated that ligandin can be further resolved by ion-exchange chromatography into dimeric proteins comprising YaYa and YaYc monomers.

Physiological importance of the glutathione S-transferases

The physiological importance of these enzymes is emphasised by their high concentration (in rat liver the enzyme group accounts for a total of 10 % of the cytosolic protein) and their wide distribution throughout the animal kingdom (Jakoby, 1978). Furthermore, glutathione S-transferase enzymes are not restricted to the liver and activity has been reported from a number of other tissues including small intestine (Clifton & Kaplowitz, 1977), kidney (Boyland & Williams, 1965; Clifton et al., 1975; Hales et al., 1978) and erythrocytes (Marcus et al., 1978). Bass et al. (1977b) have

studied the tissue distribution of ligandin in the rat using radioimmunoassay. They found ligandin in a number of different organs, including liver, kidney, proximal and distal small-intestinal mucosa, testis, ovary, adrenals, salivary glands, stomach and pancreas.

At least four physiological functions have been proposed for the glutathione S-transferases as a result of their enzyme activity and their ability to bind, covalently and non-covalently, certain compounds. First, circumstantial evidence obtained from phylogenetic studies (Levine et al., 1971), investigations into neonatal jaundice (Levi et al., 1969a, 1970) and induction of ligandin by drugs (Reyes et al., 1969, 1971) led to the hypothesis that ligandin is involved in the hepatic uptake of a large number of anions. Further, Jakoby et al. (1976b) suggested that, since all the glutathione S-transferases tested (C, B, A and AA) bound non-covalently, different non-substrate ligands, all the glutathione S-transferases were involved in hepatic anion transport. Second, glutathione S-transferases catalyse the first step in the synthesis of the mercapturic acids and are therefore involved in the excretion of potentially harmful electrophilic compounds from the body (Chasseaud, 1976; Jakoby et al., 1976a). The glutathione S-transferases appear to catalyse two main types

of reaction : replacement (substitution), typified by the conjugation of GSH with alkyl, aryl or aralky halides; and addition,typified by the conjugation of GSH with epoxides (Chasseaud, 1976). The ability of the transferases to conjugate GSH with a large number of electrophilic compounds results in their being involved in the excretion of potentially harmful compounds from the body (Chasseaud, 1976; Jakoby et al., 1976a). GSH conjugates have the requisite physicochemical properties for biliary excretion (Chasseaud, 1974) and generally will, together with their metabolites, be present in bile in relatively high concentrations. For example, the GSH, cysteinylglycine, cysteine and N-acetylcysteine (mercapturic acid) conjugates of the corresponding hydrocarbon expoxides have been detected in the bile of rats treated with certain polycyclic hydrocarbons (Boyland & Sims, 1962, 1964a, b). Thirdly, the glutathione S-transferases are considered to reduce the susceptibility of the liver to carcinogenesis through both their catalytic activity and their ability to bind, covalently, toxic compounds thereby protecting the nucleus from potential carcinogens (Jakoby & Keen, 1977; Smith et al., 1977). Fourthly, in addition to its glutathione S-transferase activity, ligandin has been reported to possess other types of enzyme activity. The reactions which ligandin is able to catalyse are therefore not restricted to mercapturic acid metabolism. Ligandin has been

shown to have 3-oxo steroid Δ^4 - Δ^5 isomerase activity (Benson et al., 1977) and glutathione peroxidase activity (Proshaska & Ganther, 1977).

Bile acids studied in this thesis

This thesis is concerned with cholic acid and lithocholic acid-binding proteins in rat liver cytosol which could be involved in the intrahepatic transport of these bile acids. Cholic acid was used for investigation since it is quantitatively the most important bile acid in the rat (Okishio & Nair, 1966). Lithocholic acid was chosen for study for several reasons; rat liver cytosol contains more binding sites for lithocholic acid than for other bile acids and binding can be studied under non-equilibrium conditions (Strange et al., 1977a). However, Strange et al. (1977a) have shown that both cholic acid and chenodeoxycholic acid can competitively inhibit lithocholic acid binding to cytosolic proteins. This suggests that rat liver cytosol contains general bile acid-binding proteins rather than a number of binding sites which are each specific for a particular bile acid. Lithocholic acid is therefore regarded as a convenient ligand which is probably handled by the same proteins as are the other bile acids. Lithocholic acid also has toxic properties and Palmer (1964)

demonstrated that it can lyse human erythrocytes in vitro . Holsti (1960) reported that rabbits which were given lithocholic acid daily (40 nmol/kg body weight) developed cirrhosis of the liver within 3 months and Fischer et al. (1974) and Stiehl et al. (1977) have confirmed that it is hepatoxic to a variety of mammalian species including the rat. Lithocholic acid appears to be most toxic to species which are unable to form sulphated derivatives (Gadacz et al., 1976). In addition Javitt & Emerman (1968) reported that lithocholic acid has cholestatic properties inhibiting the biliary secretion of taurocholic acid. Whilst lithocholic acid is present in very low concentrations in the rat the hepatic mechanisms involved in handling this potentially toxic substance and removing it from the blood are of sufficient clinical importance to merit investigation.

Objectives of the thesis

The aim of this thesis was to characterise further the interaction of cholic acid and lithocholic acid with rat liver cytosol. Cytosolic proteins were investigated to determine whether cholic acid and lithocholic acid were bound by all the glutathione S-transferases , only certain glutathione S-transferases or proteins which did not have transferase activity. The subunit compositions

of the enzymes were investigated to see if certain binding properties or enzyme characteristics could be ascribed to the presence of particular subunits.

Experiments were performed to characterise and identify the two previously described lithocholic acid-binding proteins. Their amino acid compositions, isoelectric points and monomeric compositions have been determined. The effect of phenobarbitone on their concentrations has been investigated because phenobarbitone has been reported to increase the synthesis of ligandin (Reyes et al., 1971; Fleischner et al., 1972; Arias et al., 1976). Further, glutathione S-transferase B was prepared by the procedure of Habig et al. (1976a) and ligandin by that described by Arias et al. (1976) and these two purified proteins have been compared with the lithocholic acid-binding proteins. The relationship between the two proteins was investigated by peptide "mapping".

MATERIALS AND METHODS

Chemicals

Cholic acid, (3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid) and lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid) were from Maybridge Chemical Co., Tintagel, Cornwall, U.K.. [2,4-^{3}H] Cholic acid (14 Ci/mmol) from New England Nuclear, Dreieichenhain, West Germany and [24-^{14}C] lithocholic acid (59 Ci/mol), [^{3}H] water (5 Ci/ml) glycerol tri[1-^{14}C] palmitate (50 Ci/mol) from the Radiochemical Centre, Amersham, Bucks, U.K. were used. Butan-1-ol, hydrogen peroxide and pyridine of AristaR grade, acrylamide, NN$'$-methylenebis-acrylamide, NNN$'$N$'$-tetramethylethylenediamine , ammonium persulphate, ammonium sulphate, ninhydrin, di-sodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, sodium chloride, sodium hydroxide, sucrose, acetone, ammonia solution, anisaldehyde, chloroform, diethyl ether, isopropyl ether, ethanol, formic acid, glacial acetic acid, hydrochloric acid, iso-octane, methanol and sulphuric acid of AnalaR grade, triton X-100 and toluene of scintillation grade, 2-mercaptoethanol, 2,2,4-trimethylpentane, di-iso-propyl ether, sodium dihydrogen citrate and sodium dodecyl sulphate (SDS) of laboratory grade and Folin & Ciocalteu's phenol reagent were obtained from B.D.H. Chemicals Ltd., Poole, Dorset, U.K..

The 1,2-dichloro-4-nitrobenzene was from Eastman Kodak Co., Rochester, N.Y., U.S.A. and was twice recrystallized from ethanol before use. Reduced glutathione (GSH), rat albumin, bovine serum albumin, ovalbumin, α-chymotrypsinogen A, α-chymotrypsin, trypsin, ribonuclease A, 1,4-bis[2(5-phenyloxazolyl)] benzene (POPOP), 2,5-diphenyloxazole (PPO), TEAE-cellulose and Coomassie brilliant blue G were from Sigma Co. Ltd., Poole, Dorset, U.K.. CM-Sephadex C-50, QAE-Sephadex A-50, Sephadex G-100 and Blue Dextran 2000 were from Pharmacia Fine Chemicals Ltd., London W5 5SS, U.K.. CM-cellulose (Whatman CM52) and DEAE-cellulose (Whatman DE52) were purchased from Whatman Ltd., Maidstone, Kent, U.K. and Bio-Gel A-0.5 m (200-400 mesh) was from Bio-Rad Laboratories Ltd., Bromley, Kent, U.K. Pierce-Durrum DA X2 and DC-1A ion-exchange resins were obtained from Pierce & Warriner U.K. Ltd., Chester, Cheshire, U.K.. Sodium phenobarbitone was from Evans Medical Ltd., Speke, Liverpool, U.K.. Human immunoglobulin (IgG) and albumin were obtained from the Protein Fractionation Centre, Scottish National Blood Transfusion Association, Edinburgh, Scotland, U.K.. Poly(ethylene glycol) (mol. wt. 25 000) was from Union Carbide, Southampton, Hants, U.K. and Spectrapor dialysis membrane (mol. wt. cut off of 12 000 - 14 000) was from

Spectrapor, Spectrum Medical Industries, Los Angeles, C.A., U.S.A.. Disodium phenoltetrabromophthalein sulphonate (bromosulphophthalein) was from Hynson, Westcott and Dunning Inc., Baltimore, Maryland, U.S.A. and pH 7-9 and 9-11 Ampholines were from LKB Instruments Ltd., Selsdon, South Croydon, Surrey, U.K.. The staphylococcus aureus V8 protease, thermolysin, mercaptoethanesulphonic acid, L-amino guanidinopropionic acid, L-norleucine and the amino acid standards for high-voltage paper electrophoresis ("wonder mix" which comprised lysine, histidine, arginine, glycine, valine, alanyl glycine, β-dinitrophenyl lysine, taurine, cysteic acid, aspartic acid, glutamic acid and xylene cyanol FF) were a gift from Dr. R. P. Ambler, Department of Molecular Biology, University of Edinburgh, Edinburgh.

Buffers

Several buffers were used repeatedly. The composition and the temperatures at which they were prepared are listed in Table 2.1. All the buffers used, with the exception of buffers F, I and J were prepared at room temperature (20^{o}C).

Table 2.1 Buffer compositions

Buffer	pH	Temperature buffer prepared	Composition
A	7.5	20°C	100mM-sodium phosphate
B	6.5	20°C	100mM-sodium phosphate
C	7.4	20°C	20mM-sodium phosphate containing 100mM NaCl
D	7.4	20°C	10mM-sodium phosphate
E	6.7	20°C	10mM-sodium phosphate
F	8.6	4°C	20mM-Tris/HCl
G	8.9	20°C	375mM-Tris/HCl
H	6.8	20°C	125mM-Tris/HCl
I	8.1	4°C	10mM-Tris/HCl
J	8.8	4°C	10mM-Tris/HCl

Table 2.1 Composition of buffers used and the temperatures at which they were prepared.

Analytical Methods

Radioactive Counting

This was performed in a Packard Tri-Carb liquid scintillation spectrometer (Model 3255) using a toluene: triton X (2:1 v/v) based scintillant containing 2, 5-diphenyloxazole (PPO), 4g/l and 1,4-di(2-(5-phenyloxazolyl))-benzene (POPOP), 0.2 g/l. Portions of scintillant (5 ml) were mixed with 100 µl aqueous sample in polyethylene vials and were counted with a standard deviation of less than 1%. Samples were checked for quenching by the addition of an internal standard ([^{14}C] glycerol palmitate or [^{3}H]water). The counting efficiency was determined by counting known amounts of [^{3}H] cholic acid and [^{14}C] lithocholic acid and was found to be 60% and 85% respectively.

Glutathione S-transferase activity

This was measured at 37^{o}C by the method of Habig et al. (1974b). The conjugation of GSH with either 1,2-dichloro-4-nitrobenzene or 1-chloro-2,4-dinitrobenzene was followed at 340 nm in buffers A and B respectively with a LKB Reaction Rate

Analyzer (Model 8600). Both the reaction between GSH and 1,2-dichloro-4-nitrobenzene and the reaction between GSH and 1-chloro-2, 4-dinitrobenzene were initiated by the addition of GSH. Reaction rates were corrected for the small amount (less than 5% of total rate) of non-enzyme-catalysed conjugation of GSH with 1-chloro-2, 4-dinitrobenzene.

Protein determination

Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Protein concentrations in samples eluted from columns were calculated from the extinction values at 280 nm.

Bromosulphophthalein assay

Bromosulphophthalein concentrations were measured by the absorbance values at 580 nm following addition of NaOH (100 µl; 10 M) to 250 µl portions of column fractions (Levi et al., 1969b).

Sodium determination

Na^+ concentrations in column fractions were measured

by flame photometry using an IL 343 photometer (Instrumentation Laboratory (U.K.) Ltd., Altringham, Cheshire, U.K.).

Bile acid purity

The bile acids were shown to be 99% pure by thin layer chromatography (t.l.c.) before use. The chromatograms were developed in a stationary phase (20 cm x 20 cm x 0.25 mm) consisting of silica-gel G (Merck Darmstadt : F254) by ascending chromatography. The solvent system used was iso-octane/isopropyl ether/acetic acid (2:1:1, by vol.) (Hamilton & Muldrey, 1961). Portions (10 µl; approx. 1 µCi) of the radiolabelled bile acids and non-radiolabelled bile acids (about 10 µg) were applied to the plates, at an "origin" 1 cm from the bottom of the plate, and the chromatograms were developed until the solvent front reached 1 cm from the top of the plate (O'Moore & Percy-Robb, 1973).

The purity of the non-radiolabelled bile acid samples was assessed by staining the developed plates with acetic acid/sulphuric acid/anisaldehyde (100:2:1, by vol.) and scanning the stained chromatograms at 580 nm with a vitatron TLD 100 Flying spot densitometer (Fisons Scientific Apparatus,

Loughborough, Leics., U.K.) as described by O'Moore & Percy-Robb (1973). The purity of the radiolabelled bile acid samples was assessed by cutting 40 slices (each 0.5 cm high x 2.0 cm wide) from the developed chromatograms and measuring the radioactivity contained in each silica-gel slice by liquid scintillation spectrophotometry.

Animals

Male Wistar rats (230 - 290 g) fed ad libitum, from the Animal Breeding Research Organisation, Bush Estate, Milton Bridge, Midlothian, Scotland, U.K., were used. Phenobarbitone-treated rats were given subcutaneous injections of sodium phenobarbitone in sterile water (0.5 ml; approx. 100 mg/kg body weight) for 7 successive days and were killed on the eighth day. Phenobarbitone administration resulted in a 30-50% increase in liver weight compared with untreated animals.

Preparation of Cytosol

Rats were anaesthetised with ether and the livers perfused in situ through a portal vein cannula with approx. 20 ml of ice cold

buffer C containing 250 mM sucrose, until blood free. The liver was removed, homogenized in 20 ml of the perfusion buffer and centrifuged (30 min, 4°C, 18 000 g) in a MSE Highspeed 18 (MSE Scientific Instruments, Manor Royal, Crawley, Sussex, U.K.). The supernatant was decanted off, recentrifuged (120 min, 4°C, 100 000g) in an MSE Superspeed 50 and after removal of the lipid layer the clear supernatant was stored on ice.

Analysis of cholic acid-binding by equilibrium gel-exclusion chromatography

Strange et al. (1976, 1977a) showed that although cholic acid-binding by rat liver cytosol was observed under equilibrium conditions (equilibrium dialysis) cholic acid-binding by rat liver cytosol could not be analysed under non-equilibrium conditions by gel-exclusion chromatography (zonal gel filtration) since the putative protein: cholic acid complex(es) readily dissociated during chromatography. Cholic acid-binding was therefore investigated using an equilibrium chromatographic method. The use of equilibrium chromatography was initially devised by Hummel & Dreyer (1962) and its use has been reviewed by Wood & Cooper (1970). Equilibrium chromatography avoids the problem of dissociation since the column is eluted with a constant concentration

of ligand and therefore the ligand-protein complex(es) are constantly surrounded by "free" ligand during chromatography.

Samples for analysis were concentrated by dialysis against poly(ethylene glycol) until they had a protein concentration greater than 7 mg/ml. They were then dialysed against 2 changes, each of 2 litres, of buffer C (16h, 4°C). Portions (3 ml) of cytosol (approx. 80 mg of protein) or individual glutathione S-transferase enzyme peaks (80 or 20 mg of protein), which were obtained by ion-exchange chromatography (see pp. 46-47) were diluted with an equal volume of buffer C containing 20 nM [^{3}H] cholic acid (300 d.p.s./ml). After incubation (60 min, 4°C) the mixture was applied to a Bio-Gel A-0.5 m column (2.5 cm x 38 cm) which was equilibrated and eluted (20.5 ml/h) at 4°C with buffer C containing 10 nM [^{3}H] cholic acid (150 d.p.s./ml). Fractions of 3.4 ml were collected and analysed for protein, glutathione S-transferase activity and radioactivity. The void volume, determined using Dextran Blue, was 75 ml and the salt volume, determined by using NaCl was 195 ml. The elution volume of [^{3}H] cholic acid was 200 ml. The elution volumes of immunoglobulin G (mol. wt. 180 000); albumin (mol. wt. 67 000); ovalbumin (mol. wt. 45 000); myoglobin (mol. wt. 17 800) and ribonuclease A (mol. wt. 12 700) were plotted against the logarithm

of their mol. wt. and the straight line obtained was used to estimate the mol. wt. of the bile acid-binding proteins.

Separation of the glutathione S-transferases in cytosol

Cytosol, from two livers, was dialysed (4°C, 9 h) against two changes, each of 2 litres, of buffer D. The dialysed solution (12 ml; approx. 325 mg of protein) was eluted (4°C, 16 ml/h) from a column of CM-Sephadex (2.2 cm x 15 cm) which was equilibrated with buffer D. The fraction volume was 2.7 ml. After 70 ml had been eluted, the glutathione S-transferases which were retained by the ion-exchanger were eluted with a continuous 0-80 mM NaCl gradient in buffer D (Strange et al., 1977b). Fractions of 2.7 ml were collected and analysed for glutathione S-transferase activity by measuring the conjugation of GSH with either 1-chloro-2, 4-dinitrobenzene or 1,2-dichloro-4-nitrobenzene.

The fractions which eluted (130-180 ml) from CM-Sephadex were of particular interest and were subjected to further chromatography. These fractions were combined and concentrated to about 5 ml by dialysis, at 4°C, against poly(ethylene glycol).

After concentration this fraction was dialysed against 2 changes, each of 2 litres, of buffer F. This solution was applied to a DEAE-Sephadex column (2.2 cm x 15 cm) which was equilibrated and eluted (20 ml/h, 4°C) with buffer F. A continuous 0-500 mM NaCl gradient in buffer F was established after 95 ml eluted from the column (Strange et al., 1977b). Fractions of 3.3 ml were collected and analysed for glutathione S-transferase activity.

Analysis of lithocholic acid-binding by cytosol

The lithocholic acid-binding capacity of cytosol was studied by gel-exclusion chromatography. 500 nmol Lithocholic acid and 5.1 nmol [^{14}C] lithocholic acid (0.3 μCi) were added to 5 ml portions (40 mg of protein) of cytosol and mixed by inversion. After incubation (60 min, 4°C), the mixture was eluted (22.2 ml/h), using buffer C, from a Bio-Gel A-0.5 m column (2.5 cm x 38 cm). Fractions of 3.7 ml were collected and analysed for protein, glutatione S-transferase activity and radioactivity. The void volume, determined using Dextran Blue, was 75 ml, the salt volume, determined using NaCl was 195 ml and the elution volume of [^{14}C] lithocholic acid was 230 ml.

The lithocholic acid-binding activity of different glutathione S-transferases was studied by CM-Sephadex chromatography. Cytosol (12 ml; approx. 325 mg of protein) was dialysed (4°C, 9h) against 2 changes, each of 2 litres, of buffer D and the dialysed material was then incubated (60 min, 4°C) with 20 nmol [^{14}C] lithocholic acid (1.2 μCi). This mixture was eluted from CM-Sephadex as described.

Discontinuous SDS/polyacrylamide gel electrophoresis

This was performed at room temperature (20°C) in the presence of 0.1% (w/v) sodium dodecyl sulphate (SDS) using vertical slab gels (0.075 cm x 16.5 cm x 18 cm) in an IN/96 electrophoresis apparatus obtained from Raven Scientific Ltd., Haverhill, Suffolk, U.K.. The electrophoretic system used was that described by Laemmli (1970). This comprised a 14 cm long resolving gel of either 11.5% or 16.5% (w/v) polyacrylamide containing buffer G and a 1 cm high stacking gel of 3% (w/v) polyacrylamide containing buffer H which was formed on top of the resolving gel. Samples were prepared for electrophoresis by heating at 85°C for 10 min in an aqueous solution containing 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue and 10% (w/v) sucrose as described by Maizel (1971).

Portions (40 µl; 5-30 µg of protein) of these mixtures were run through the stacking gel at 6 W and through the resolving gel at 2W - 4W. Electrophoresis was stopped when the bromophenol blue dye marker had reached 0.5 - 3.0 cm from the end of the resolving gel. The gels were stained (2h, 20°C) in a 0.2% (w/v) solution of Coomassie brilliant blue in water/methanol/acetic acid (50:50:7, by vol.) and destained in water/methanol/acetic acid (88:5:7, by vol.). After slicing, the gels were scanned at 580 nm using a Vitatron TLD 100 Flying spot densitometer from Fisons Scientific Apparatus, Loughborough, Leics., U.K.. Fig. 2.1 shows an SDS/polyacrylamide gel of whole hepatic cytosol from normal and phenobarbitone-treated rats. Fig. 2.2 shows the densitometric scan obtained from the gel slice of the normal rat cytosol shown in Fig. 2.1.

The Ya, Yb and Yc monomers present in the Y fraction (Bass et al., 1977a) were identified as follows. Cytosol (3 ml, 40 mg of protein) was eluted (4°C, 22 ml/h) from a column of Bio-Gel A-0.5 m (2.5 cm x 38 cm). The fraction volume was 3.7 ml. The void volume (Dextran Blue), was 75 ml and the salt volume (Na^+), was 195 ml. The 3 fractions which contained the maximum glutathione S-transferase activities

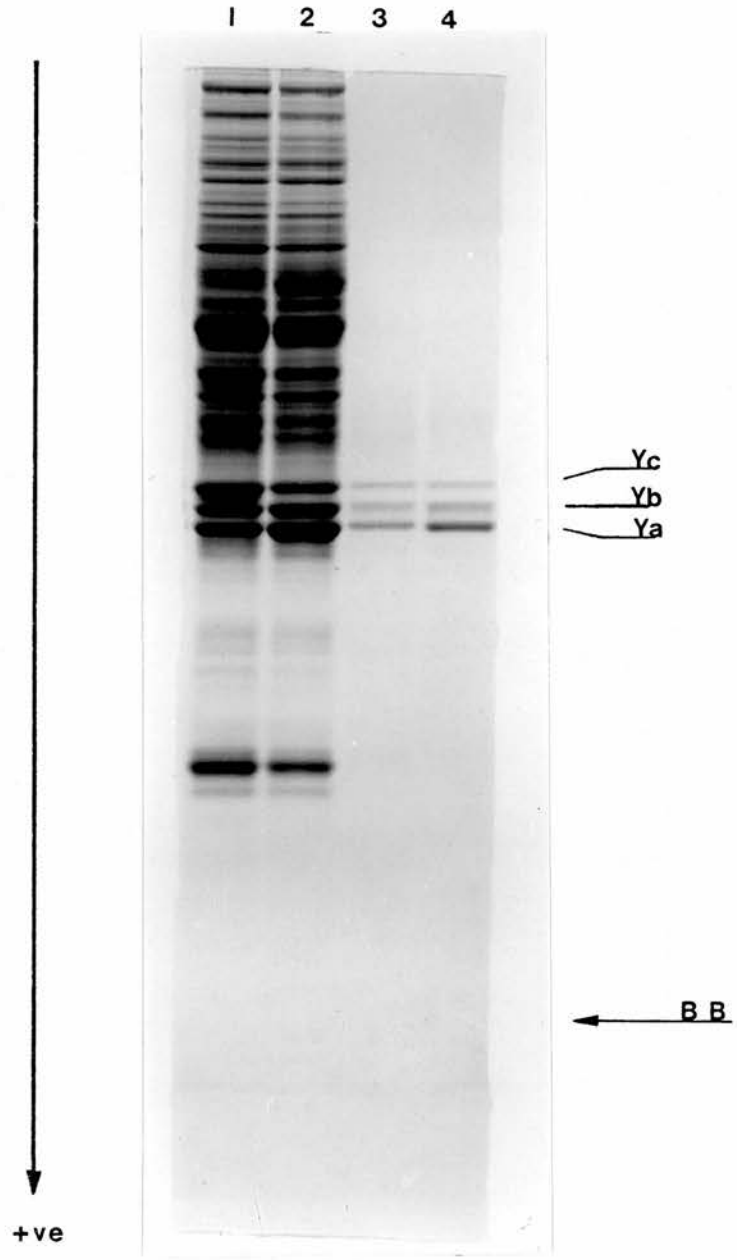


Figure 2.1

Figure 2.1 Discontinuous SDS/polyacrylamide gel electrophoresis of cytosol and "Y fractions" from normal and phenobarbitone-treated rats. The samples were applied to the gel (11.5% resolving gel) from left to right; 1, cytosol from a normal rat (60 μg of protein); 2, cytosol from a phenobarbitone-treated rat (60 μg of protein); 3, "Y fraction" from a normal rat (2.5 μg of protein) and 4, "Y fraction" from a phenobarbitone-treated rat (2.5 μg of protein). The samples were applied at the top and were run towards the anode (bottom). The position of the bromophenol blue dye marker is indicated (B.B.).

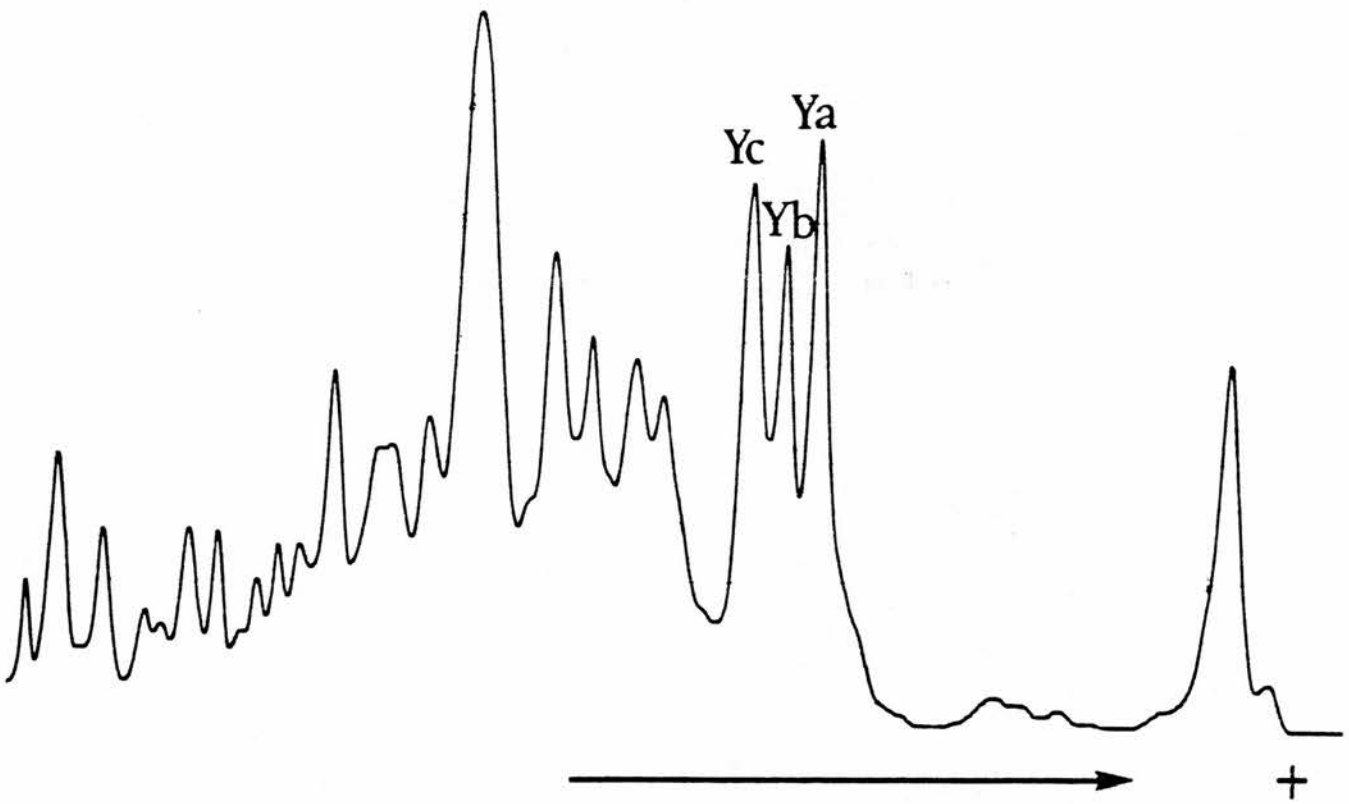


Figure 2.2 SDS/polyacrylamide gel scan of hepatic cytosol from normal rats. The gel pattern obtained from normal rat cytosol in Fig. 2.1 (track 1) (11.5% resolving gel) was scanned at 580 nm using a Vitatron TLD 100 Flying spot densitometer. The top of the gel is on the left and the direction of electrophoresis (towards the anode) is shown. The Ya, Yb and Yc bands are indicated.

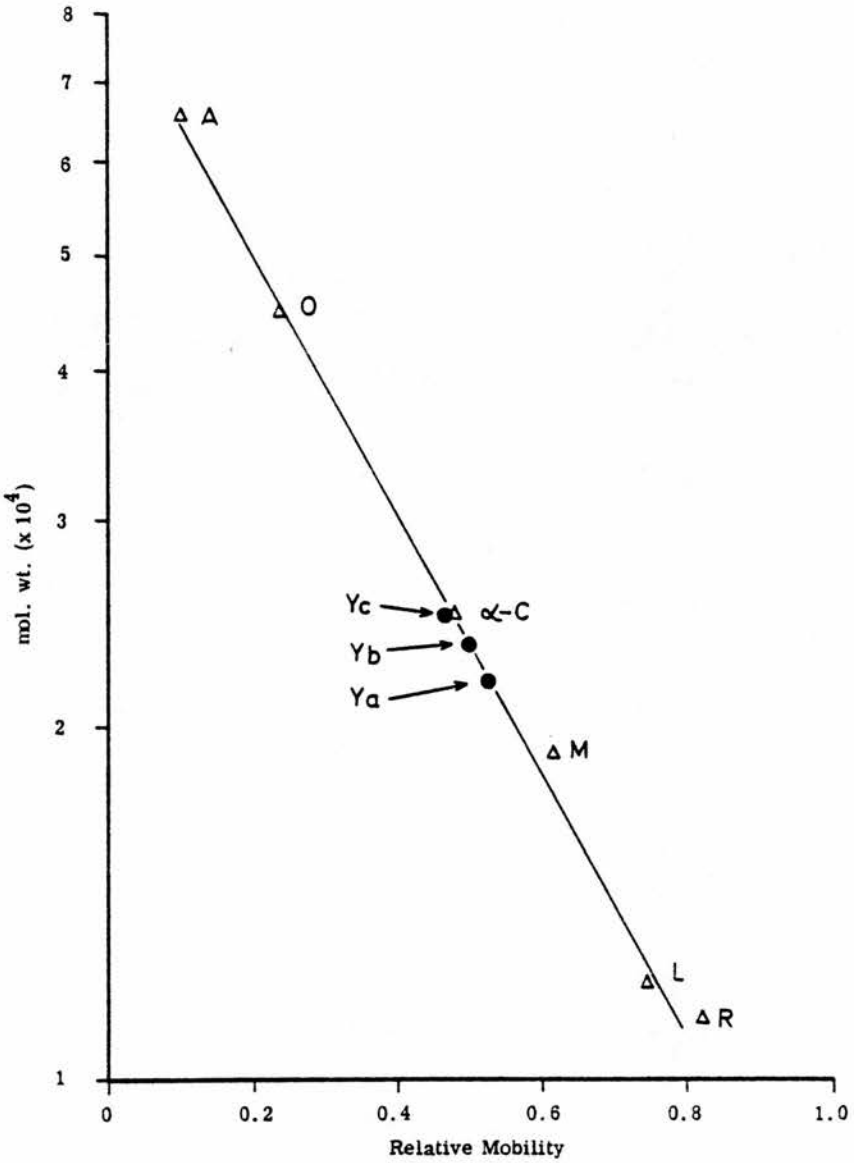

8
7
6
5
4
3
2
1
mol. wt. (x 10⁴)
A
O
Yc
Yb
Ya
α-C
M
L
R
0
0.2
0.4
0.6
0.8
1.0
Relative Mobility

Figure 2.3

Figure 2.3 Plot of mol. wt. of marker proteins against their electrophoretic mobility. Portions of rat albumin (A), ovalbumin (O), *a*-chymotrypsinogen (*a*-C), myoglobin (M), lysozyne (L) and ribonoclease A (R) were prepared for electrophoresis and samples (approx. 5 µg of protein) of each were applied to SDS/polyacrylamide gels (11.5% resolving gel). The electrophoretic mobility of each protein was compared with the bromophenol blue marker dye and its relative mobility plotted against its log. mol. wt. The relative mobilities of the Ya, Yb and Yc bands are indicated.

(elution volume 124 - 136 ml) were combined and a portion (approx. 20 μg of protein) of this preparation was examined by discontinuous SDS/polyacrylamide gel electrophoresis. Densitometry of the electrophoretic pattern demonstrated that 85% of the total protein present was recovered in 3 bands (Ya, Yb and Yc). The mol. wt. of the Ya, Yb and Yc bands were calculated by comparing their electrophoretic mobilities against rat albumin (mol. wt. 67 000), ovalbumin (mol. wt. 45 000), α-chymotrypsinogen (mol. wt. 25 000), myoglobin (mol. wt. 17 800), lysozyme (mol. wt. 14 300) and ribonuclease A (mol. wt. 12 700). The relative mobility of each protein compared with the bromophenol blue "tracking-dye" was calculated and plotted against the logarithm of the mol. wt. (Fig. 2.3). The graph was found to be linear as reported by Shapiro et al. (1967). From this graph the mol. wt. of Ya, Yb and Yc was estimated as 22 000, 23 500 and 25 000 respectively, which is in good agreement with the values of 23 000 (Ya), 24 000 (Yb) and 25 000 (Yc) reported by Bass et al. (1977a).

Subunit composition of the glutathione S-transferases

Before discontinuous SDS/polyacrylamide gel electrophoresis,

the glutathione S-transferases were partially purified. Two portions of cytosol (each 5 ml; approx. 180 mg of protein) were eluted (22 ml/h) with buffer C, from the Bio-Gel column (2.5 cm x 38 cm). The enzyme-containing fractions which eluted between 124 - 136 ml were combined (about 85% of the protein in this mixture migrated with the Ya, Yb and Yc monomer bands). Ammonium sulphate was added to the combined enzyme-containing eluate and the precipitate, formed between 55-85% saturation, was collected, redissolved in 12 ml of buffer D and dialysed against 2 changes, each of 2 litres, of the same buffer (4°C, 15 h)(Dixon & Webb, 1964; Strange et al., 1977b). The dialysed solution was eluted from CM-Sephadex as described and the fractions which eluted from CM-Sephadex between 130-180 ml were rechromatographed on DEAE-Sephadex as described. Portions (10 µg of protein) of each enzyme-containing peak were examined by discontinuous SDS/polyacrylamide gel electrophoresis as described.

Polyacrylamide gel isoelectric focussing

This was performed using 6% (w/v) polyacrylamide slab gels (12 cm x 11.5 cm x 0.2 cm) containing 12.5% (w/v) sorbitol and 0.3% (w/v) ampholines pH 7-9 and 2% (w/v) ampholines pH 9-11 on

an LKB 2117 Multiphor (8°C) with an LKB 2103 power supply as described by Karlsson et al., 1973. The cathode electrode solution was 1.0 M NaOH and the anode electrode solution was a 0.1% (w/v) solution of ampholines pH 7 - 9. The gel was run for 60 min to allow a pH gradient to form before the samples (20 µl; approx. 25 µg of protein) were applied with LKB electrofocussing strips (10 mm x 6.5 mm) (Vesterberg, 1973). The pH range obtained after electrophoresis (3h; 20 W - 30 W) was determined by taking 5 mm slices from the gel after each run and eluting the carrier ampholines with degassed deionised water. The pH's of these ampholine-containing solutions were measured at 20°C. The gels were then stained (60 min, 60°C) in a 0.1% (w/v) solution of Coomassie brilliant blue in methanol/water (15:31, v/v) containing 0.15 M sulphosalicylic acid and 0.65 M trichloroacetic acid as described (Karlsson et al., 1973). The gels were destained in water/ethanol/acetic acid (66:26:8, by vol.).

Preparation of glutathione S-transferase B

This was prepared as described by Habig et al. (1974b, 1976a) and Table 2.2 presents a summary of the purification procedure. Livers from 2 rats were frozen in a beaker placed in a mixture of

Purification procedure for glutathione S-transferase B

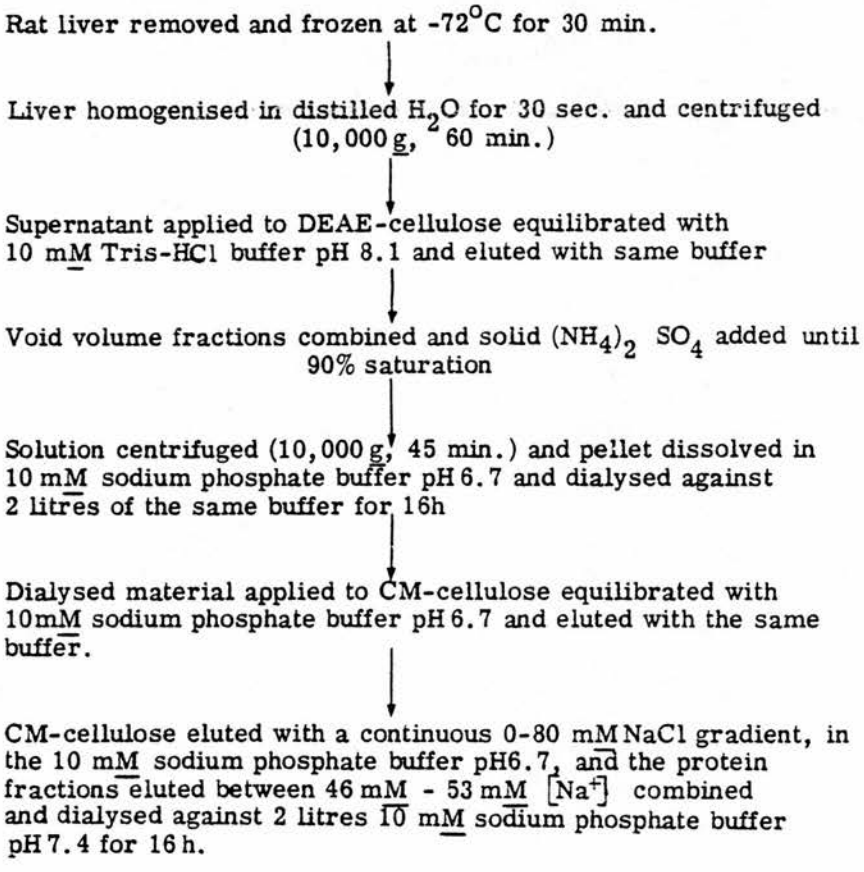


Table 2.2 Purification scheme for glutathione S-transferase B.

solid CO_2 and ethanol (approx. -72°C). After 30 min the frozen livers were removed, added to 40 ml of distilled water (4°C) and crushed with a pestle. This preparation was blended for 30 s in a Sunbeam liquidiser Model PB-P (Sunbeam Corporation Ltd., Maribyrnong, Victoria, Australia) and the resulting solution was centrifuged (1 h, 4°C, 10 000g) in an M.S.E. Highspeed 18 centrifuge. The lipid layer was removed and 16 ml of the supernatant applied to a DEAE-cellulose column (2.2 cm x 15 cm) which was equilibrated and eluted (22 ml/h, 4°C) with buffer I. The enzyme-containing fractions, eluted between 29 - 65 ml, were combined. Solid $(NH_4)_2SO_4$ was added, with stirring, at 20°C until 90% saturation was reached (Dixon & Webb, 1964). After standing (30 min, 20°C), the solution was centrifuged (10 000 g, 45 min, 20°C). The supernatant was discarded, the precipitate redissolved in 10 ml of buffer E and the solution dialysed (16 h, 4°C) against 2 litres of the same buffer. The dialysed solution was applied to a CM-cellulose column (2.2 cm x 15 cm) equilibrated and eluted (22 ml/h, 4°C) with buffer E. The fraction volume was 3.7 ml. After 120 ml of eluant had been collected a continuous 0-80 mM NaCl gradient in the same buffer was initiated (Habig et al., 1974b). Five peaks of enzyme activity, able to catalyse the conjugation of GSH with 1-chloro-2,

4-dinitrobenezene, were eluted at Na^+ concentrations of 15, 37, 50, 59 and 70 mM. Only the enzyme-containing peaks eluted at Na^+ concentrations of 37 and 59 mM were able to catalyse the conjugation of GSH and 1,2-dichloro-4-nitrobenzene indicating that these peaks contain transferases C and A respectively (Habig et al., 1974b). The enzyme-containing peak which eluted between these transferases, at a Na^+ concentration of 50 mM, is glutathione S-transferase B (Habig et al., 1974b).

Preparation of ligandin

Ligandin was prepared essentially by the method of Arias et al. (1976) and Table 2.3 presents a summary of the purification procedure. After perfusion in situ with approx. 20 ml of ice-cold buffer D, 100 g of liver was homogenised in 300 ml of buffer D containing 250 mM sucrose. The homogenate was centrifuged (100 000 g, 90 min, 4°C) in an M.S.E. Superspeed 50 ultracentrifuge and the resulting supernatant dialysed against 5 litres of buffer J (16 h, 4°C). The dialysed material (80 ml) was eluted (25 ml/h) from a column of TEAE-cellulose (2.5 cm x 100 cm) equilibrated with buffer J. The first protein peak, eluted between 350 - 500 ml, was combined, concentrated to 10 ml by dialysis against poly(ethylene glycol) and redialysed

Purification procedure for ligandin

Rat livers perfused *in situ* with 10 mM sodium phosphate buffer pH 7.4, removed and homogenised in the same buffer containing 250 mM sucrose.

↓

Homogenate centrifuged 100,000 g for 90 min.

↓

Supernatant dialysed against 5 litres of 10 mM Tris-HCl buffer pH 8.8 for 16 h

↓

Dialysed material applied to TEAE-cellulose equilibrated and eluted with 10 mM Tris-HCl buffer pH8.8

↓

Void volume fractions combined, concentrated to 10 ml and redialysed against 2 litres of 10 mM sodium phosphate buffer pH 7.4 for 16 h

↓

Bromosulphophthalein added to the dialysed material and the mixture eluted from Sephadex G-100.

↓

Fractions containing protein-bound bromosulphophthalein combined, concentrated to 5 ml and redialysed against 2 litres of 10 mM Tris-HCl buffer pH 8.8 for 16 h

↓

Dialysed material applied to QAE-Sephadex equilibrated and eluted with 10 mM Tris-HCl buffer pH 8.8

↓

Void volume fractions combined and dialysed against 2 litres of 10 mM sodium phosphate buffer pH 7.4 for 16 h

Table 2.3 Purification scheme for ligandin.

(16 h, 4°C) against 2 litres of buffer D. The resulting solution was incubated (60 min, 4°C) with 20 mg of bromosulphophthalein and eluted (24 ml/h) with buffer D from a column of Sephadex G-100 (2.5 cm x 100 cm) (Kirsch et al., 1975). The six fractions (elution volume 265-300 ml; approx. mol. wt. 45 000) containing the maximum amount of protein-bound bromosulphophthalein were combined and concentrated to approx. 5 ml of dialysis against poly(ethylene glycol). After dialysis (16 h, 4°C) against 2 litres of buffer J this solution was eluted (30 ml/h) from a column of QAE-Sephadex A-50 (1.5 cm x 60 cm) equilibrated with buffer J. Fractions eluted between 30 - 60 ml contained ligandin (Bass et al., 1977a).

Purification of the two lithocholic acid-binding proteins LBa and LBb

The lithocholic acid-binding proteins LBa and LBb were purified essentially as described by Strange et al. (1977b) and Table 2.4 presents a summary of the purification procedure.

Solution 1 Cytosol from 20 livers was dialysed (4°C, 18 h) against 2 changes, each of 5 litres, of buffer D. The dialysed solution (250 ml, approx. 1 g of protein) was eluted (5o ml/h) from a CM-Sephadex C-50 column (4.4 cm x 20 cm) with buffer D and 8.9 ml fractions were collected. A continuous 0-80 mM NaCl

Purification procedure for LBa and LBb.

Rat livers perfused in situ with 10mM sodium phosphate buffer pH7.4 containing 250mM sucrose, removed and homogenised in the same buffer.

↓

Homogenate centrifuged 18,000g for 30min and the supernatant recentrifuged 100,000g for 120min.

↓

Supernatant dialysed against 2 changes, each of 2 litres, of 10mM sodium phosphate buffer pH7.4 for 18h.

↓

Dialysed material applied to CM-Sephadex equilibrated with 10mM sodium phosphate buffer pH7.4 . CM-Sephadex eluted with a 0-80mM NaCl gradient, in the 10mM sodium phosphate buffer pH7.4, and the protein fractions eluted between 25mM - 75mM $[Na^+]$ combined.

↓

Solid $(NH_4)_2SO_4$ added to the combined fractions and the material precipitated between 55% - 85% saturation combined, centrifuged (50,000g, 30min) and the pellet dissolved in 10mM sodium phosphate buffer pH7.4

↓

Redissolved material eluted from Sephadex G-100 with 10mM sodium phosphate buffer. Fractions containing glutathione S-transferase activity combined and dialysed against 2 changes, each of 20mM Tris-HCl buffer pH8.6

↓

Dialysed material applied to DEAE-Sephadex equilibrated and eluted with 20mM Tris-HCl buffer pH8.6

↓

Void volume fractions combined and dialysed against 2 changes, each of 2 litres, of 10mM sodium phosphate buffer pH7.4 for 18h.

↓

Dialysed material applied to CM-Sephadex equilibrated with 10mM sodium phosphate buffer pH7.4 . CM-Sephadex eluted with a 0-80mM NaCl gradient in the 10mM sodium phosphate buffer pH7.4 and the fractions eluted between 35mM - 45mM and 54mM - 61mM $[Na^+]$ separately combined.

Table 2.4 Purification scheme for LBa and LBb.

gradient, in the same buffer, was initiated after elution of 530 ml; the fractions which eluted between 25-75 mM Na^+ (elution volume 550 - 610 ml) were combined, giving Solution 1 (Fig. 2.4).

Solution 2 Solid $(NH_4)_2SO_4$ was added (20°C), with stirring, to solution 1 until 55% saturation was achieved (Dixon & Webb, 1964). This solution was left standing for 30 min (20°C) before the precipitate was removed by centrifugation (5000 g, 15 min, 20°C) and discarded. More $(NH_4)_2SO_4$ was added to the resulting supernatant until 85% saturation was obtained. This solution was also left standing for 30 min (20°C) before centrifugation (50 000 g, 30 min, 20°C) in an M.S.E. Superspeed 50 ultracentrifuge. The supernatant was discarded and the precipitate redissolved in 10 ml of buffer D (Solution 2).

Solution 3 The redissolved material was applied to a Sephadex G-100 column (2.5 cm x 95 cm) which was equilibrated and eluted (31 ml/h) with buffer D. The void volume (Dextran Blue), was 200 ml and the salt volume (Na^+), was 480 ml. Fractions of 5.2 ml were collected. The 7 fractions (elution volume 265 - 301 ml) which contained most glutathione S-transferase activity (measured with GSH and 1-chloro-2, 4-dinitrobenzene) were combined and dialysed (4°C, 18 h) against two changes, each of

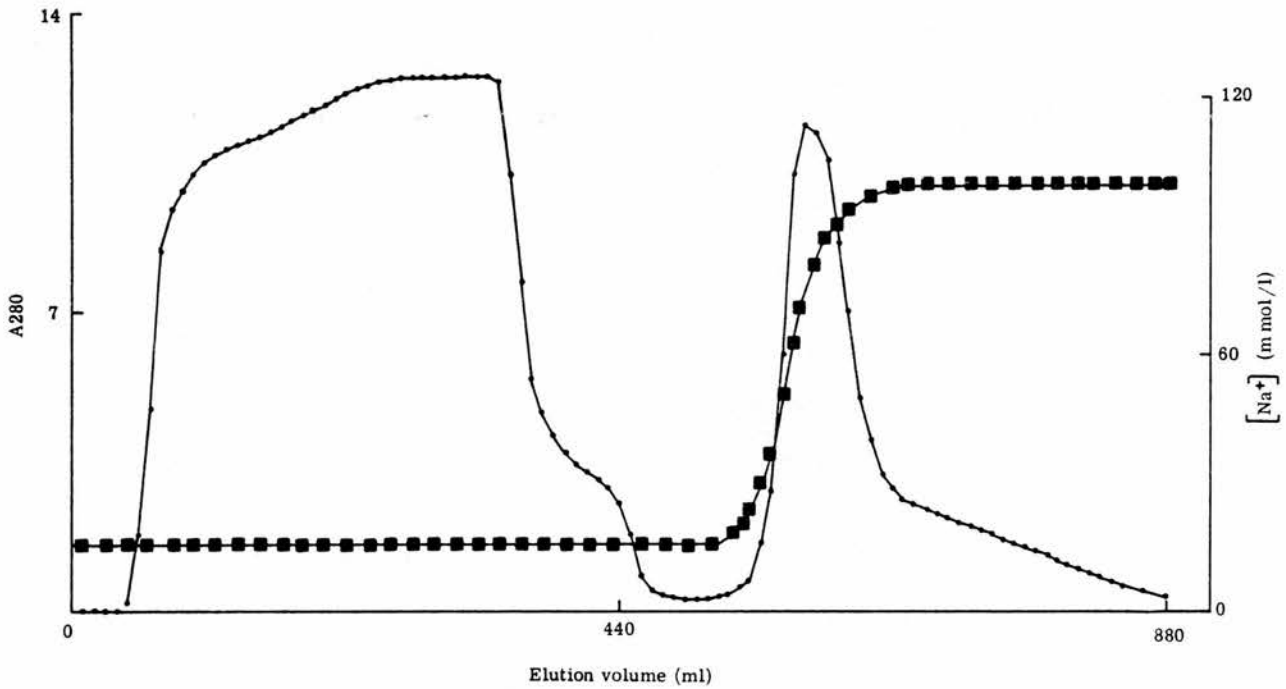


Figure 2.4 Purification of LBa and LBb (preparation of Solution 1). Cytosol (250 ml, approx. 1g of protein) was eluted from a CM-Sephadex column (4.4 cm x 20 cm) as described). Fractions of 8.9 ml were collected and the A_{280} (●) and the Na^+ concentrations (■) determined. The fractions which contained 25 - 75 mM $[Na^+]$ were combined, giving Solution 1.

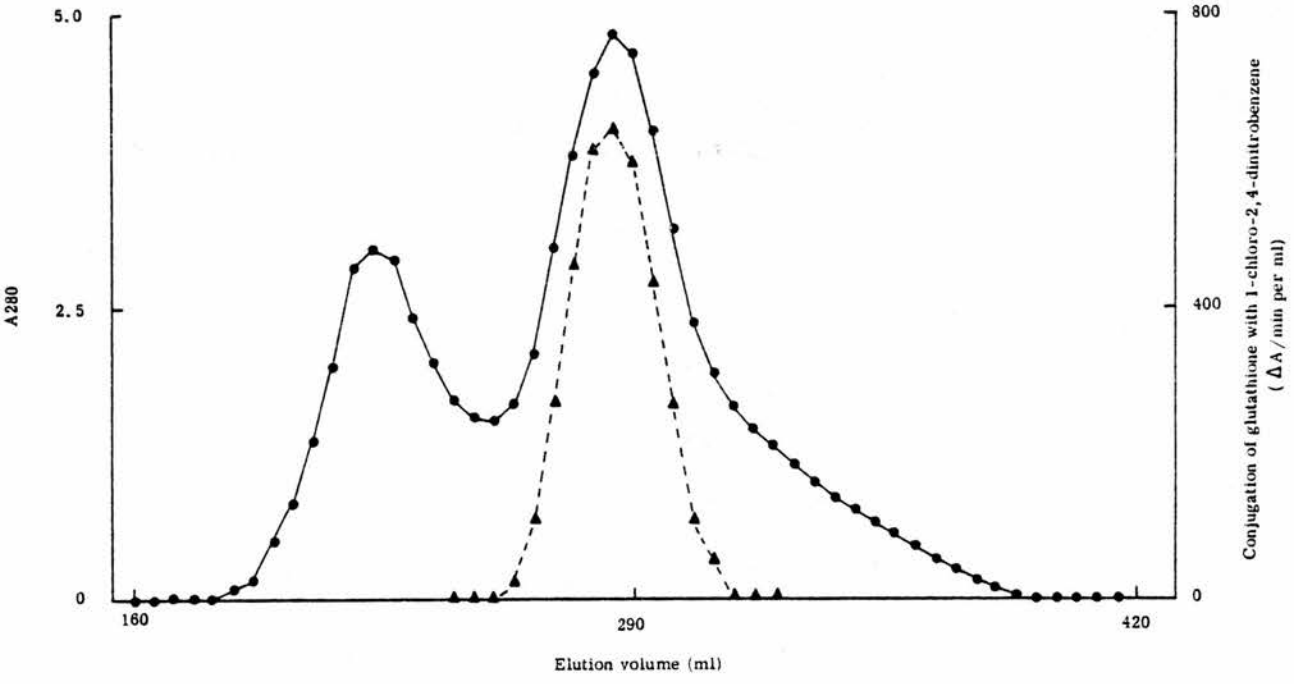


Figure 2.5 Purification of LBa and LBb (preparation of Solution 3). The protein fraction in Solution 1 which precipitated between 55 - 85% ammonium sulphate saturation was collected and dissolved in 10 ml of buffer D, giving Solution 2. This solution was eluted from a Sephadex G-100 column (2.5 cm x 95 cm). Fractions of 5.2 ml were collected and the A_{280} (●) and glutathione S-transferase activities (▲) were measured. Fractions which eluted 265 - 301 ml were combined, giving Solution 3.

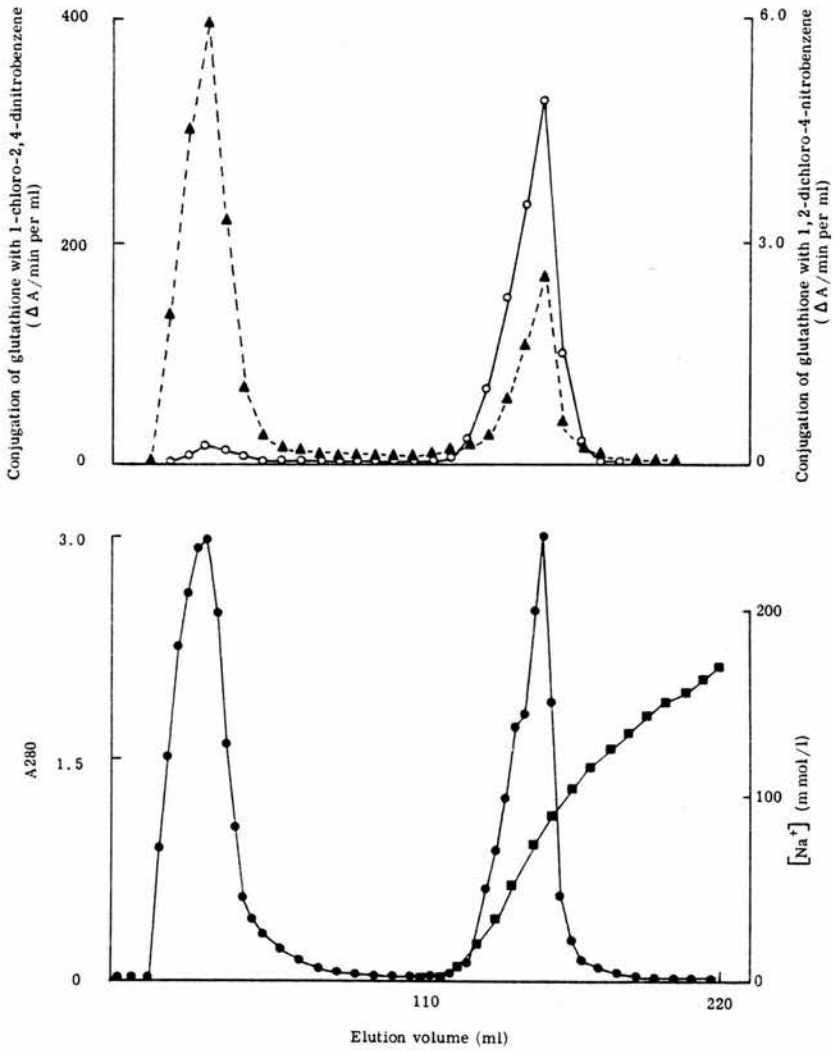


Figure 2.6 Purification of LBa and LBb (preparation of Solution 4). Solution 3 was eluted from a DEAE-Sephadex column (2.2 cm x 15 cm) as described. Fractions of 3.4 ml were collected and the A_{280} (●) and the Na^+ concentration (■) were determined. The glutathione S-transferase activity was measured with 1-chloro-2, 4-dinitrobenzene (▲) and 1,2-dichloro-4-nitrobenzene (○) as substrates. Fractions eluted between 14 - 38 ml were combined and dialysed as described, giving Solution 4.

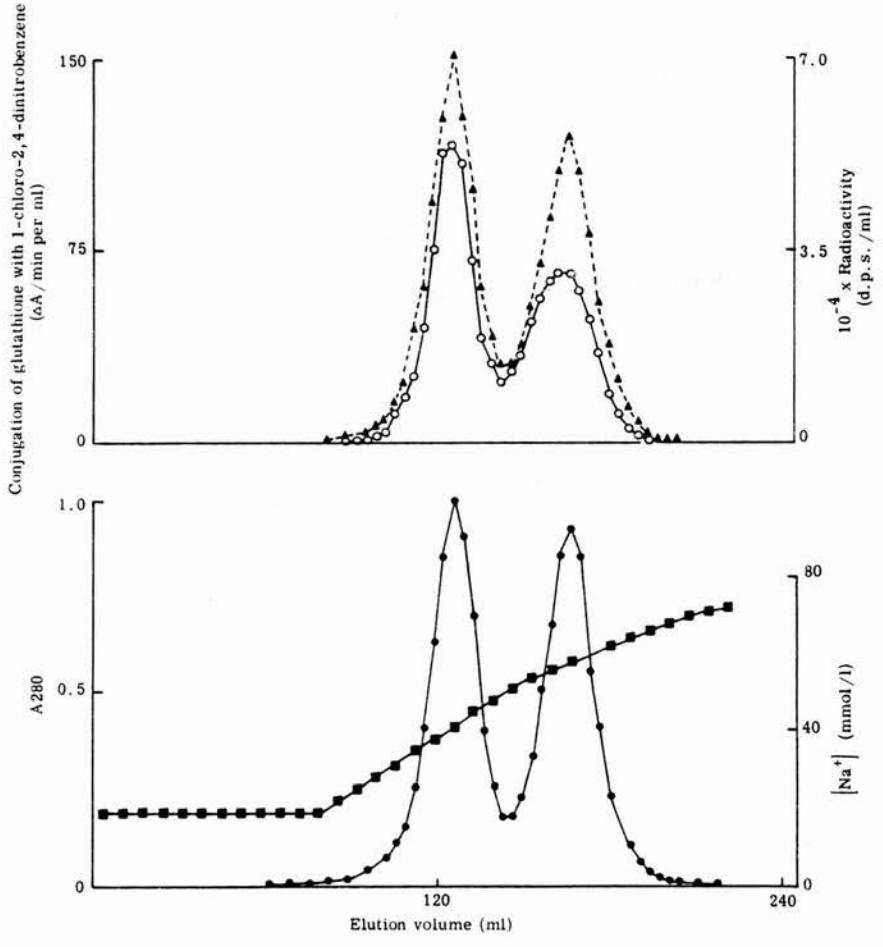


Figure 2.7 Purification of LBa and LBb (resolution of the two proteins). Solution 4 was eluted with [^{14}C]lithocholic acid from a column of CM-Sephadex (2.2 cm x 20 cm) as described. Fractions of 3.4 ml were collected and the A_{280} (●), Na^+ concentration (■), radioactivity (○) and glutathione S-transferase activi ty, with 1-chloro-2, 4-dinitrobenzene as substrate (▲), were measured. Fractions which eluted between 115 - 133 ml (LBa) and 156 - 173 ml (LBb) were combined.

2 litres, of buffer F (Solution 3) (Fig. 2.5).

Solution 4 Solution 3 was applied to a column (2.2 cm x 15 cm) of DEAE-Sephadex A-50 equilibrated and eluted at 4°C with buffer F. The flow rate was 20.4 ml/h and the fraction volume 3.4 ml. A continuous 0-200 mM NaCl gradient was established in buffer F after 110 ml was collected. Fractions eluted between 14 - 38 ml were combined and dialysed (4°C, 18h) against two changes, each of 2 litres, of buffer D giving Solution 4 (Fig. 2.6). Solution 4 was incubated (4°C, 60 min) with 17 nmol [^{14}C]lithocholic acid (50 µl; 1 µCi) and applied to a column (2.2 cm x 20 cm) of CM-Sephadex C-50 equilibrated and eluted with buffer D. The flow rate was 20.4 ml/h and the fraction volume 3.4 ml. A continuous 0.80 mM NaCl gradient was established in buffer D after 80 ml was collected (Fig. 2.7). The fractions eluted between 115 - 133 ml and 156 - 173 ml were separately combined and dialysed against two changes, each of 2 litres, of 50 mM ammonium acetate buffer, pH 8.5. These contained LBa and LBb respectively.

Analysis of amino acid compositions

To allow a direct comparison between the amino acid compositions

of the two purified lithocholic acid-binding proteins, LBa and LBb, to be made, the different steps involved in the analyses were performed simultaneously, on both proteins, under identical conditions.

Portions (0.7 mg of protein) of LBa and LBb were evaporated to dryness under vacuum in a desiccator, over H_2SO_4, at room temperature. These samples were hydrolysed at 105°C in evacuated sealed Pyrex glass tubes with 0.4 ml of 6 M HCl for 24 h or 96 h as described by Ambler & Brown (1967). After hydrolysis the glass tubes were opened and the samples were desiccated under vacuum, over NaOH pellets. The dried samples were then redissolved in 0.35 ml 200 mM trisodium citrate/HCl buffer, pH 2.2, containing 0.5 mM L-amino guanidinopropionic acid and 0.5 mM L-norleucine as internal standards.

The amino acid compositions were determined using a Beckman-Spinco Model 120-C Amino Acid Analyser (Benson & Patterson, 1965). This analyser employs two ion-exchange columns: one column (0.9 cm x 5.0 cm, which contained Pierce-Durram DA-X2 ion-exchange resin) separates the basic amino acids and ammonia and the other column (0.9 cm x 50 cm, which contained Pierce-Durram DC-1A ion-exchange resin) separates the acidic and the neutral amino acids. To enable the complete amino acid compositions (basic amino

acids plus acidic and neutral amino acids) of LBa and LBb to be determined both hydrolysed proteins were divided into two 0.15 ml portions. One portion was eluted (70 ml/h, 53°C) from the short column (0.9 cm x 5.0 cm) with 116 mM trisodium citrate buffer, pH 5.2. The other portion was applied to the long column (0.9 cm x 50 cm) which was first eluted (70 ml/h, 53°C) with 67 mM trisodium citrate/HCl buffer, pH 3.28, for about 1 h (until proline, glycine and alanine had eluted) and was then eluted (70 ml/h, 53°C) with 67 mM trisodium citrate/HCl buffer, pH 4.25. The recoveries of the basic amino acids, which eluted from the short column (0.9 cm x 5.0 cm) and the neutral and acidic amino acids which eluted from the long column (0.9 cm x 50 cm) were checked by comparing the elution of the two internal standards. L-amino guanidinopropionic acid was used to check the amino acid recovery from the short column (0.9 cm x 5.0 cm); it eluted between NH_3 and arginine. L-norleucine was used to check the amino acid recovery from the long column (0.9 cm x 50 cm); it eluted between leucine and tyrosine. The recoveries were found to be 85-98%.

Certain amino acids (asparagine, glutamine, cysteine cystine and tryptophan) are destroyed during acid hydrolysis. Asparagine is converted to aspartic acid and glutamine is converted to glutamic acid during acid hydrolysis. These two amino acids are

responsible for the NH_3 produced by chemical hydrolysis of proteins. However since no distinction was made between the aspartic acid (Asp.) originally present in LBa and LBb and the asparagine (Asn.) which has been subsequently converted to aspartic acid during hydrolysis the aspartic acid recovered from the long column (0.9 cm x 50 cm) during analysis was designated Asx, rather than Asp. Likewise the glutamic acid recovered during analysis of LBa and LBb was designated Glx, rather than Glu.

Since cysteine and cystine are acid-labile, the amounts of these amino acids in LBa and LBb were determined after first oxidising the proteins with performic acid (Hirs, 1967; Schroeder, 1968). Oxidation with performic acid converts both cystine and cysteine to cysteic acid and methionine to methionine sulphone. Cysteic acid, unlike cystine and cysteine, is not destroyed during hydrolysis in 6 M HCl and therefore the content of cystine and/or cysteine was determined by measuring the cysteic acid content after performic acid oxidation. Portions (0.7 mg of protein) of LBa and LBb, which had been evaporated to dryness, were treated with 0.4 ml 22 M performic acid (120 min, $0^{\circ}C$) and the reaction products were desiccated. These samples were then hydroysed with 0.4 ml 6 M HCl (24h, $105^{\circ}C$) before being desiccated and the dried products dissolved in 0.2 ml 200 mM trisodium citrate/HCl buffer, pH 2.2, containing 0.5 mM L-amino guanidinopropionic acid and 0.5 mM L-norleucine.

A portion (0.15 ml; approx. 0.3 mg of protein) of the redissolved material was eluted from the long column (0.9 cm x 50 cm) of the Amino Acid Analyser. Cysteic acid is the first amino acid to elute from the long column.

The tryptophan content of LBa and LBb was determined by hydrolysis with 3 M mercaptoethanesulphonic acid (Penke et al., 1974). Portions (o.7 mg of protein) of LBa and LBb, which had been evaporated to dryness, were treated with 0.4 ml of 3 M mercaptoethanesulphonic acid for 24 h. These hydrolysates were then desiccated under vacuum, over H_2SO_2 and dissolved in 0.35 ml 200 mM trisodium citrate/HCl buffer, pH 2.2, containing 0.5 mM L-amino guanidinopropionic acid and 0.5 mM L-norleucine. Two 0.15 ml portions were analysed. One portion was eluted from the short column and one portion was eluted from the long column of the Amino Acid Analyser as described. Tryptophan eluted from the long column after phenylalanine.

The results from different analyses were corrected to minimize the effect of pipetting inaccuracies. This correction factor was applied to the recoveries of all the analyses and was the ratio of the sums of the recoveries of glycine, alanine, leucine, aspartic acid (Asx), glutamic acid (Glx), phenylalanine, lysine and arginine to the sum of these

amino acids in the 24 h sample (Ambler & Brown, 1967). These amino acids were chosen since they are stable during hydrolysis and their recoveries after different analyses should be very similar. No corrections were made for the hydrolytic loss of serine and threonine, nor were corrections made for the increased recovery of valine and isoleucine with prolonged hydrolysis.

Peptide "mapping" of LBa and LBb by limited proteolytic digestion in the presence of SDS

The method used was that described by Cleveland et al. (1977). The two purified lithocholic acid-binding proteins (approx. 1 mg of protein/ml) were each heated to 95°C, for 2 min, in the presence of 0.2%(w/v) SDS and 1 mM EDTA. These mixtures were cooled (37°C, 10 min) and proteolytic digestions were carried out at 37°C, by addition of various amounts of either α-chymotrypsin or staphylococcus aureus V8 protease (10 µl containing 14, 1.4, 0.14, 0.014 or 0.0014 µg of protein) to portions (50 µl approx. 50 µg of protein) of the purified lithocholic acid-binding proteins (LBa and LBb). After 45 min, 2-mercaptoethanol, SDS, sucrose and bromophenol blue were added to final concentrations of 1%, 2%, 10% and 0.002% respectively and the digestions terminated by heating (95°C, 10 min). Portions (50 µl) of the digest, containing 25 µg of digested lithocholic

acid-binding protein and 7.0 - 0.0007 μg of either of the proteolytic enzymes, were analysed by discontinuous SDS/polyacrylamide gel electrophoresis using a 3% stacking gel and 16.5% polyacrylamide resolving gel as described. For controls either *a*-chymotrypsin or staphylococcus aureus V8 protease were incubated alone at the highest concentration used for digestion (140 μg/ml) and 7.0 μg of the proteolytic enzyme was applied to the gel.

Peptide "mapping" of LBa and LBb by total proteolytic digestion

Portions (0.5, 1.0 or 1.5 mg) of the lithocholic acid-binding proteins LBa and LBb were dried under vacuum in a desiccator over H_2SO_4. The two proteins were prepared for proteolysis by oxidation with performic acid (120 min, 0°C) and the reaction products desiccated under vacuum. The two proteins were dissolved in 100 μl 0.1 M NH_3 before 0.3 ml of 200 mM ammonium acetate buffer, pH 8.5, was added and the pH adjusted to 9-10 with 0.1 M NH_3.

Digestion of LBa and LBb was carried out at 37°C for 6 h with either trypsin, chymotrypsin or thermolysin using a protein/proteolytic enzyme ratio of 50:1 (w/w) (Bhargava et al., 1978). After digestion the reaction mixture was lyophilised and

stored under vacuum at room temperature.

The freeze-dried digestion products were examined by high-voltage electrophoresis after dissolution of the freeze-dried powder in 100 µl 0.1 M NH_3. This solution was centrifuged (4 000 g, 2 min, 20°C) and the supernatant applied to Whatman 3 MM paper. Pieces of Whatman 3 MM chromatography paper (56 cm long, 25 - 42 cm wide) were used throughout. High-voltage paper electrophoresis was carried out in apparatus similar to that described by Michl (1951). The buffer systems and coolants at pH 6.5, 3.5 and 2.0 were as described by Ambler (1963). In all the runs (45 min at 3000 v) a parallel separation of a mixture of amino acids including arginine, histidine, E-DNP-lysine, aspartic acid, glutamic acid and xylene cyanol FF ("Wondermix") and a spot of red Pentel pen was performed on the same paper (Milstein & Milstein, 1968). After each run the paper was examined under ultraviolet light (340 nm) before ninhydrin treatment, as the tryptophan-containing peptides fluoresce (Ambler, 1963). Peptides were visualised by dipping the paper in 0.2% (w/v) ninhydrin solution in acetone to which had been added (just before use) 3% (v/v) collidine. The paper was then dried at room temperature, for 5 min, and then heated, at 70°C, for 10 min.

The multidimensional tryptic peptide "maps" of LBa and LBb were formed by a combination of electrophoresis and chromatography. All the steps involved in the "mapping" were performed simultaneously on both proteins under identical conditions. A diagramatic representation (which is not drawn to scale) of the procedures involved in constructing these "maps" is shown in Fig. 2.8. In the first dimension the soluble peptides were separated into the basic, neutral and acidic peptides by high-voltage paper electrophoresis at pH 6.5. During electrophoresis at pH 6.5 the basic peptides migrate towards the cathode, the acidic peptides migrate towards the anode and the neutral peptides migrate as a single band which is slightly displaced from the origin (towards the cathode) by endosmosis. The basic and acidic peptides, which were partially resolved by electrophoresis at pH 6.5, were separated in the second dimension by electrophoresis at pH 3.5. This was carried out by cutting these peptides from the electrophoresis paper after the first dimension, rotating them through 90^{o} and sewing them onto fresh paper so that migration in the second dimension was at right angles to that in the first. The basic peptides from LBa and LBb were sewn onto a single piece of chromatography paper in a "tail-to-tail" orientation which resulted in the production of inverted repeat V-shaped patterns after migration in the second dimension. The acidic peptides from LBa and LBb were also sewn onto a single piece of chromatography paper in a "tail-to-tail" manner and were

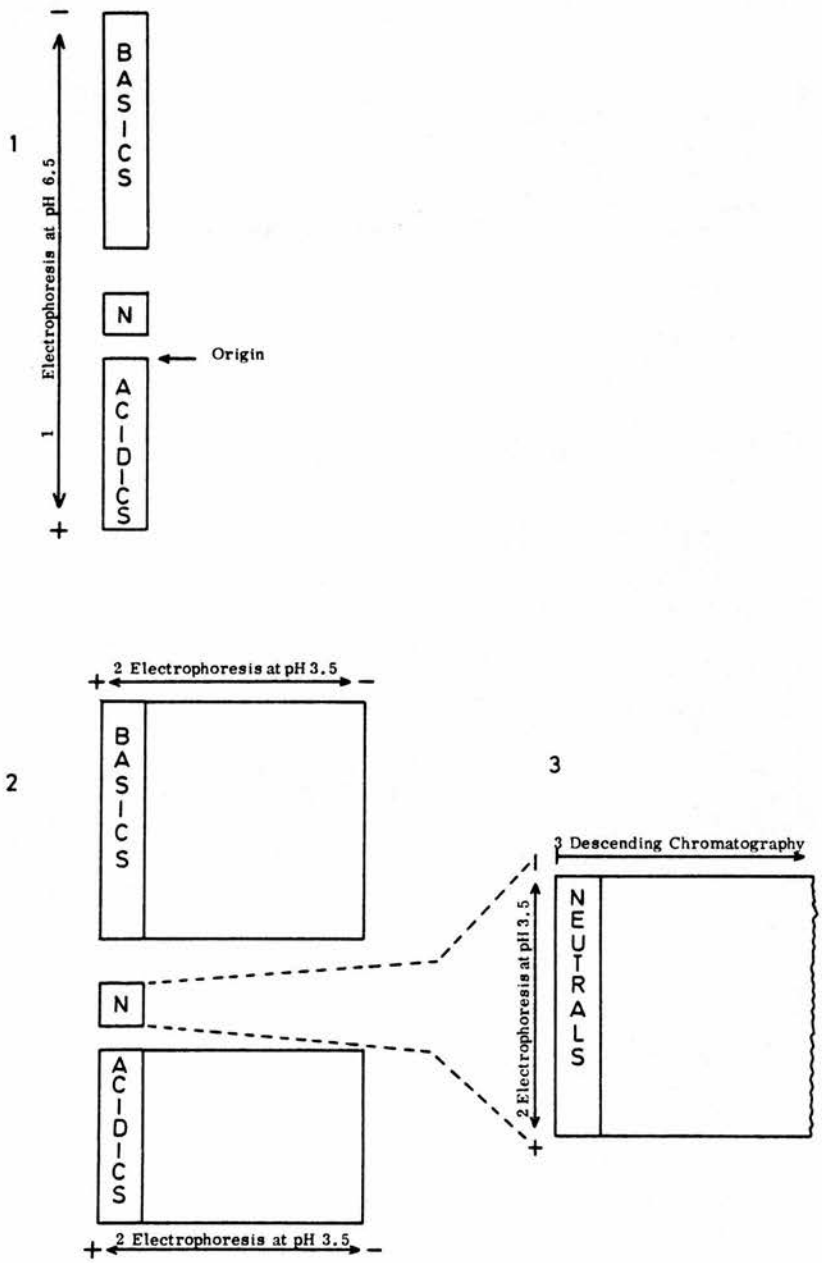

1
1 Electrophoresis at pH 6.5
BASICS
N
Origin
ACIDICS
2
2 Electrophoresis at pH 3.5
BASICS
N
ACIDICS
2 Electrophoresis at pH 3.5
3
3 Descending Chromatography
2 Electrophoresis at pH 3.5
NEUTRALS

Figure 2.8

Figure 2.8 Scheme for constructing the multi-dimensional peptide "maps" of LBa and LBb. For the sake of simplicity the diagram only shows the construction of a multi-dimensional "map" for a single protein. The peptide "maps" of LBa and LBb were constructed in parallel. The tryptic digests of LBa and LBb were separated in the first dimension (1) by electrophoresis at pH 6.5 into the basic, neutral (N) and acidic peptides. These were further separated by (2) electrophoresis at pH 3.5 and (3) descending chromatography using butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) as shown. The diagram is not drawn to scale.

separately subjected to electrophoresis at pH 3.5. Since after electrophoresis at pH 6.5 (the first dimension) the neutral peptides, from LBa and LBb, migrated as single spots, they were sewn onto chromatography paper and separated by electrophoresis at pH 3.5 in the same orientation as the first dimension. This resulted in the neutral peptides being resolved into a single column after the second dimension. The neutral peptides were then further separated by descending chromatography using butan-1-ol/acetic acid/water/ pyridine (15:3:12:10 by vol.) for 16 h in a third dimension. This was carried out at right angles to the direction of migration of the two high-voltage paper electrophoresis runs (Fig. 2.8).

Peptide nomenclature

To facilitate the comparison between the tryptic digests of LBa and LBb each peptide spot, obtained after the two and three dimensional "mappings" was assigned a number. The peptides in the acidic, basic and neutral "maps" were designated A, B and N respectively. The peptides which appeared to be common to both proteins were then numbered first, according to their mobility in the pH 6.5 electrophoretic system (the most acidic ones were numbered first). The unique peptides which were found in only one protein were numbered second. The peptides were then designated a or b according to their protein of origin. Using this nomenclature

the peptides in the acidic peptide "map" of LBa and LBb, which migrated furthest from the origin towards the anode, during electrophoresis at pH 6.5 were designated Ala and Alb in the final two dimensional "map". Likewise, in the basic peptide "map", the peptides from LBa and LBb which, after the first dimension, were recovered nearest the anode (i.e. migrated least) were designated Bla and Blb in the final two dimensional "map". In the peptide "map" which was constructed using descending chromatography in the final dimension (the neutral peptide "map") the peptides were numbered according to their mobility; the peptides which migrated least, and were found nearest the origin were numbered first.

RESULTS

Aims of the experiments

In this thesis the interactions between cholic acid and rat liver cytosol and between lithocholic acid and rat liver cytosol were investigated. Cholic acid-binding was studied by an equilibrium method using gel-exclusion chromatography. Lithocholic acid-binding was studied by non-equilibrium methods using gel-exclusion chromatography and ion-exchange chromatography. Two lithocholic acid-binding proteins from rat liver cytosol have previously been described and both proteins were shown to have glutathione S-transferase activity (Strange et al., 1977b). Cytosolic proteins were studied to determine whether cholic acid and lithocholic acid were bound by all the glutathione S-transferases, only certain glutathione S-transferases or by proteins which had no transferase activity. The subunit compositions of the transferases were investigated to see if a particular monomer could be responsible for bile acid-binding activity. Further, experiments were also performed to characterise and identify the two previously described lithocholic acid-binding proteins which also possessed glutathione S-transferase activity. In this thesis these two proteins were called LBa and LBb.

Cholic acid-binding to liver cytosol

The binding of cholic acid to cytosolic preparations was studied using an equilibrium method (see pp. 44-46). Using this method cystolic proteins were incubated with [^{3}H]cholic acid and applied to a gel-exclusion column which had previously been equilibrated with the same ligand. A constant "baseline" of radioactivity was therefore eluted from the column; the concentration of [^{3}H]cholic acid eluted at the beginning of each run (pre-void volume, 0-70 ml) was equal to that eluted at the end of each run (post-salt volume, 230-300 ml). Protein-bound [^{3}H]cholic acid eluted as a peak of radioactivity which was observed above this "baseline" level. In cases where cholic acid-binding was observed the [^{3}H]cholic acid which was in free solution in the test sample, eluted from the column at the salt volume as a trough of radioactivity, since the concentration of the free ligand in these samples was less than the concentration of the ligand with which the column was equilibrated. In these instances, the areas of the "trough" and "peak", although inverted, should have been equal since the rate at which the ligand was applied to the column was constant throughout each experiment. Under these circumstances, the binding proteins picked up more [^{3}H]cholic acid as they passed down the column, until they were in equilibrium with the free ligand given by the equilibrium solution (eluting buffer).

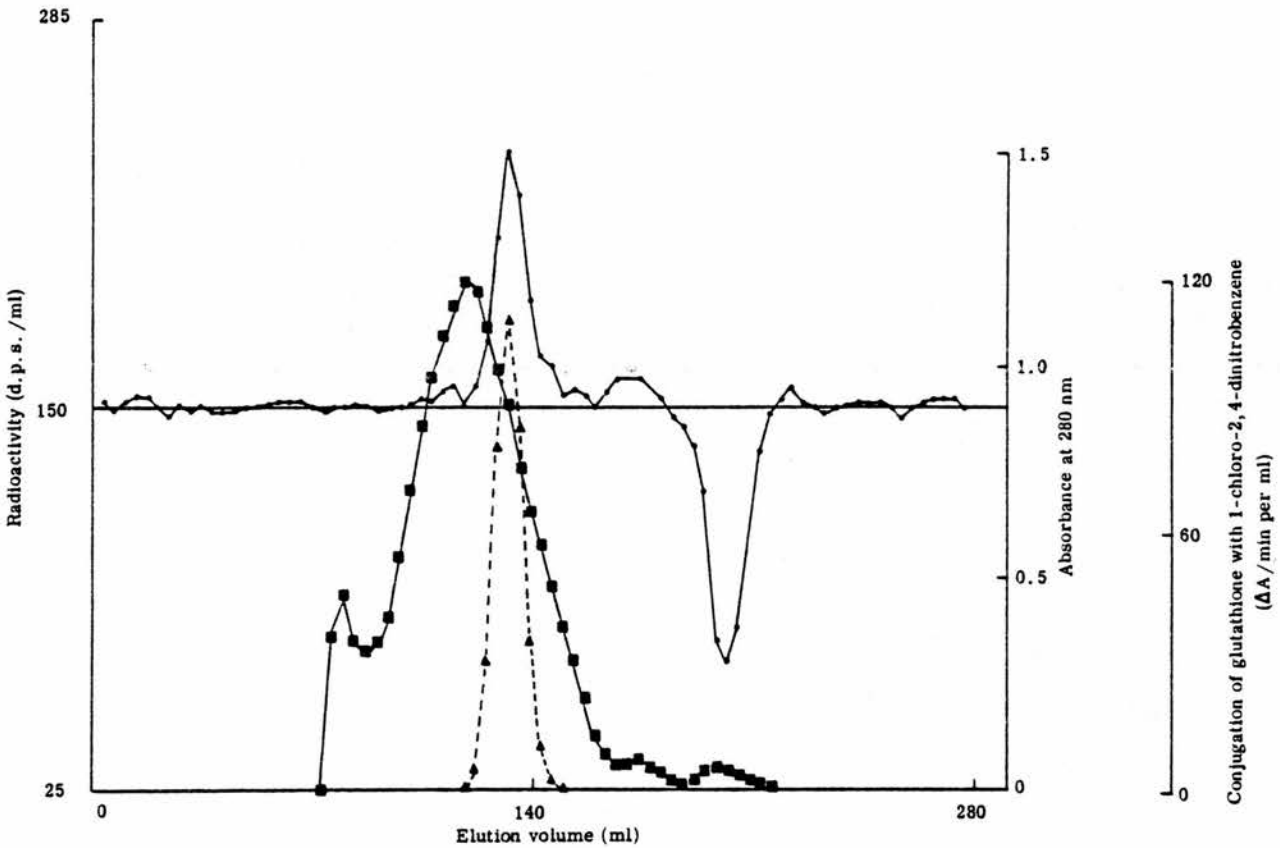


Figure 3.1 Elution pattern of cytosol from Bio-Gel equilibrated with [^{3}H]cholic acid. Rat liver cytosol (approx. 150 mg of protein) was dialysed against buffer C (4°C, 18 h, 2 litres) and eluted from a Bio-Gel column equilibrated with [^{3}H]cholic acid as described. Fractions (3.4 ml) were collected and the A_{280} (■), the radioactivity (●) and glutathione S-transferase activity with 1-chloro-2,4-dinitrobenzene (▲) were measured. The solid horizontal line represents the radioactivity in the eluting buffer.

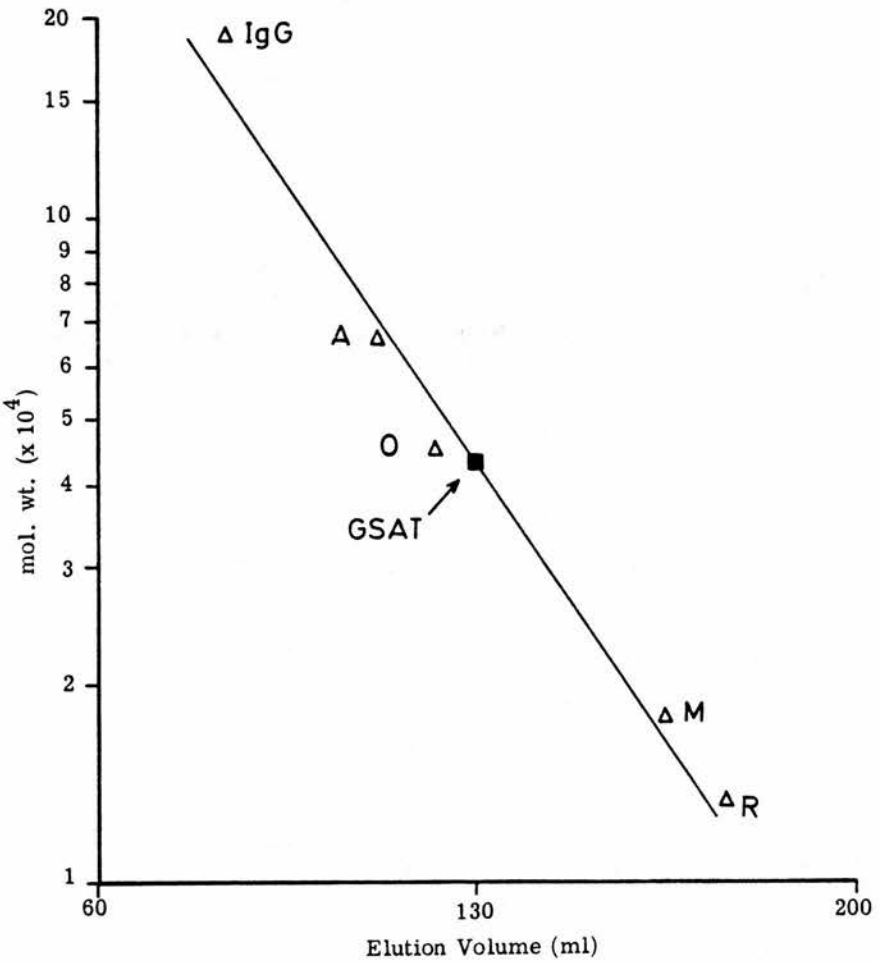


Figure 3.2 Bio-Gel mol. wt. calibration curve. The mol. wt. of the cholic acid-binding component(s) (■), and the glutathione S-transferases (GSAT) in rat liver cytosol were estimated by comparing their elution volume from the Bio-Gel A-0.5 m column (2.5 cm x 38 cm) with the elution volumes of standards (20 mg of protein), immunoglobulin G (IgG), rat albumin (A), ovalbumin (O), myoglobin (M) and ribonuclease A (R).

The elution pattern of cytosol from a Bio-Gel column equilibrated with [^{3}H]cholic acid (Fig. 3.1) demonstrated that bound cholic acid coeluted with glutathione S-transferase activity. The mol. wt. of the binding component(s) was determined by comparing the elution volume of bound [^{3}H]cholic acid with that of various protein standards. Fig. 3.2 shows that both glutathione S-transferase activity and cholic acid-binding activity eluted from Bio-Gel with proteins of mol. wt. about 43 000. However, since several distinct enzymes, of similar mol. wt., have glutathione S-transferase activity the enzyme mixture was further examined for cholic acid-binding activity.

Cholic acid-binding to individual glutathione S-transferase enzymes

A partial separation of the transferases in liver cytosol was achieved by a combination of CM-Sephadex and DEAE-Sephadex chromatography as described on pp. 46-47.

CM-Sephadex resolved rat liver cytosol into 5 peaks of glutathione S-transferase activity (measured by the conjugation of 1-chloro-2, 4-dinitrobenzene and GSH) (Fig. 3.3). These peaks were designated 1-5 by their elution volumes; peak 1, 15-40 ml; peak 2, 43-85 ml; peak 3, 91-115 ml; peak 4, 130-180 ml and peak 5, 197-241 ml. Enzyme activity in peak 4 included a shoulder

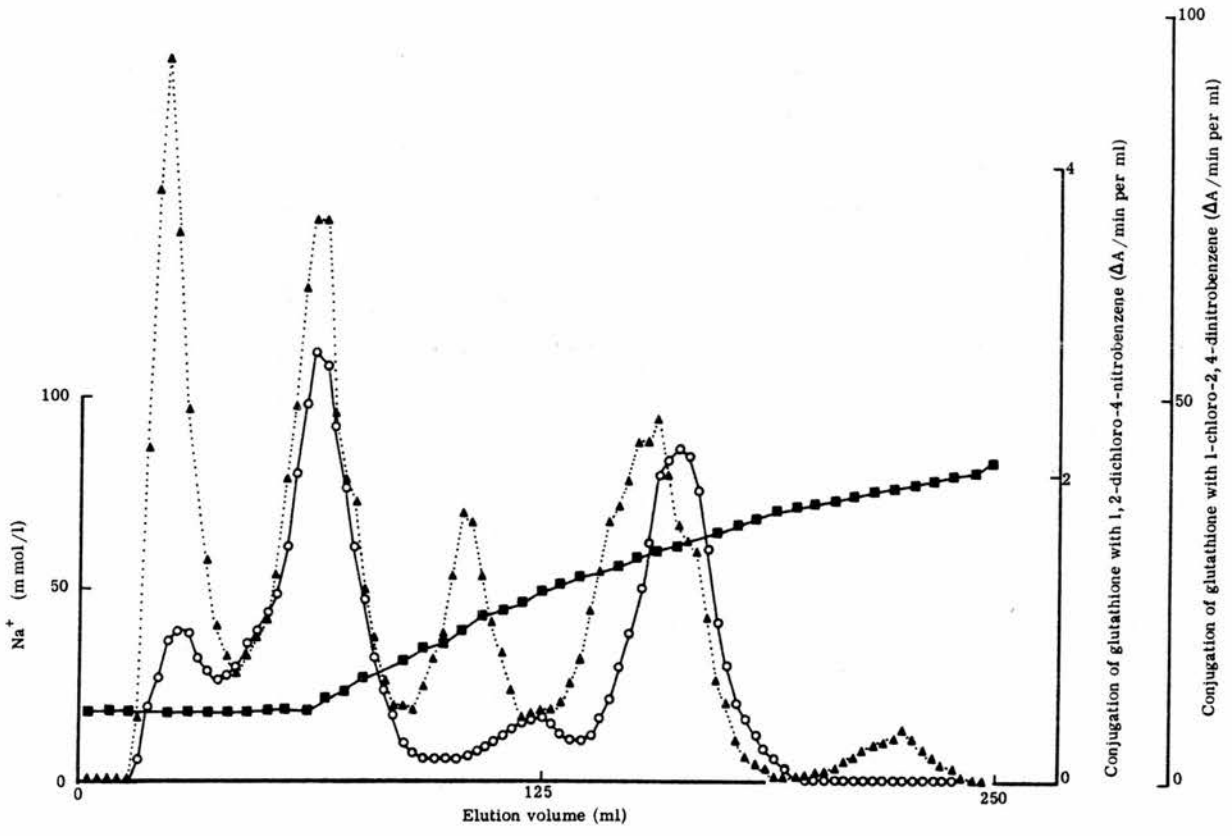


Figure 3.3 Elution pattern of cytosol from normal rats from CM-Sephadex. Rat liver cytosol (approx. 325 mg of protein) from normal rats was dialysed against buffer D and eluted from a column (2.2 cm x 15 cm) of CM-Sephadex C-50. Fractions (2.7 ml) were collected and the Na^+ concentrations (■) determined. Glutathione S-transferase activity was also measured using 1-chloro-2,4-dinitrobenzene (▲) or 1,2-dichloro-4-nitrobenzene (○) as substrate.

indicating the presence of at least two enzymes.

Peaks 1, 2 and part of peak 4 (elution volume 155-180 ml) were able to catalyse the conjugation of GSH with 1,2-dichloro-4-nitrobenzene. A small peak, containing 1,2-dichloro-4-nitrobenzene:GSH conjugating activity, was eluted between peaks 3 and 4 (elution volume 115-130 ml). Enzyme activity in peak 4 was partially resolved by CM-Sephadex chromatography since the initial fractions (elution volume 130-154 ml) were unable to catalyse the conjugation of GSH with 1,2-dichloro-4-nitrobenzene whereas the later fractions (elution volume 155-180 ml), were able to catalyse the conjugation of GSH with both 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene.

The combined elution profile of the 1-chloro-2,4-dinitrobenzene:GSH and 1,2-dichloro-4-nitrobenzene:GSH conjugating activities from CM-Sephadex demonstrated the presence of at least 7 glutathione S-transferases in hepatic cytosol; 6 were able to catalyse the conjugation of 1-chloro-2, 4-dinitrobenzene and GSH.

The glutathione S-transferases which eluted from CM-Sephadex in peak 4 (elution volume 130-180 ml) were further resolved by DEAE-Sephadex chromatography into 2 peaks of activity (Fig. 3.4); these were

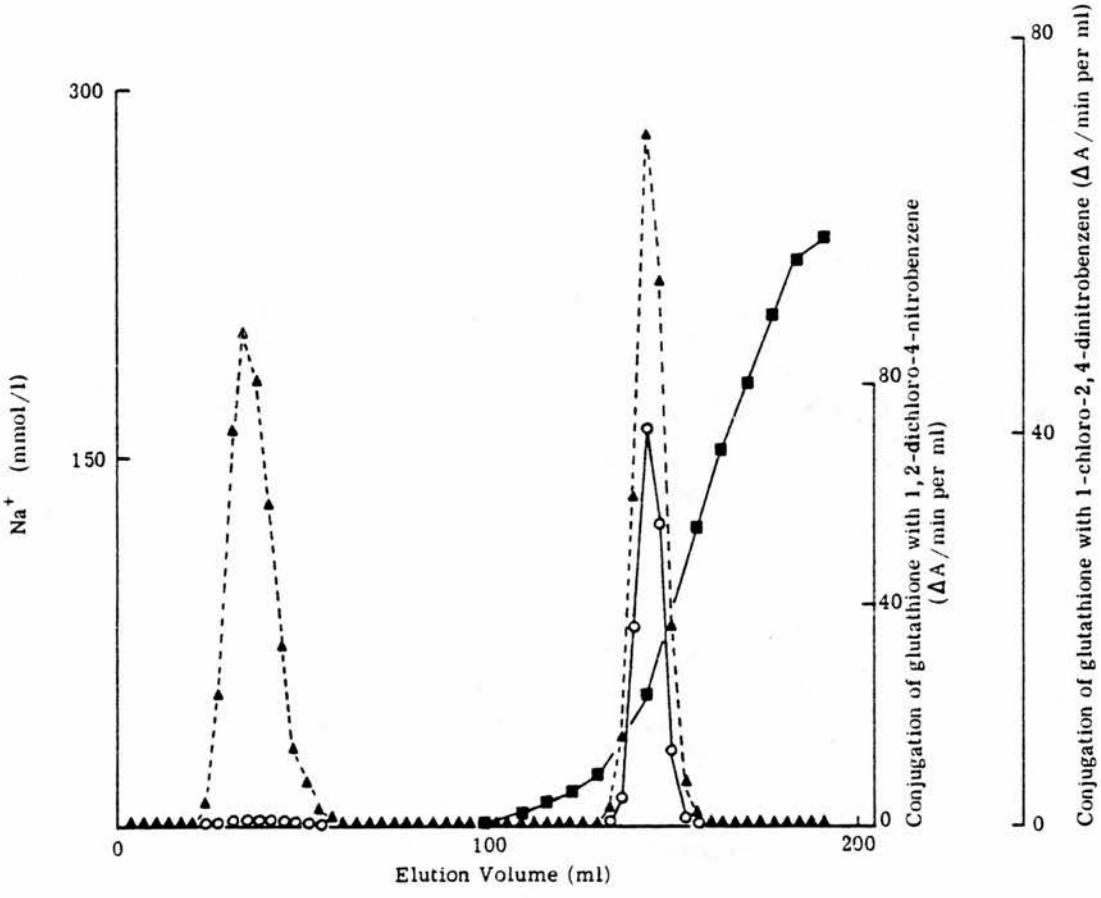


Figure 3.4 DEAE-Sephadex chromatography of peak 4. Cytosol was eluted (20.4 ml/h, 4°C) from a CM-Sephadex column (2.2 cm x 15 cm) and peak 4 (elution volume 130-180 ml) was combined and concentrated. After dialysis against buffer I, peak 4 was eluted (20.4 ml/h, 4°C) from a DEAE-Sephadex column (2.2 cm x 15 cm) Fractions (3.4 ml) were collected and the glutathione S-transferase activity with 1-chloro-2,4-dinitrobenzene (▲) and 1,2-dichloro-4-nitrobenzene (○) and the Na^+ concentration (■) were measured.

designated 4(i) (elution volume 20-53 ml) and 4(ii) (elution volume 127-153 ml). Peak 4(i) was able to catalyse the conjugation of GSH with only 1-chloro-2,4-dinitrobenzene whilst peak 4(ii) was able to catalyse the conjugation of GSH with both 1-chloro-2, 4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene. On the basis of substrate specificity, peak 4(i) probably eluted from CM-Sephadex in the initial fractions of peak 4 (130-154 ml) and peak 4(ii) in the later fractions of peak 4 (155-180 ml). In this thesis the transferases were defined as peaks 1, 2, 3, 4(i), 4(ii) and 5 by their elution order from the ion-exchangers CM-Sephadex and DEAE-Sephadex as shown in Table 3.1.

Peaks 1, 2, 3, 4(i), 4(ii) and 5 were individually combined and analysed for cholic acid-binding activity by an equilibrium chromatographic method as described. Binding by peaks 1, 3, 4(i) and 5 was associated with enzyme activity (Fig. 3.5a & b). Cholic acid-binding by peak 1 was not restricted to enzyme containing fractions, but was also found in fractions with the same elution volume as rat albumin (110 ml). Peak 1 also contained a component of mol. wt. about 17 000 which bound cholic acid. This may be the low mol. wt. lithocholic acid-binding protein previously described by Strange et al. (1977a). No cholic acid-binding was demonstrated in peaks 2 or 4(ii) despite the recovery of substantial enzyme activity.

Table 3.1 Nomenclature used to define the glutathione S-transferase containing peaks. The glutathione S-transferases from rat liver cytosol were initially resolved by CM-Sephadex chromatography into 5 peaks of GSH:1-chloro-2,4-dinitrobenzene conjugating activity (Fig. 3.3). These enzyme-containing peaks were designated 1-5 by their elution order from CM-Sephadex. Peak 4 was further resolved into 2 peaks of GSH:1-chloro-2,4-dinitrobenzene conjugating activity (Fig. 3.4). These enzyme-containing peaks were designated 4(i) and 4(ii) by their elution order from DEAE-Sephadex.

Peak	Elution volume from CM-Sephadex (ml)	Elution volume from DEAE-Sephadex (ml)
1	15-40	N.D.
2	43-85	N.D.
3	91-115	N.D.
4	130-180	
4(i)	130-154	20-53
4(ii)	155-180	127-153
5	197-241	N.D.

(N.D., not determined).

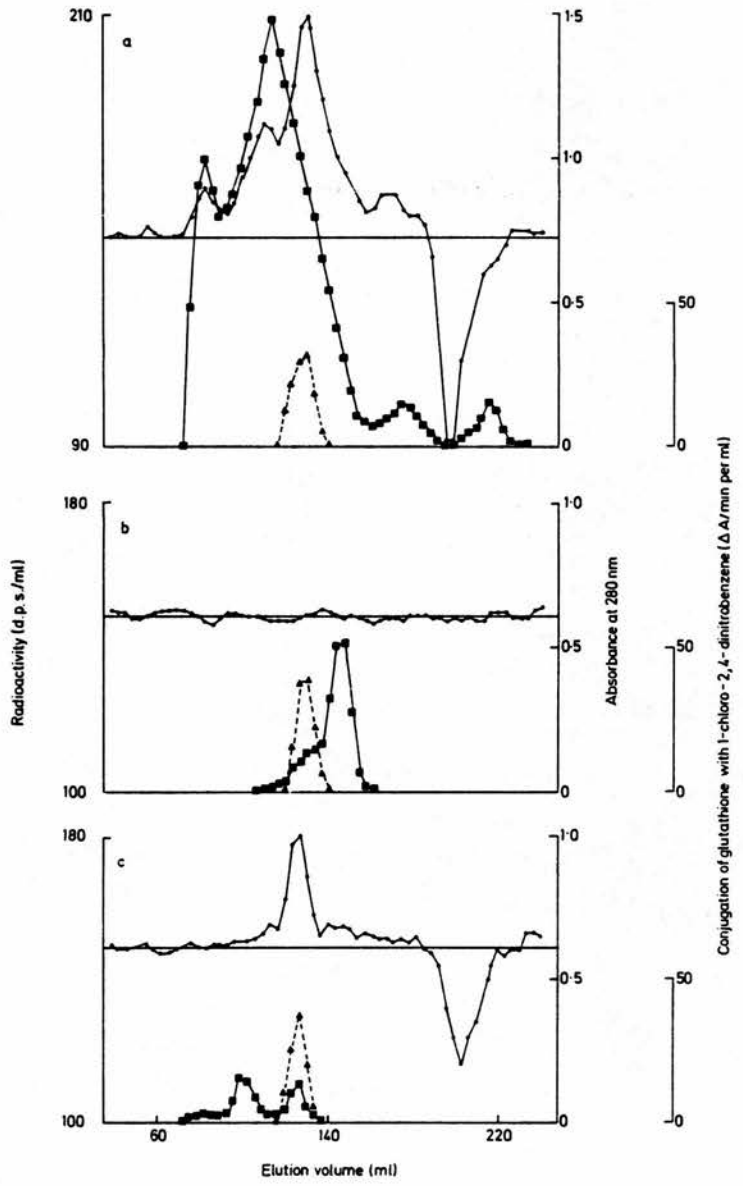

a
b
c
210
90
180
100
180
100
1·5
1·0
0·5
0
50
0
1·0
0·5
0
50
0
1·0
0·5
0
50
0
60
140
220
Elution volume (ml)
Radioactivity (d.p.s./ml)
Absorbance at 280 nm
Conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (ΔA/min per ml)

Figure 3.5a

Figure 3.5a Elution pattern of individual glutathione S-transferase peaks from Bio-Gel equilibrated with [^{3}H]cholic acid. The transferase peaks 1, 2 and 3 obtained after ion-exchange chromatography were concentrated and dialysed against buffer C. They were then eluted (20.5 ml/h, 4^{o}C) from a Bio-Gel A-0.5 m column (2.5 cm x 38 cm) equilibrated with [^{3}H] cholic acid; peak 1, a; peak 2, b and peak 3, c. Fractions (3.4 ml) were collected and the A_{280} (■), radioactivity (●) and glutathione S-transferase activity with 1-chloro-2,4-dinitrobenzene (▲) were measured. The solid horizontal line represents the radioactivity in the eluting buffer.

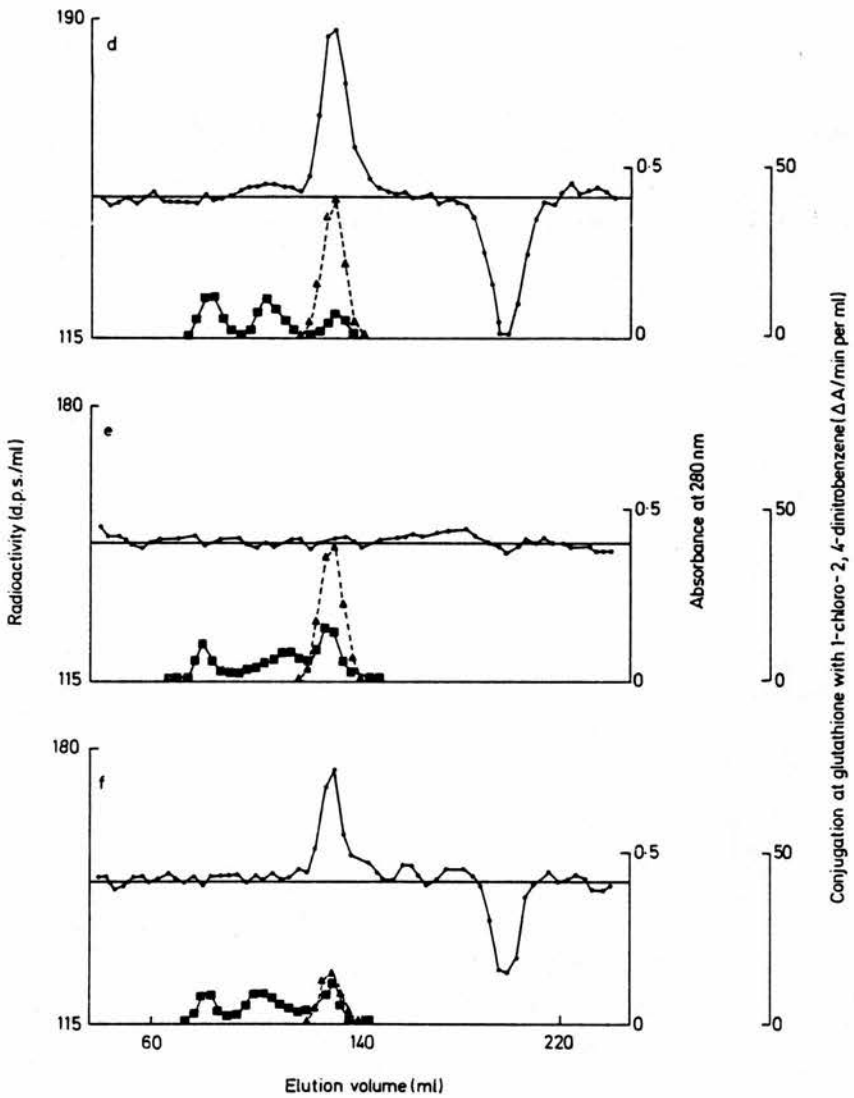

190
d
0·5
50
115
0
0
180
e
0·5
50
115
0
0
180
f
0·5
50
115
0
0
60
140
220
Elution volume (ml)
Radioactivity (d.p.s./ml)
Absorbance at 280 nm
Conjugation at glutathione with 1-chloro-2, 4-dinitrobenzene (ΔA/min per ml)

Figure 3.5b

Figure 3.5b Elution pattern from Bio-Gel of the individual glutathione S-transferase peaks equilibrated with [^{3}H]cholic acid. The transferase peaks 4(i), 4(ii) and 5 obtained after ion-exchange chromatography were concentrated and dialysed against buffer C. They were then eluted (20.5 ml/h, 4^{o}C) from a Bio-Gel A-0.5 m column (2.5 cm x 38 cm) equilibrated with [^{3}H]cholic acid; peak 4(i), d; peak 4(ii), e and peak 5, f. Fractions (3.4 ml) were collected and the A_{280} (■), the radioactivity (●) and glutathione S-transferase activity with 1-chloro-2, 4-dinitrobenzene (▲) were measured. The solid horizontal line represents the radioactivity in the eluting buffer.

Cholic acid-binding, other than that found in the albumin-containing fractions from peak 1, therefore, appears to be associated with proteins which coelute with glutathione S-transferase activity. The only transferase which has previously been reported to bind cholic acid is ligandin (Tipping et al., 1976).

Lithocholic acid-binding to liver cytosol

Gel-exclusion chromatography of a mixture of cytosol and lithocholic acid from a calibrated Bio-Gel column, demonstrated the presence of two types of binding species (Fig. 3.6). The larger binding component (mol. wt. about 43 000) eluted with glutathione S-transferase activi ty whilst the smaller binding component had a mol. wt. of about 17 000. The 43 000 mol. wt. component bound 0.67±0.09 μmol lithocholic acid/g supernatant protein (mean±S.E.M., n = 3) and the 17 000 mol. wt. component 1.67±0.10 μmol lithocholic acid/g supernatant protein (mean±S.E.M., n = 3).

Lithocholic acid-binding to the glutathione S-transferases

To identify the lithocholic acid-binding glutathione S-transferases, cytosol (12 ml; approx. 325 mg protein), which had been dialysed against buffer D, was incubated (60 min, 4°C) with [^{14}C]lithocholic acid

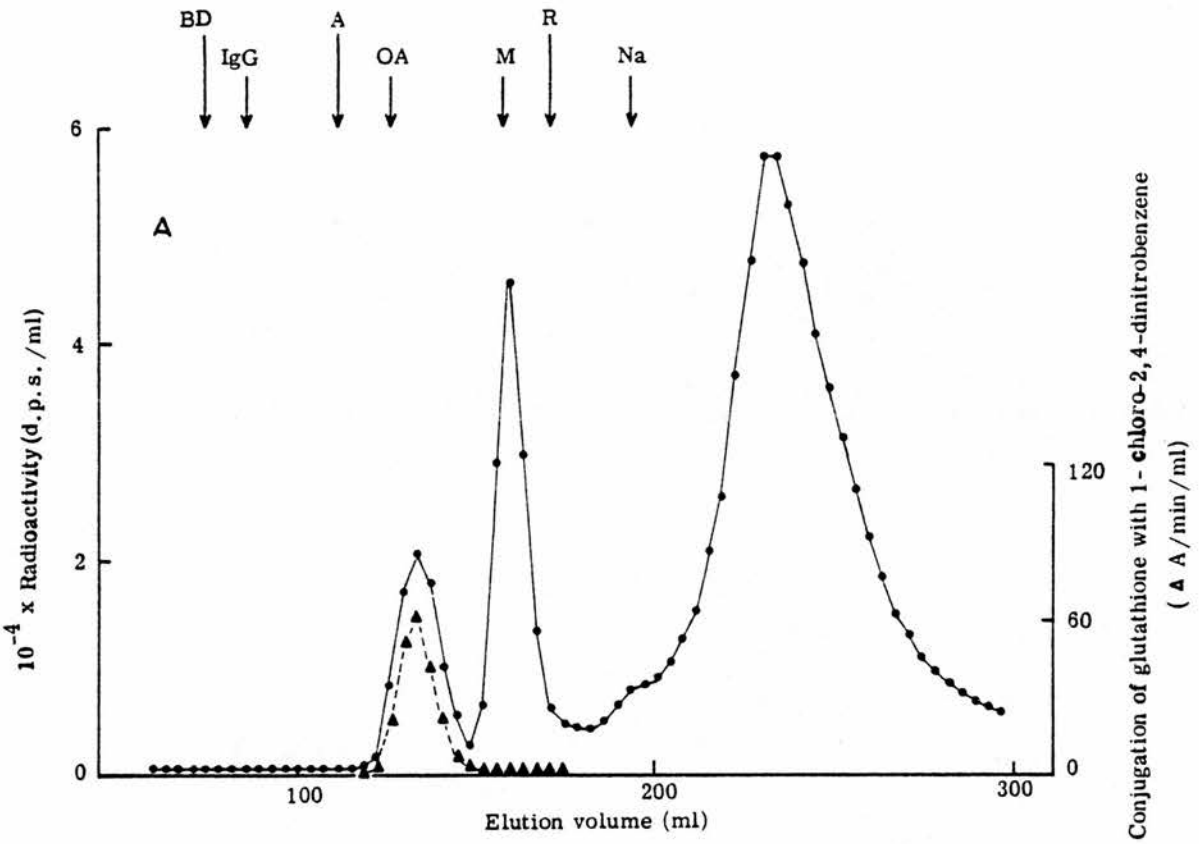


Figure 3.6 Elution pattern of cytosol and [^{14}C]lithocholic acid from Bio-Gel. Rat liver cytosol (approx. 40 mg of protein) was incubated (60 min, 4^{o}C) with [^{14}C]lithocholic acid (0.3μCi, approx. 0.5 μmol) and eluted from a Bio-Gel A-0.5 m column (2.5 cm x 38 cm). Fractions (3.7 ml) were collected and the radioactivity (●) and glutathione S-transferase activity with 1-chloro-2, 4-dinitrobenzene (▲) were measured. The elution volumes of Dextran Blue (BD), immunoglobulin G (IgG), rat albumin (A), ovalbumin (OA), myoglobin (M), ribonuclease (R) and Na^{+} (Na) are shown.

(luCi; 16 nmol) and the mixture eluted from a CM-Sephadex column (2.2 cm x 15 cm). Preincubation of cytosol with [^{14}C]lithocholic acid did not alter the transferase elution pattern from CM-Sephadex. The radioactivity eluted with enzyme activity in peaks 1 and 3 and also with the initial fractions of peak 4 (elution volume 130-154 ml, peak 4(i)) (Fig. 3.7).

Elution of [^{14}C]lithocholic acid alone from the CM-Sephadex column resulted in elution of the radioactivity between 20-40 ml suggesting that at least some of the lithocholic acid associated with peak 1 may be in free solution. The presence of a 43 000 mol. wt. binding component in these fractions was shown as follows; cytosol was eluted from the CM-Sephadex column and the fractions from peak 1 were combined and dialysed against two changes, each of 2 litres, of buffer C (16 h, 4^{o}C). A portion of this solution (40 mg of protein) was incubated (60 min, 4^{o}C) with [^{14}C]lithocholic acid (luCi; 16 nmol) and eluted from the Bio-Gel column. [^{14}C]Lithocholic acid eluted with a component of 43 000 mol. wt. (binding capacity 38 nmol/g of protein) and also with a component of 17 000 mol. wt. (binding capacity 120 nmol/g of protein). The higher mol. wt. binding component eluted with glutathione S-transferase activity. Although a relatively smaller amount of lithocholic acid was associated with the 17 000 mol. wt. binding component obtained from

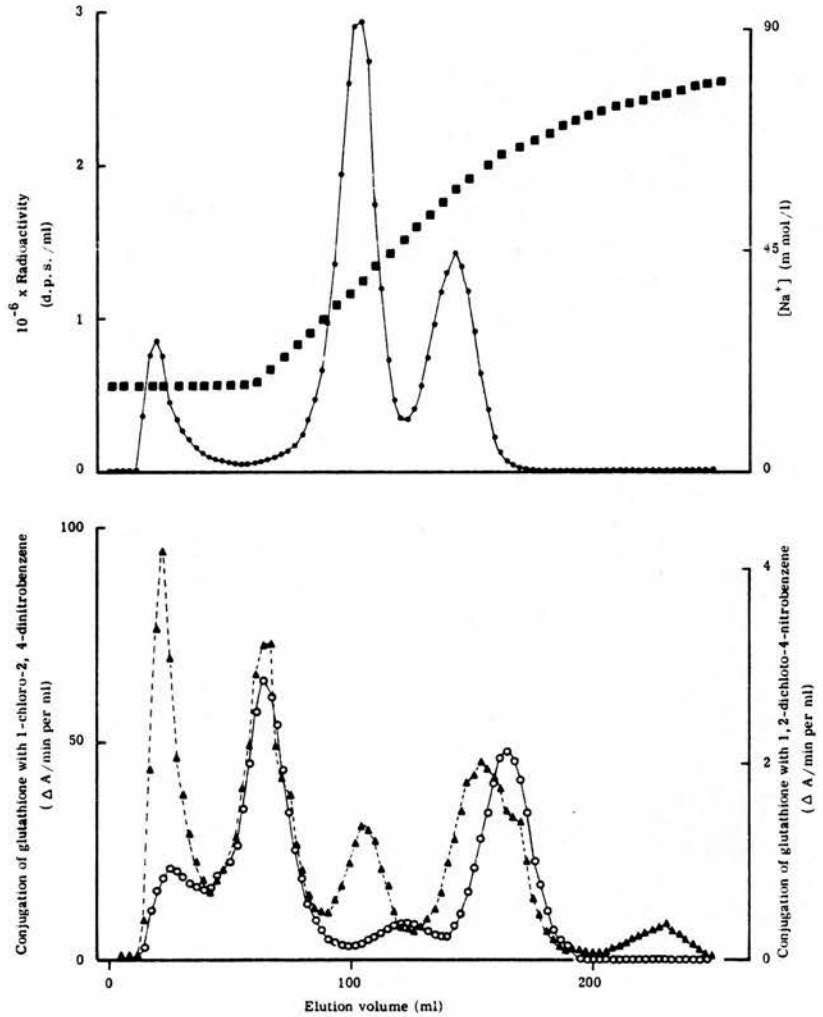

3
2
1
0
90
45
0
10^{-6} x Radioactivity (d.p.s./ml)
$[Na^+]$ (m mol/l)
100
50
0
4
2
0
Conjugation of glutathione with 1-chloro-2, 4-dinitrobenzene (Δ A/min per ml)
Conjugation of glutathione with 1, 2-dichloto-4-nitrobenzene (Δ A/min per ml)
0
100
200
Elution volume (ml)

Figure 3.7

Figure 3.7 Elution pattern of cytosol and [^{14}C]lithocholic acid from CM-Sephadex. Rat liver cytosol (approx. 325 mg of protein) was dialysed against buffer D, incubated with [^{14}C]lithocholic acid and eluted from CM-Sephadex as described. Fractions (2.7 ml) were collected and the radioactivity (●) and Na^+ concentrations (■) were determined. Glutathione S-transferase activity was also measured using 1-chloro-2, 4-dinitrobenzene (▲) or 1, 2-dichloro-4-nitrobenzene (○) as substrates.

the transferase peak 1 which was obtained after CM-Sephadex chromatography (elution volume from CM-Sephadex 15-40 ml) than was associated with the 17 000 mol. wt. binding component which was obtained from whole cytosol this difference may only reflect loss of the component during dialysis (membrane cut off 12 000 - 14 000). When this experiment was repeated using samples from the peak 3 transferase and the peak 4 (i) transferase (Table 3.1) the 17 000 mol. wt. component was not present; radioactivity was associated only with glutathione S-transferase activity. Peaks 3 and 4(i), which eluted from the CM-Sephadex column on the NaCl gradient at Na^+ concentrations of 40 mM and 57 mM, contain the two previously described lithocholic acid-binding proteins (Strange et al., 1977b).

Subunit composition of the glutathione S-transferases

The glutathione S-transferases were partially purified by a combination of gel-exclusion chromatography and ammonium sulphate fractionation before being separated by CM-Sephadex and DEAE-Sephadex chromatography. The partial purification is described on pp. 55-56. It involved eluting cytosol from a Bio-Gel column and combining the fractions which contained maximum transferase activity. Ammonium sulphate was added to the enzyme containing eluate and the fraction which precipitated between 55-85% saturation was combined. This fraction was eluted from CM-Sephadex and the transferase peaks 1-5 were

Table 3.2 Elution characteristics, subunit compositions and cholic acid-binding by glutathione S-transferases from rat liver cytosol. The glutathione S-transferases in rat liver cytosol were separated by a combination of CM-Sephadex and DEAE-Sephadex chromatography. Their monomer composition was determined by SDS/polyacrylamide gel electrophoresis and their ability to bind cholic acid by an equilibrium chromatographic method.

Peak	Elution volume (ml) from CM-Sephadex	Elution volume (ml) from DEAE-Sephadex	Percentage subunit composition			Cholic acid binding
			Ya	Yb	Yc	
1	15-40		5	25	70	Yes
2	43-85		5	95	0	No
3	91-115		90	5	5	Yes
4	130-180					
4(i)	130-154	20-53	50	0	50	Yes
4(ii)	155-180	127-153	0	100	0	No
5	197-241		0	0	100	Yes

individually pooled. The subunit compositions of peaks 1, 2, 3 and 5 were determined after CM-Sephadex chromatography. Peak 4 was further resolved into peaks 4(i) and 4(ii) by DEAE-Sephadex chromatography before analysis. Portions (10-20 µg of protein) of each transferase peak (1-5) were analysed by discontinuous SDS/polyacrylamide gel electrophoresis. The gels were scanned at 580 nm, as described, and the percentage monomer composition of each peak was calculated (Table 3.2). Peak 1 probably comprises a mixture of transferases since it contains mainly unequal amounts of the Yb and Yc monomers. Peak 2 appears to comprise a YbYb dimer. Peak 3 comprises a YaYa dimer. Peak 4(i) appears to comprise a YaYc dimer and peak 4(ii) a YbYb dimer. Peak 5 probably comprises a YcYc dimer. Although peaks 2 and 4(ii) eluted differently from CM-Sephadex both appeared to comprise a YbYb dimer.

Effect of phenobarbitone on the glutathione S-transferases

Habig et al. (1974b, 1976a) have prepared 6 of the glutathione S-transferases and termed them E and D (eluted together) and C, B, A and AA by their order of elution from CM-cellulose. Transferases B, A and AA eluted with a NaCl gradient. On the basis of elution volume, substrate specificities and cholic acid-binding activity, either of the two lithocholic acid-binding proteins (LBa and LBb) eluted

in peaks 3 and 4(i) could be ligandin.

Phenobarbitone administration has been reported to increase the concentration of hepatic ligandin (Arias et al., 1976) and the effect of this agent on the elution profile of the glutathione S-transferases from CM-Sephadex was studied (Fig. 3.8). A comparison of the elution profiles obtained from normal rats (Fig. 3.3) and from phenobarbitone-treated rats (see p.43) (Fig. 3.8) shows that the elution volumes of the glutathione S-transferase peaks were not changed by phenobarbitone treatment. Transferase activity in peaks 1 and 2, measured using either 1-chloro-2,4-dinitrobenzene or 1,2-dichloro-4-nitrobenzene was unaffected by phenobarbitone treatment. Transferase activity in peak 3 was increased approx. 3.5-fold when measured using 1-chloro-2,4-dinitrobenzene as the substrate and transferase activity in peak 4 towards both substrates was increased approx. 2-fold. Transferase activity in peak 5, however, measured with 1-chloro-2,4-dinitrobenzene and, GSH was similar in both groups of animals. The enzyme activities in both the first and second parts of peak 4 were increased by phenobarbitone treatment.

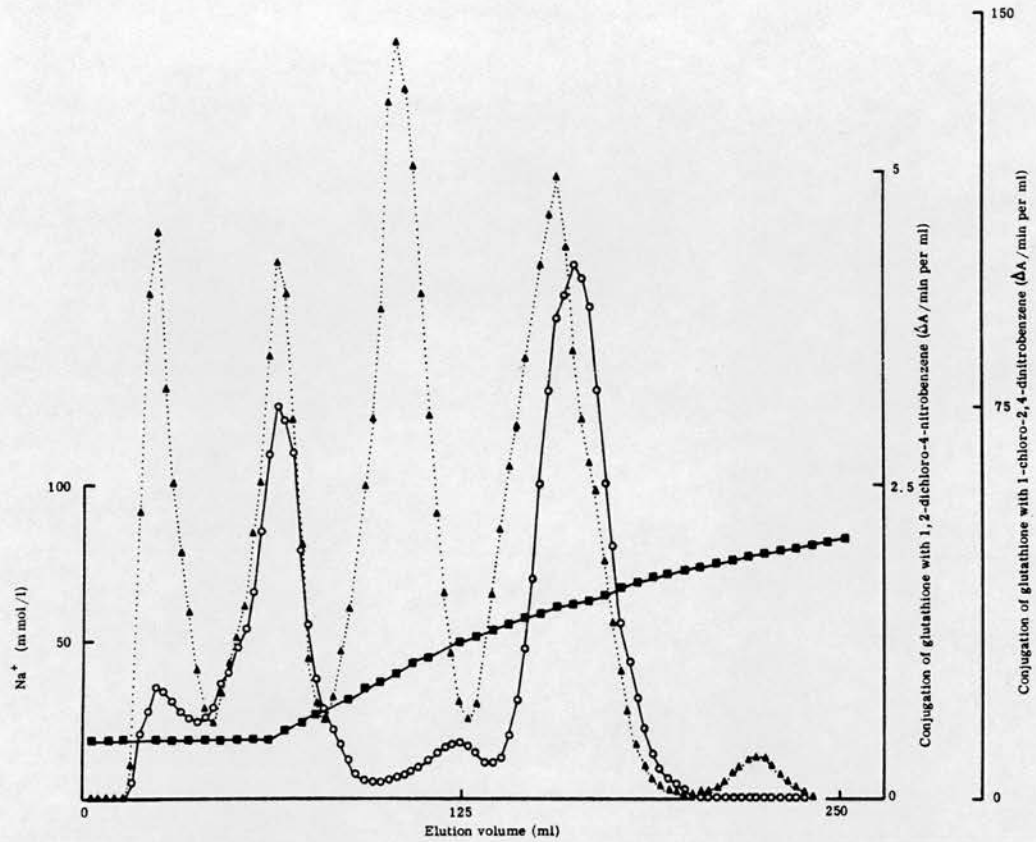


Figure 3.8 Elution pattern from CM-Sephadex of cytosol from phenobarbitone-treated rats. Rat liver cytosol (approx. 325 mg of protein) from phenobarbitone-treated rats was dialysed against buffer D and eluted from a column (2.2 cm x 15 cm) of CM-Sephadex C-50. Fractions (2.7 ml) were collected and the Na^+ concentrations (■) were determined. Glutathione S-transferase activity was also measured using 1-chloro-2,4-dinitrobenzene (▲) or 1,2-dichloro-4-nitrobenzene (○) as substrates.

Effects of phenobarbitone on the subunit composition of the glutathione S-transferases

The subunit composition data in Table 3.2 suggested that transferase peaks 3, 4(i) and 4(ii) comprise YaYa, YaYc and YbYb respectively. The effect of phenobarbitone on the concentration of these bands was studied.

Equal portions of cytosol (approx. 325 mg of protein) from control and phenobarbitone-treated rats were chromatographed on columns of CM-Sephadex. Electrophoresis of portions (15 µl approx. 10 µg of protein) of fractions of peak 3 (91, 96, 99, 103, 107, 115 and 118 ml) obtained from control animals demonstrated that the concentration of the Ya band across the peak increased with enzyme activity; 70% of the protein migrated with the Ya band in the fraction which contained most enzyme activity (Fig. 3.9). This provides further evidence that a YaYa dimer is responsible for the enzyme activity in peak 3 (Fig. 3.3) and is in agreement with the results in Table 3.2. Phenobarbitone-treatment resulted in a 3-fold increase in the amount of the Ya monomer. Only small amounts of the Yb and Yc monomers were found in control animals, the amounts of these monomers were not changed by phenobarbitone treatment.

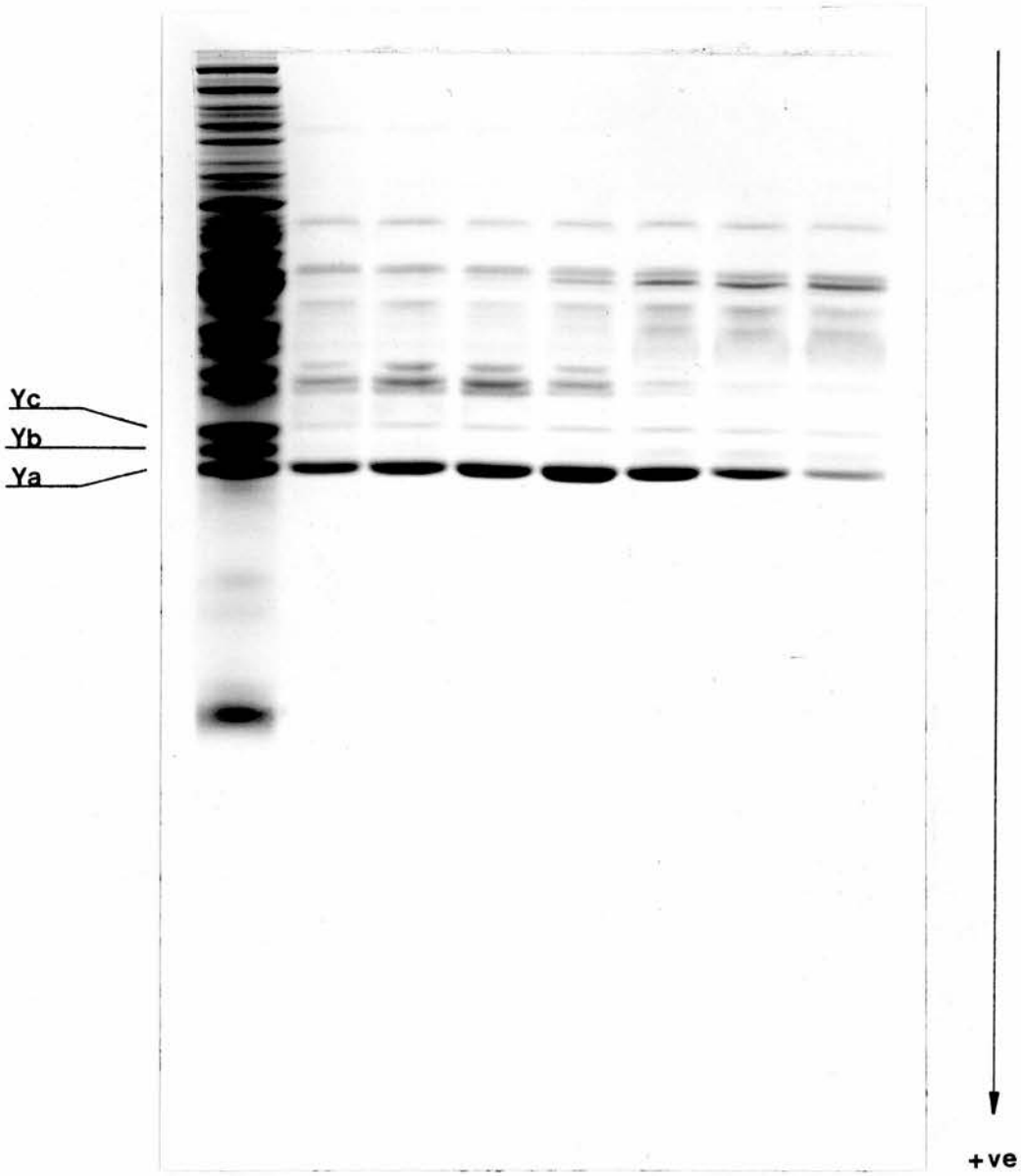


Figure 3.9

Figure 3.9 Examination of the subunit composition of peak 3 using SDS/polyacrylamide gel electrophoresis. Peak 3 was prepared by CM-Sephadex chromatography of hepatic cytosol. The fractions which eluted at 91, 96, 99, 103, 107, 115 and 118 ml across peak 3 were collected and 25 µl (approx. 15 µg of protein) was taken for electrophoresis. The samples were applied from left to right: whole hepatic cytosol and fractions eluted at 91, 96, 99, 103, 107, 115 and 118 ml. The origin is at the top of the gel and samples were run down towards the anode. The Ya (mol. wt. 22 000), Yb (mol. wt. 23 500) and Yc (mol. wt. 25 000) bands are indicated.

For normal rats, electrophoresis of portions (15 µl, approx. 15 µg of protein) of fractions from peak 4 (136, 141, 147, 157, 165 and 173 ml) (Fig. 3.10) demonstrated a change in the Ya, Yb, Yc subunit composition (Fig. 3.11). The initial fractions contained primarily Ya and Yc, in equal concentrations, whilst the later fractions contained predominantly Yb. The Yb content changed from approx. 16% (elution volume 136 ml) to 55% (elution volume 173 ml) of the total Ya Yb Yc protein; this suggested that a Ya Yc dimer is responsible for the enzyme and lithocholic acid-binding activities in the initial fractions of peak 4 (peak 4(i)), and a YbYb dimer for the enzyme activity in the later fractions (peak 4(ii)) and agrees with the data in Table 3.2. After phenobarbitone treatment the concentration of the Ya, Yb and Yc bands were each increased approx. 2-fold in all the fractions across peak 4 (Fig. 3.11). The concentration of other bands was unchanged. Phenobarbitone treatment therefore appears to induce both of the two partially resolved glutathione S-transferases in peak 4.

Peak 3 and the initial fractions of peak 4, which contain the two lithocholic acid-binding proteins LBa and LBb, are both induced by phenobarbitone treatment and could therefore be ligandin. Ligandin has been described as a YaYc dimer (Daniel et al., 1977; Bhargava et al., 1978) which is consistent with its being eluted in

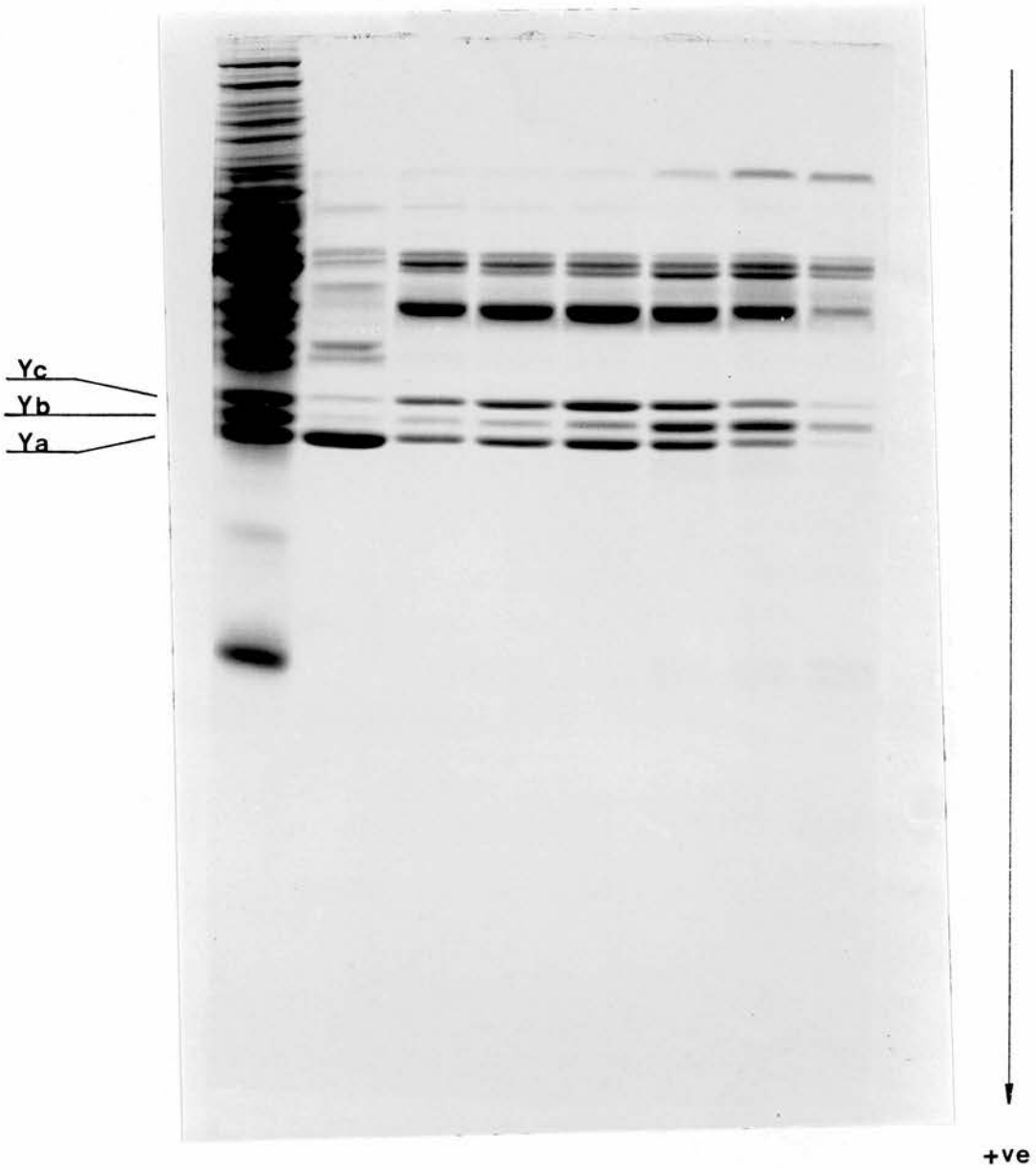


Figure 3.10

Figure 3.10 SDS/polyacrylamide gel electrophoresis of peaks 3 and 4. Peaks 3 and 4 were prepared by CM-Sephadex chromatography; the three fractions which eluted in peak 3 with maximum enzyme activity were combined and fractions which eluted at 136, 141, 147, 157, 165 173 ml across peak 4 were collected and 25 µl (approx. 20 µg of protein) was removed from each fraction and prepared for electrophoresis. The samples were applied from left to right: whole hepatic cytosol, the 3 combined fractions (eluted 99-107 ml) from peak 3, and fractions eluted at 136, 141, 147, 157, 165 and 173 ml. The origin is at the top of the gel and samples were run down towards the anode. The Ya (mol. wt. 22 000), Yb (mol. wt. 23 500) and Yc (mol. wt. 25 000) bands are shown.

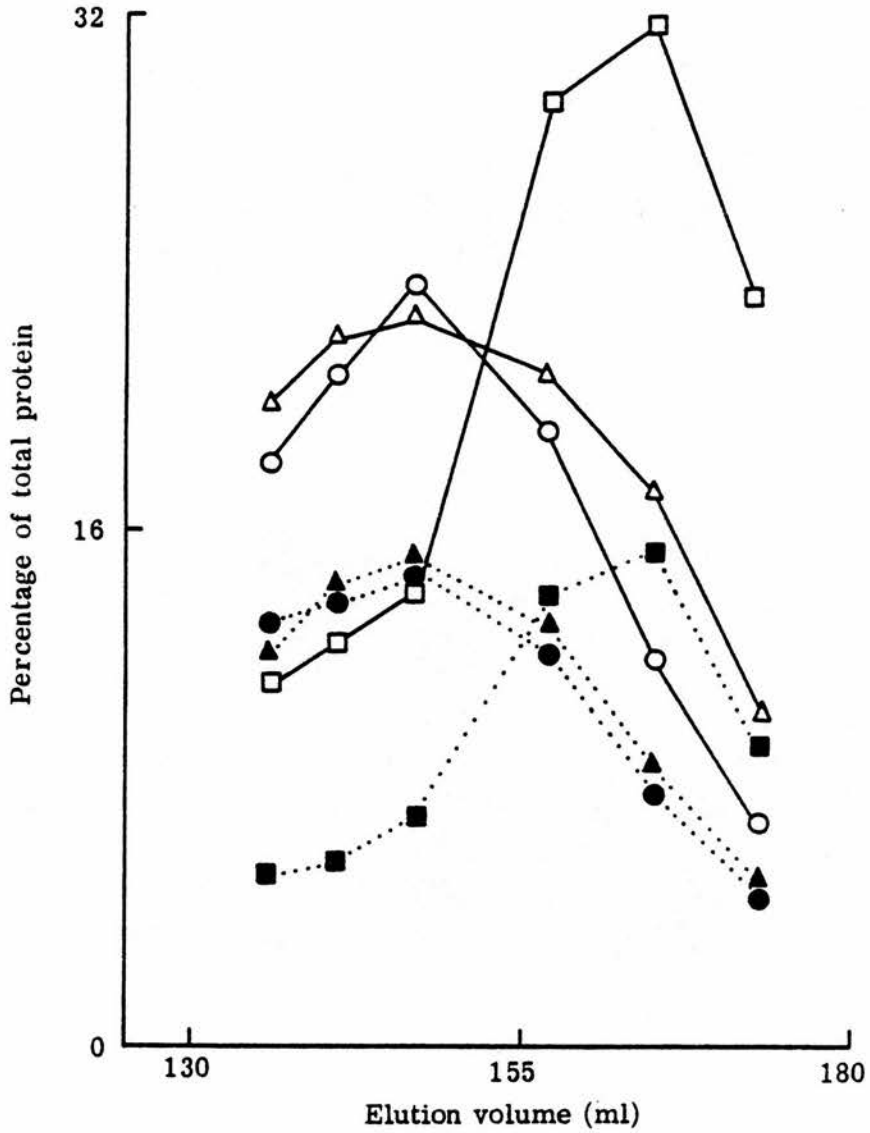

32
16
0
Percentage of total protein
130
155
180
Elution volume (ml)

Figure 3.11

Figure 3.11 Phenobarbitone induction of peak 4 proteins. Peak 4 was prepared by CM-Sephadex chromatography of whole cytosol. Electrophoresis of samples (25 µl) from fractions which eluted at 136, 141, 147, 157, 165 and 173 from both phenobarbitone-treated and untreated rats was performed as described. The relative concentration in the peak 4 fractions of Ya (▲---▲), Yb (■---■) and Yc (●---●) in untreated rats and Ya (Δ——Δ), Yb (□——□) and Yc (○——○) in phenobarbitone-treated rats was determined by densitometry and expressed as a percentage of the total protein in each fraction.

peak 4. However, Bass et al. (1977a) have shown that their preparation of ligandin contained predominantly YaYa protein which is consistent with it being eluted in peak 3 while Carne et al. (1979) prepared ligandin which could be resolved into a YaYa protein and a YaYc protein. The identity of the two bile acid-binding proteins LBa and LBb was therefore further investigated by preparing independently ligandin (see Table 2.3) and glutathione S-transferase B (see Table 2.2) and studying the elution behaviour of the two purified proteins from CM-Sephadex.

Elution of ligandin and glutathione S-transferase B from CM-Sephadex

Chromatography of ligandin from CM-Sephadex resulted in elution of a peak (85-120 ml) of protein and enzyme activity (measured with 1-chloro-2, 4-dinitrobenzene and GSH) at a Na^+ concentration of 40 mM (Fig. 3.12). Glutathione S-transferase B was eluted (130-155 ml) from CM-Sephadex as a single peak of protein and enzyme activity (measured with 1-chloro-2, 4-dinitrobenzene and GSH) at a Na^+ concentration of 57 mM (Fig. 3.12). The glutathione S-transferase activity of ligandin and glutathione S-transferase B using 1, 2-dichloro-4-nitrobenzene was less than 0.3% of that with 1-chloro-2,4-dinitrobenzene. When either ligandin or glutathione S-transferase B was incubated ($4^{\circ}C$, 60 min) with [^{14}C]lithocholic acid (1μCi; 16 nmol)

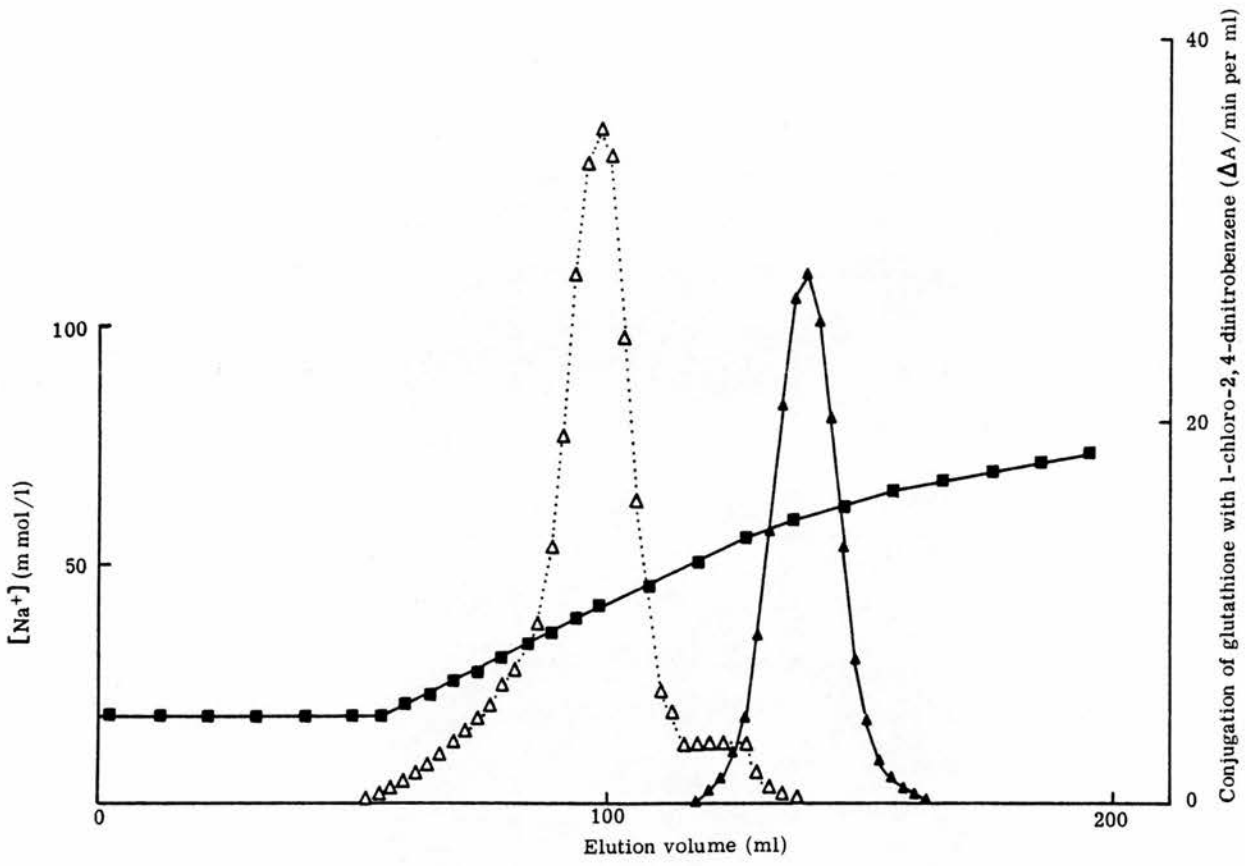


Figure 3.12 CM-Sephadex chromatography of glutathione S-transferase B and ligandin. Glutathione S-transferase B and ligandin were prepared and 5 ml (approx. 1 mg) of each preparation was eluted (16.0 ml/h) from CM-Sephadex C50. Fractions (2.7 ml) were collected and the Na^+ concentration (■) determined. The results from the separate chromatography of the two protein preparations are combined. GSH: 1-chloro-2, 4-dinitrobenzene conjugating activities in the eluate from chromatographed ligandin (Δ) and glutathione S-transferase B (▲) are shown.

before elution from CM-Sephadex , single peaks of radioactivity eluted with the protein peaks indicating that both ligandin and glutathione S-transferase B bind lithocholic acid.

Subunit composition of LBa and LBb

The two lithocholic acid-binding proteins, LBa (ligandin) and LBb (glutathione S-transferase B), were prepared as described (Table 2.4). SDS/polyacrylamide gel electrophoresis of portions (15 μg of protein) of the two binding proteins showed that the LBa protein migrated as a single band, which was identified as the Ya monomer and the LBb protein migrated as two bands, identified as the Ya and Yc monomers (Fig. 3.13). In both cases there was no evidence that any protein in either of the samples was excluded from the resolving gel. Densitometry of the gel pattern obtained from LBa indicated that 98-99% of the sample applied migrated with the Ya monomer. Densitometry of the gel pattern obtained from LBb indicated that the Ya and Yc monomers contained equal amounts of protein. No other protein bands were detected even when the amount of protein applied was increased to 25 μg.

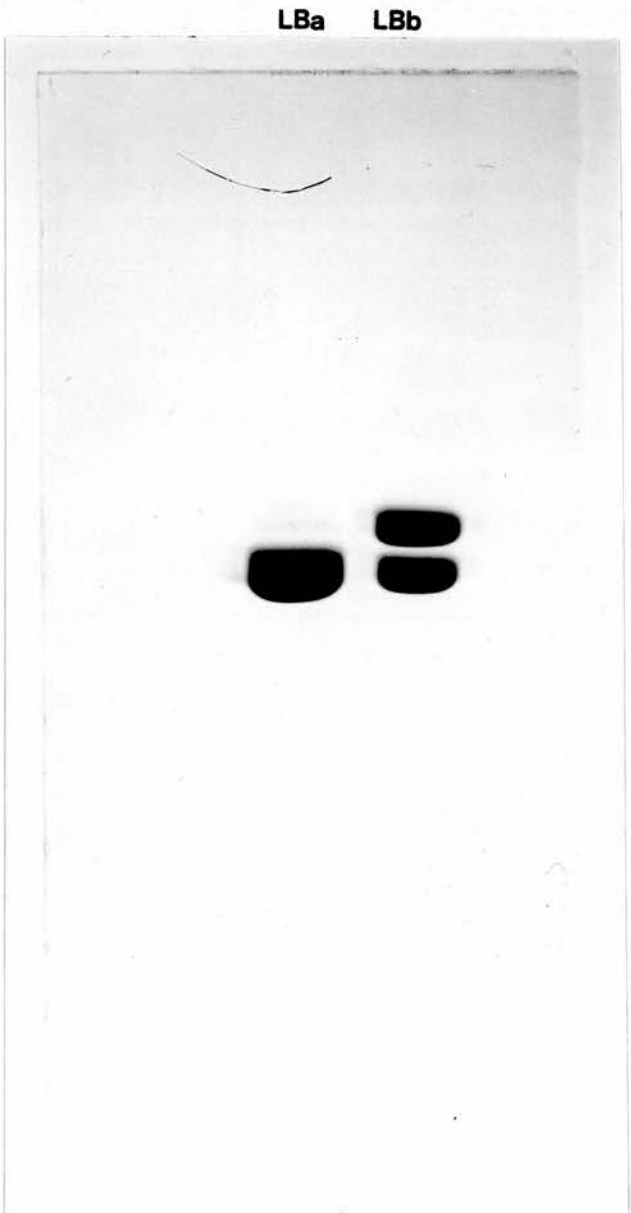


Figure 3.13

Figure 3.13 SDS/polyacrylamide gel electrophoresis of the two lithocholic acid-binding proteins (LBa and LBb). LBa and LBb were purified and prepared for SDS/polyacrylamide gel electrophoresis. Portions of each protein (15 μg) were applied to the gels (11.5% polyacrylamide resolving gel) and were run from the cathode (top) to the anode (bottom). Purified protein which eluted from CM-Sephadex at a Na^+ concentration of 40 mM (LBa) was applied to the left hand side of the gel and protein which eluted at a Na^+ concentration of 57 mM (LBb) was applied to the right hand side of the gel.

Isoelectric focussing of LBa and LBb

Thin-layer polyacrylamide gel isoelectric focussing of portions (25 μg of protein) of LBa and LBb was performed as described (pp. 56-57). This showed that each focussed as single bands in the pH 7-10 range. There was no evidence, in either preparation, that protein was excluded from the polyacrylamide gel at the point of application. Densitometry of the gel pattern obtained from either binding protein failed to demonstrate the presence of other protein bands. The pI for LBa was pH 8.7 and that for LBb pH 8.6 (Fig. 3.14).

Purity of LBa and LBb

The combination of SDS/polyacrylamide gel electrophoresis and polyacrylamide gel isoelectric focussing indicated that the preparations of LBa and LBb were highly purified. Although LBa was not completely homogeneous at least 98% of the stainable protein migrated as a single band when examined using the two electrophoretic systems. SDS/polyacrylamide gel electrophoresis indicated that LBb comprises two non-identical monomers; at least 99% of the protein migrated with the two monomeric bands. Isoelectric focussing also showed that at least 99% of the stainable protein in the

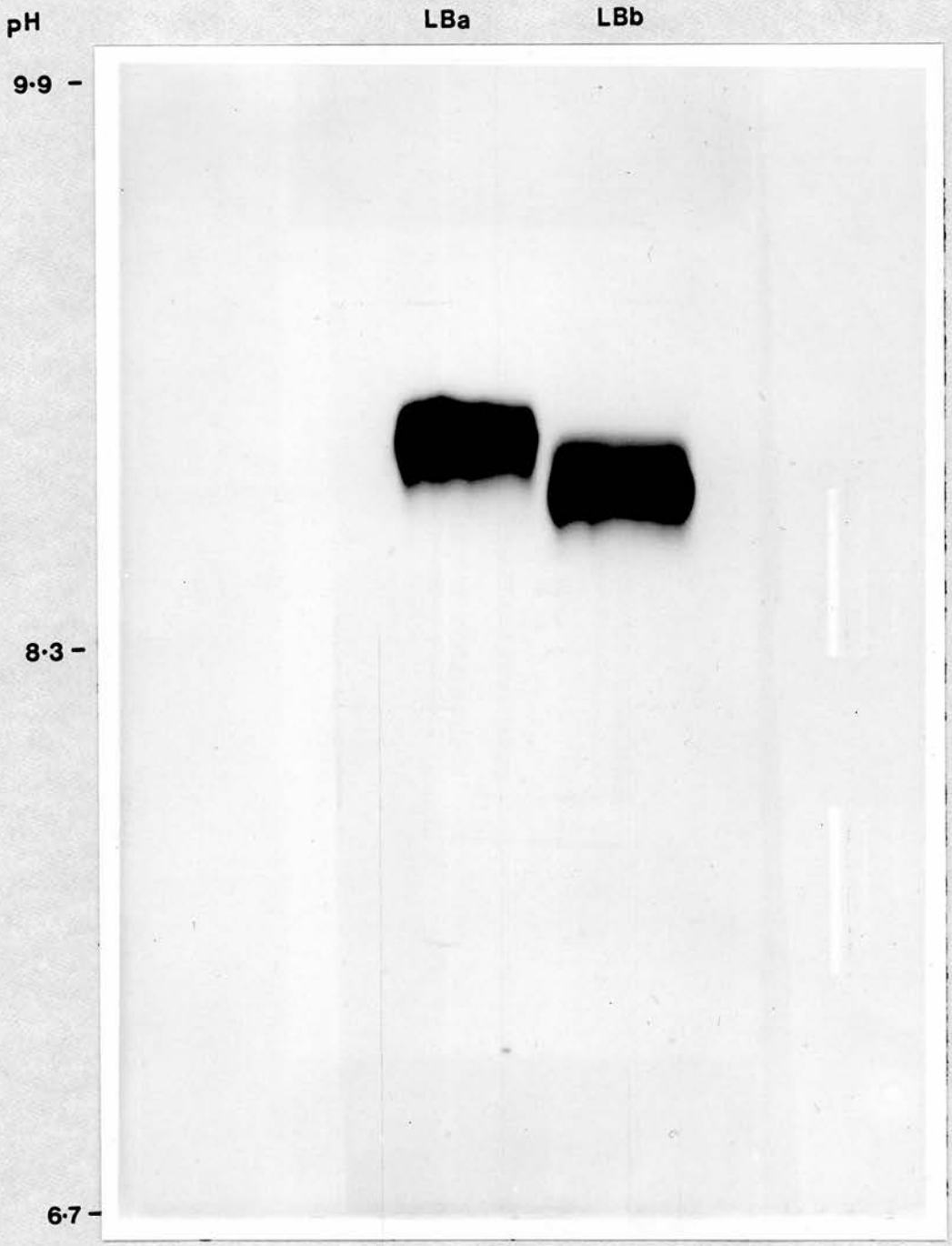


Figure 3.14

Figure 3.14 Thin-layer polyacrylamide gel isoelectric focussing of LBa and LBb. LBa and LBb were prepared as described and portions of each protein (25 μg) were applied to the gels using electrofocussing strips (in the photograph LBa is on the left hand side and LBb is on the right hand side). After electrophoresis (3 h) the pH range obtained was determined by eluting the carrier-ampholines from gel slices as described. The pH at the cathode (top) was 9.9 and at the anode (bottom) 6.7.

sample from LBb migrates as a single band.

Amino acid compositions

The amino acid compositions of LBa (ligandin) and LBb (glutathione S-transferase B) were determined to see whether the two proteins possessed similar compositions, and could therefore be coded for by the same gene(s), or different compositions, and could therefore be the products of separate genes. To enable this comparison to be made the two proteins were simultaneously subjected to identical analytical procedures.

Table 3.3 presents the quantitative data obtained from the amino acid analysis of LBa and LBb. No significant differences between the compositions of the two proteins were detected. The largest differences observed between the two proteins, after the 24 h HCl hydrolysis, were in the recoveries of leucine and lysine. However the differences were within the 3% error limits which Schroeder (1969) suggested was the precision of the method when carrying out duplicate determinations. Both proteins contained large amounts of aspartic acid(Asx), glutamic acid (Glx), alanine, leucine and lysine; in both cases these amino acids accounted for more than 50% of the total amino acids recovered from the hydrolysate. No glucosamine or galactosamine was recovered from either LBa or LBb.

	Amino acids recovered after hydrolysis (μ mol)							
	LBa				LBb			
	24 h	96 h	Ox	Mesa	24 h	96 h	Ox	Mesa
Lys	0.185	0.186			0.191	0.188		
His	0.030	0.025			0.028	0.027		
Arg	0.110	0.112			0.114	0.118		
NH_3	0.304	0.401			0.318	0.381		
Asx	0.183	0.186	0.190	0.186	0.182	0.185	0.193	0.191
Thr	0.057	0.039	0.054	0.053	0.059	0.041	0.055	0.054
Ser	0.085	0.039	0.083	0.083	0.084	0.045	0.079	0.081
Glx	0.220	0.233	0.221	0.222	0.219	0.240	0.226	0.221
Pro	0.106	0.101	0.093	0.099	0.110	0.106	0.097	0.095
Gly	0.097	0.100	0.103	0.102	0.095	0.096	0.103	0.099
Ala	0.143	0.153	0.151	0.140	0.146	0.150	0.149	0.146
$\frac{1}{2}$ Cys			0.018*				0.019*	
Val	0.102	0.124	0.115	0.089	0.099	0.121	0.117	0.090
Met	0.058	0.057	0.053*	0.060	0.059	0.058	0.054*	0.062
Ile	0.090	0.60	0.089	0.087	0.087	0.058	0.090	0.087
Lue	0.249	0.245	0.234	0.271	0.256	0.255	0.243	0.262
Tyr	0.071	0.062		0.082	0.073	0.064		0.079
Phe	0.080	0.079	0.077	0.080	0.082	0.078	0.078	0.078
Trp				0.002				0.002
Correction Factor	1.00	1.05	0.96	0.98	1.00	0.98	1.07	1.03

Table 3.3 Amino acid composition of LBa and LBb. Samples of LBa and LBb were hydrolysed in either 6M - HCl for 24 h and 96 h or 3M -mercaptoethanesulphonic acid for 24 h (Mesa). Portions of LBa and LBb were also treated with performic acid and the oxidixed protein hydrolysed in 6M -HCl for 24 h (Ox). The results obtained are presented in μmol of amino acid recovered from the column. Different samples were normalized on Gly, Ala, Leu, Asx, Glx, Phe, Lys and Arg as described and the correction factor applied to each sample is indicated. In the Ox samples Cys and Met were recovered as cysteic acid and methionine sulphone and the corresponding recoveries are marked with an asterisk (*).

These are identifiable as they elute from the long column (0.9 cm x 50 cm) of the Beckman-Spinco Amino Acid Analyser after the acidic and neutral amino acids.

A comparison between the analysis of the 24 h and the 96 h HCl-hydrolysis of either protein revealed striking differences in the recoveries of threonine, serine, valine and isoleucine. However, in each case, the recoveries of these amino acids after 96 h HCl-hydrolysis, from LBa and LBb, was similar and no obvious differences between the two proteins were observed. The loss of threonine from both LBa and LBb between the 24h hydrolysis and the 96 h hydrolysis was 32%. Both proteins therefore lost threonine to the extent of 11% per 24 h of hydrolysis. The loss of serine from LBa and LBb between the 24 h hydrolysis and the 96 h hydrolysis was 54% and 46% respectively. LBa and LBb therefore lost serine to the extent of 18% and 15% respectively per 24 h of hydrolysis. In both instances the hydrolytic loss of threonine and serine was greater than expected. Normally threonine and serine are destroyed to the extent of 5% and 10% respectively per 24 h of hydrolysis (Schroeder, 1969). However, this result may only reflect the individual behaviour of different proteins.

A lower recovery of isoleucine was also obtained from the 96 h hydrolysis than from the 24 h hydrolysis of either protein (33% loss in both proteins). This was an unexpected result as the recovery

of this amino acid normally increases with prolonged hydrolysis, and the isoleucine content of proteins is normally calculated by extrapolation to infinite hydrolysis time (Schroeder, 1969). This finding may be due to the recovery of an additional peak, which eluted between methionine and isoleucine, which was found in the 96 h hydrolysates. This peak was found in both LBa and LBb and was provisionally identified as allo-isoleucine (Dr. R. P. Ambler, personal communication).

In both proteins the recovery of valine was found to increase between the 24 h hydrolysis and the 96 h hydrolysis. This increase was calculated as 22% and 23% for LBa and LBb respectively over the 72 h period.

Performic acid treatment of LBa and LBb and subsequent hydrolysis revealed no difference in the cysteine and/or cystine content of the two proteins. However, since this approach does not permit cysteine or cystine to be distinguished from each other, this method does not remove the possibility that differences exist between LBa and LBb in the oxidation state of these residues.

The use of mercaptoethanesulphonic acid to enable the tryptophan content of the two proteins to be determined also revealed no differences between the two proteins.

Table 3.4 shows the amino acid compositions of LBa and LBb. The results were calculated from the 24 h hydrolyses using the mol. wts. which were determined by SDS/polyacrylamide gel electrophoresis. They are expressed as moles of amino acid/44 000 g protein for LBa and as moles of amino acid/47 000 g protein for LBb. The actual mol. wt. of LBa and LBb calculated from the amino acid values are 44 010 and 47 040 respectively (Asx, Glx and $\frac{1}{2}$Cys were assigned mol. wts. of 133, 147 and 121 respectively). Table 3.4 shows that the amino acid compositions of the two proteins were very similar when expressed as a percentage of the total amino acids recovered. However, small differences between LBa and LBb were observed when the results were expressed as residues/mol of transferase. These differences were most noticable in the case of residues such as lysine, arginine, alanine and leucine, which were present in large amounts. The results of Table 3.4 can only be regarded as approximate since no corrections were made to the data to allow for the loss of serine or threonine (by extrapolation to zero hydrolysis time) or the increase in the recovery of valine with prolonged hydrolysis (by extrapolation to infinite hydrolysis time). These corrections were not made since analyses were performed on samples from only two different hydrolysis times. This provided insufficient data to permit reliable extrapolations to be made. However the amino acid compositions of LBa and LBb were calculated to enable

Amino Acid	Amount of amino acid as % of total recovered		Residues/mol	
	LBa	LBb	LBa	LBb
Lys	9.8	10.0	38	41
His	1.6	1.5	6	6
Arg	5.8	6.0	22	25
Asx	9.7	9.5	38	39
Thr	3.0	3.1	12	13
Ser	4.5	4.4	17	18
Glx	11.7	11.5	45	47
Pro	5.6	5.8	22	24
Gly	5.1	5.0	20	21
Ala	7.6	7.7	29	32
$\frac{1}{2}$ Cys	1.0	1.0	4	4
Val	5.4	5.2	21	21
Met	3.1	3.1	12	13
Ile	4.8	4.6	18	19
Leu	13.2	13.4	51	55
Tyr	3.8	3.8	15	16
Phe	4.2	4.3	16	18
Trp	0.1	0.1	-	-
			Total 386	412

Table 3.4 Amino acid composition of LBa and LBb. The data presented in Table 3.3 were used to calculate the percentage amino acid compositions of the two proteins and also the number of moles of each amino acid per mol. of LBa or LBb as described. The compositions were not corrected for the increased or decreased recoveries of Ser, Thr, Val and Ile.

an estimate to be made of the number of peptides, which should be obtained after proteolytic digestion of these two proteins. Further, the amino acid compositions were determined to allow comparisons to be made with the published data of Habig et al. (1974b), Ketterer et al. (1975) and Kirsch et al. (1975).

The amino acid analyses did not reveal any major differences between LBa and LBb and therefore both proteins may be the product of the same gene(s). Further, the amino acid compositions and SDS/polyacrylamide gel electrophoresis data suggests that the differences between the two proteins resides in only one of the two subunits which LBa (YaYa) and LBb (YaYc) comprise. It is unlikely that this difference is due to the oxidation state of the cysteine/cystine residues in the two proteins since the subunit compositions were unchanged when 2-mercaptoethanol was either omitted, or its concentration increased to 5% (v/v) in the electrophoresis sample mixture. The difference between LBa (ligandin) and LBb (glutathione S-transferase B) may however be the result of a post-translational modification to either the Ya or Yc monomer or both, rather than the two monomers being coded separately. The nature of this postulated modification is not known but it is unlikely that it is due to the addition of carbohydrate since no glucosamine or galactosamine residues were recovered from the hydrolysates of either

protein. LBa and LBb were therefore further examined for differences in the primary structure of the two proteins.

Peptide "mapping" of LBa and LBb using SDS/polyacrylamide gels

Evidence for differences in the primary structure of these two binding proteins was looked for by peptide "mapping". Peptide "maps" were constructed by two different methods. The first method used was that described by Cleveland et al. (1977) and involved forming partial digests of LBa and LBb, the products of which were large enough to be separated, according to size, by SDS/polyacrylamide gel electrophoresis. The second method used, was that described by Ambler (1963), involved allowing the proteolytic digestion of LBa and LBb to continue to completion. The digestion products were then separated, according to charge, by high-voltage paper electrophoresis in either one or two dimensions.

"Cleveland gels" of LBa and LBb digestion products were constructed following partial digestion with the proteolytic enzymes chymotrypsin and staphylococcus aureus V8 protease. Limited proteolysis of LBa and LBb with chymotrypsin followed by SDS/polyacrylamide gel electrophoresis revealed two major digestion products which were common to both proteins (Fig. 3.15). Comparison

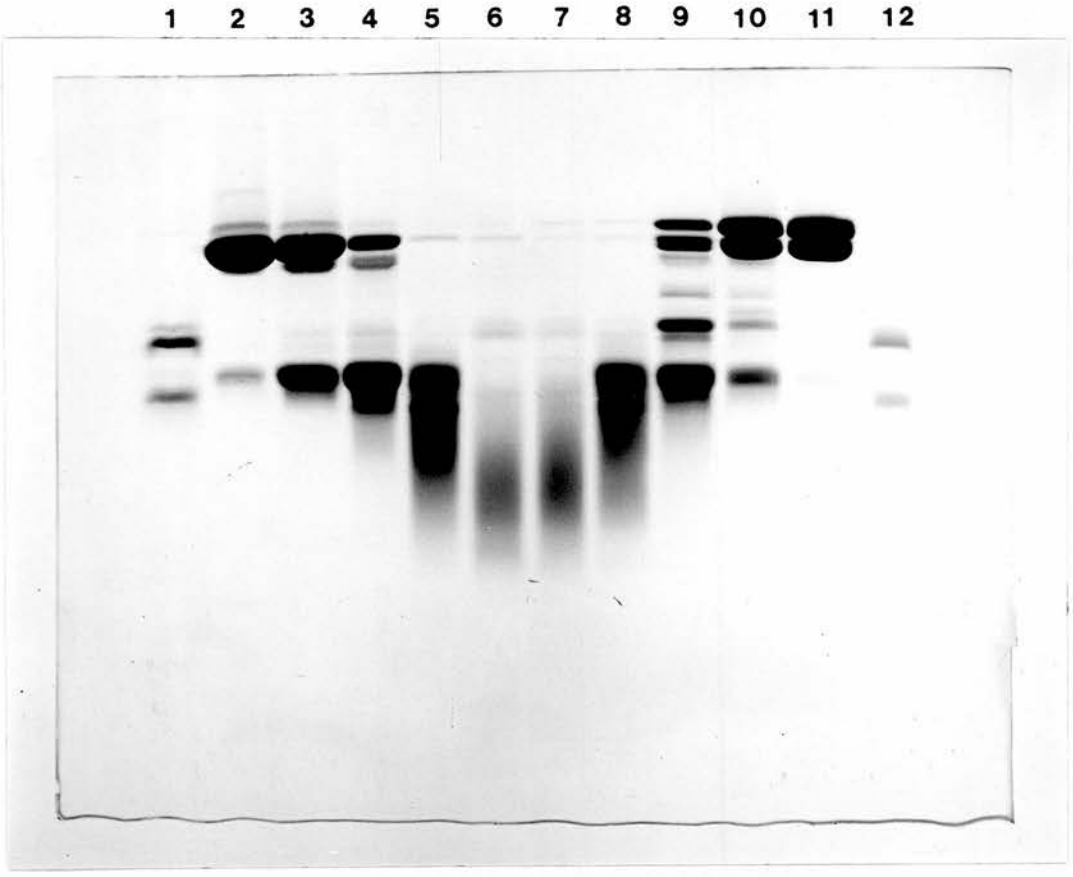


Figure 3.15

Figure 3.15 Partial digestion of LBa and LBb with chymotrypsin. Portions (50 μg) of the lithocholic acid-binding proteins were digested with varying amounts of α-chymotrypsin as described. After 45 min digestion, the reaction mixtures were applied to an SDS/polyacrylamide gel and run from the cathode (top) to the anode (bottom).

The samples applied (left to right) were; 1: Chymotrypsin (7 μg), 2-6: Lithocholic acid-binding protein LBa (Ya Ya)(25 μg) and 0.0007 μg, 0.007 μg, 0.07 μg, 0.7 μg, 7 μg chymotrypsin respectively, 7-11: Lithocholic acid-binding protein LBb (Ya Yc)(25 μg) and 7 μg, 0.7 μg, 0.07 μg, 0.007 μg, 0.0007 μg chymotrypsin respectively, 12: Chymotrypsin (7 μg).

Chymotrypsin, treated in the same way as the lithocholic acid-binding proteins, was dissociated into its subunits (mol. wt. approx. 17 000 and 11 000 respectively (Schroeder, 1968)) and was used as a mol. wt. marker (channels 1 and 12).

of the mobility of these two products with that of the subunits of chymotrypsin showed them to have mol. wt. about 10 000 and 13 000. Figure 3.15 however, also revealed digestion products of mol. wt. about 19 000 and 17 000 which are found in LBb but not in LBa.

Limited proteolysis of LBa and LBb with staphylococcus aureus V8 protease followed by SDS/polyacrylamide gel electrophoresis revealed three major digestion products which were common to both proteins (Fig. 3.16). These had mol. wt. of about 9000, 12 000 and 15 000. The recovery of these products from LBa and LBb was different. The 9000 mol. wt. fragment was recovered in highest concentration from LBa. The 12 000 mol. wt. fragment was recovered in highest concentration from LBb. The 15 000 mol. wt. fragment was recovered in equal amounts from both proteins. Figure 3.16 also revealed digestion products of mol. wt. about 8500, 16 000 and 20 000 which were found in the digests from LBa but not from LBb.

Although these two "Cleveland gels" revealed structural differences between the two proteins, the gels also suggested that these proteins possessed a degree of structural similarity since in both the V8 protease and chymotrypsin digests LBa and LBb yielded

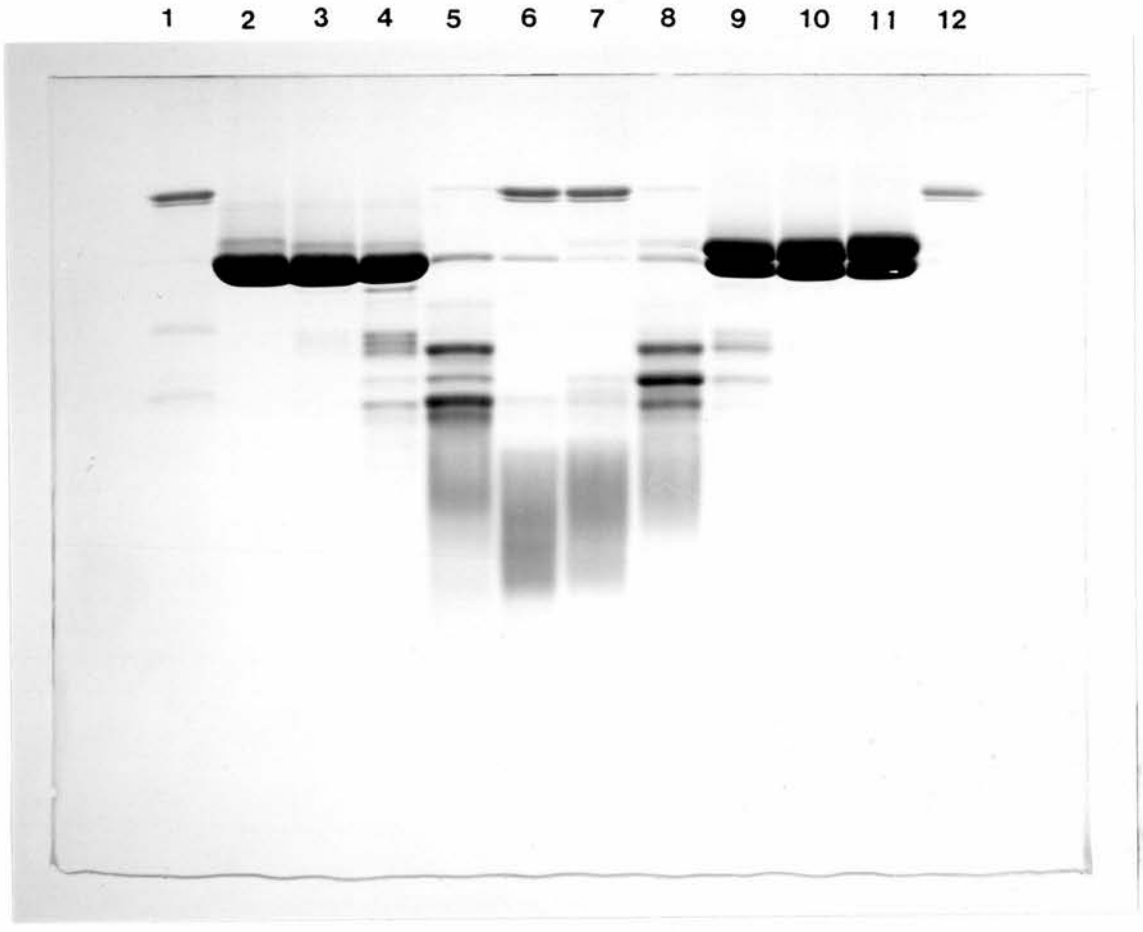


Figure 3.16 Partial digestion of LBa and LBb with V8 protease. Portions (50 μg) of LBa and LBb were digested with varying amounts of staphylococcus aureus V8 protease as described. After 45 min the digestion products were applied to an SDS/polyacrylamide gel and run from the cathode (top) to the anode (bottom).

The samples applied (left to right) were ; 1, V8 protease (7 μg); 2-6, LBa (25 μg) and 0.0007 μg, 0.007 μg, 0.07 μg, 0.7 μg, 7 μg V8 protease respectively; 7-11, LBb (25 μg) and 7 μg, 0.7 μg, 0.07 μg, 0.007 μg , 0.0007 μg V8 protease respectively; 12, V8 protease (7 μg).

digestion products of similar mol. wt.. However the data obtained from these "Cleveland gels" is only qualitative since the digests, which were used for the comparisons, were not allowed to go to completion. To enable a more definitive comparison between the two proteins to be made LBa and LBb were analysed by two dimensional peptide "mapping" procedures. In these experiments the proteolytic digestions were allowed to go to completion and all the steps were performed simultaneously on both proteins under identical conditions.

Peptide "mapping" of LBa and LBb using high-voltage paper electrophoresis

Before preparing the multi-dimensional peptide "maps" LBa and LBb were separately digested with thermolysin, chymotrypsin and trypsin with a view to finding the proteolytic enzyme whose specificity revealed the largest number of structural differences between the two binding-proteins. The products from the 6 digests were analysed in parallel by high-voltage paper electrophoresis at pH 6.5 . Examination of these one dimensional peptide "maps" indicated that thermolysin, chymotrypsin and trypsin each revealed minor differences between LBa and LBb (Fig. 3.17). Although different peptide "patterns" were observed it was not possible to determine whether one protein produced more peptides than the other

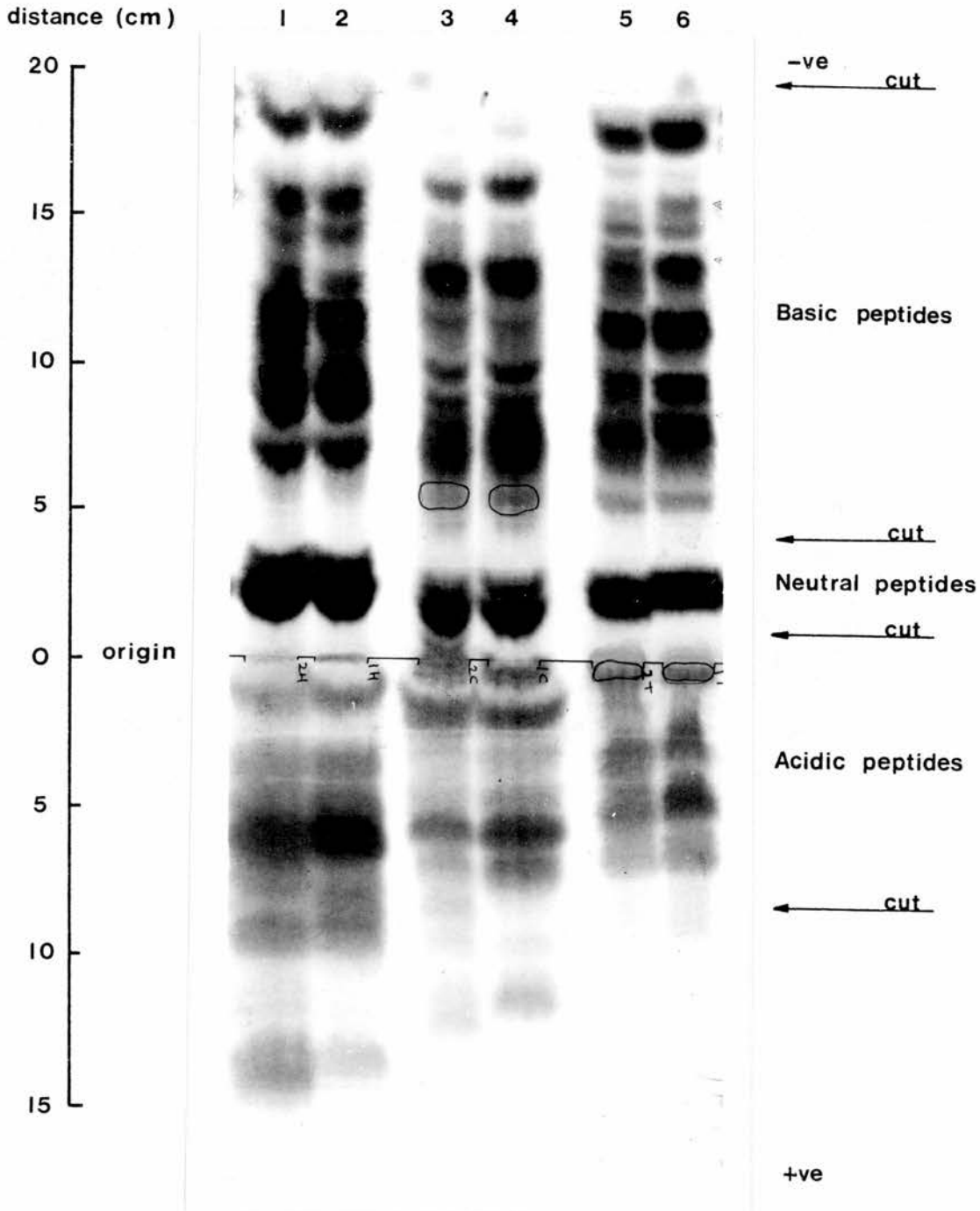

distance (cm)
1
2
3
4
5
6
20
15
10
5
0
origin
5
10
15
-ve
cut
Basic peptides
cut
Neutral peptides
cut
Acidic peptides
cut
+ve

Figure 3.17

Figure 3.17 One dimensional "map" of thermolysin, chymotrypsin and trypsin digests of LBa and LBb. Portions of LBa and LBb (1 mg of protein) were digested at 37°C with portions of thermolysin, chymotrypsin or trypsin (20 μg of protein). After 6 h the digests were freeze-dried. The lyophilised digests were then redissolved in 100 μl of 0.1 M NH_3 and applied to Whatman 3MM chromatography paper from left to right as follows; 1, thermolysin digest of LBb; 2, thermolysin digest of LBa; 3, chymotrypsin digest of LBb; 4, chymotrypsin digest of LBa; 5, trypsin digest of LBb and 6, trypsin digest of LBa. These samples were analysed by high-voltage electrophoresis at pH 6.5. The origin is marked and the cathode is at the top and the anode at the bottom of the photograph. The peptides were divided into three groups which were designated the basic, neutral and acidic peptides, according to their electrophoretic mobility at pH 6.5 (Fig. 2.8).

To enable the two dimensional tryptic "maps" to be constructed, these three groups were separated by cutting the paper after the first dimension at the positions indicated.

or whether both proteins produced the same number of peptides, some of which had a different mobility from their counterparts in the other protein, since the peptides in these one dimensional "maps" were incompletely resolved. The electrophoretic mobility of the "difference peptides" (or position where the peptide pattern differed in the two proteins) was expressed in terms of the distance and direction each migrated from the origin. These peptides were compared with amino acid standards run in parallel. The basic amino acid markers, lysine, arginine and histidine migrated 20 cm, 17.5 cm and 9.5 cm respectively from the origin towards the cathode (–VE) and were therefore designated positions -20 cm, -17.5 cm and -9.5 cm respectively. The "neutral" (monoaminomonocarboxylic) amino acids migrated 2.5 cm from the origin towards the cathode (–VE) and were therefore assigned the position -2.5 cm. The acidic amino acids, glutamic acid and aspartic acid migrated 16 cm and 17.8 cm respectively, from the origin towards the anode (+VE) and were designated positions +16 cm and +17.8 cm (Fig. 3.17).

High-voltage paper electrophoresis of the thermolysin digests of LBa and LBb revealed differences in the pattern obtained from the basic peptides (position on "map" - 15 cm) and the acidic peptides (position + 15.5 cm). Differences in the LBa and LBb "map" patterns were obtained after digestion by chymotrypsin. These differences

were observed in the neutral peptides ("map" position -0.5 cm) and further differences were observed in the acidic peptides (positions +5.5 cm, +8.0 cm, +9.5 cm and +13 cm). Analysis of the tryptic digests of LBa and LBb revealed a large number of differences in the basic peptides (positions -17.5 cm, -15 cm, -10 cm and -9.5 cm). A slight difference in the migration of the neutral peptides (which produced a single broad band with a "map" position between -2 cm and -3.75 cm) was also observed. The neutral peptides which were obtained from LBa migrated 0.25 cm further from the origin than the equivalent peptides from LBb. Further differences were also observed in the pattern produced by the acidic peptides at positions +2.5 cm and +5.0 cm. No differences were observed in any digests between the tryptophan containing peptides (identified by examination of the electrophoresis paper under U.V. light) obtained from LBa or LBb. On the basis of these results trypsin was chosen as the proteolytic enzyme to construct the two dimensional "maps" of LBa and LBb since its specificity of action revealed the largest number of structural differences between the two proteins.

Two dimensional peptide "maps" of LBa and LBb

The two dimensional peptide "maps" of LBa and LBb were constructed by high-voltage paper electrophoresis at pH 6.5 in the

first dimension and at pH 3.5 in the second dimension (Fig. 2.8). After electrophoresis in the first dimension, of portions (1.5 mg of protein) of the tryptic digests, the electrophoresis paper was cut, as indicated in Fig. 3.17, into sections containing the basic peptides, the neutral peptides and the acidic peptides. The cutting was facilitated by running along with the 1.5 mg portions of LBa and LBb, 0.5 mg portions of LBa and LBb digests. These 0.5 mg portions were stained, after electrophoresis, with ninhydrin and served as markers enabling the peptides on the unstained paper to be divided (into the "neutrals","basics" and "acidics") without cutting through any peptides. The two pieces of paper containing the acidic peptides from LBa and LBb were sewn on to a single piece of electrophoresis paper end-to-end such that the least acidic peptides (which were still at the original origin) from LBa and LBb were adjacent and the direction of migration in the second dimension was at right angles to the direction of migration in the first dimension. The basic peptides from LBa and LBb were also sewn in a similar manner onto a single piece of electrophoresis paper. This enabled these two types of peptides from LBa and LBb to be directly compared and resulted in the formation of mirror image "maps" of the acidic and basic peptides.

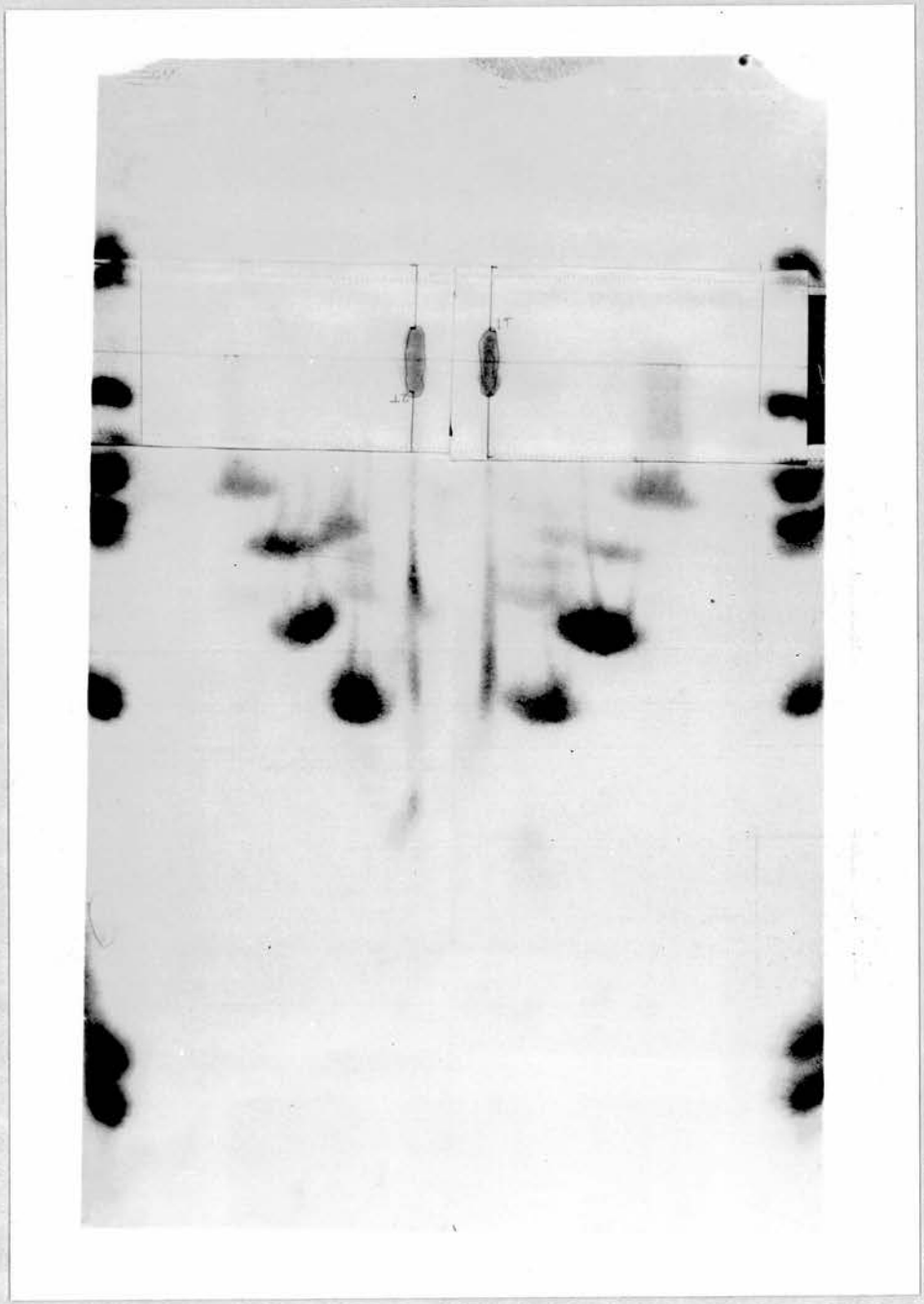


Figure 3.18a

Figure 3.18a Two dimensional "map" of the acidic peptides from tryptic digests of LBa and LBb. The tryptic digests of portions (1.5 mg of protein) of LBa and LBb were prepared as described. The peptides were separated by high-voltage paper electrophoresis at pH 6.5 in the first dimension and the acidic peptides were cut out of the paper as described. These peptides were separated in the second dimension by high-voltage electrophoresis at pH 3.5. The peptides from LBa are on the right hand half of the "map" and the peptides from LBb are on the left hand half of the "map" as indicated (1T and 2T respectively). A diagramatic representation of the "map" is shown in Fig. 3.18b.

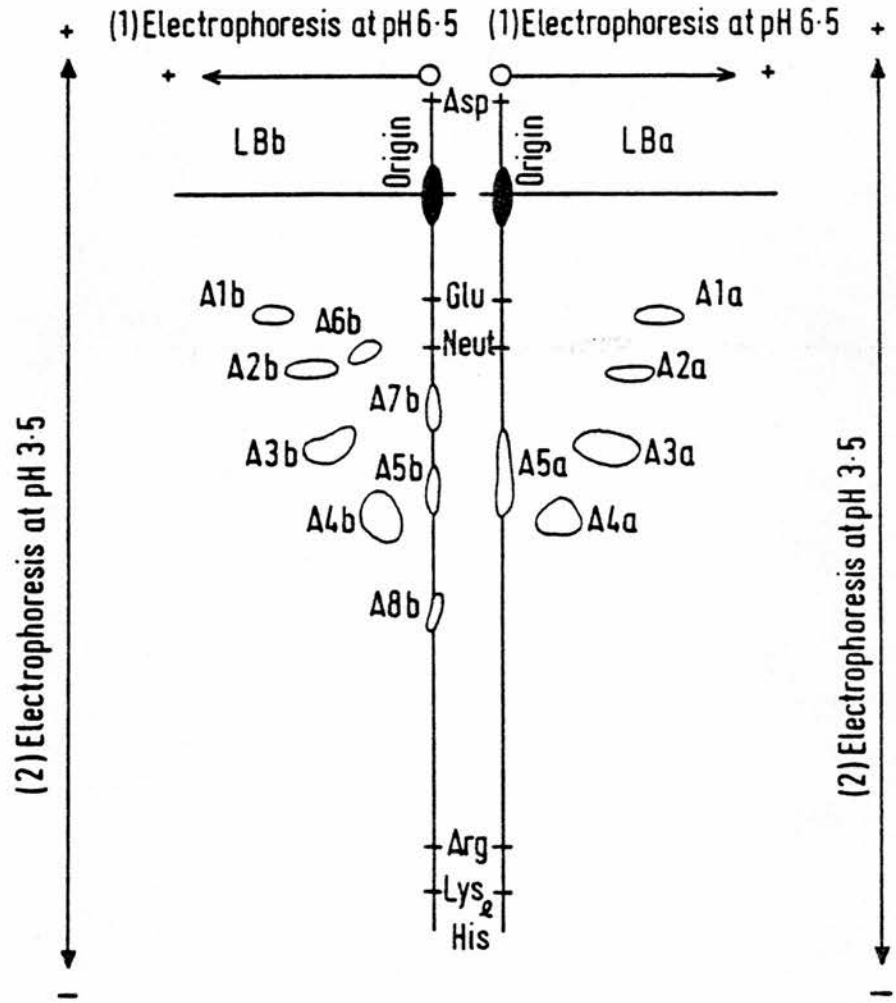


Figure 3.18b Diagramatic representation of the two dimensional "map" of the acidic peptides from tryptic digests of LBa and LBb. The acidic peptides from LBa and LBb were separated as described in Fig. 3.18a. Definition of the symbols is given in the text.

Fig. 3.18a shows the two dimensional "map" of the acidic peptides from LBa and LBb and Fig. 3.18b shows a diagramatic representation of the same "map". The peptide nomenclature, which was devised to enable the multi-dimensional "maps" from LBa and LBb to be compared, is described and is based on the mobility of the peptides. In this nomenclature the peptides which were common to both proteins were designated first and the unique peptides designated second. Fig. 3.18b shows that at least 5 peptide spots (A1a-A5a and A1b-A5b) appear to be common to both proteins. LBb also appeared to contain several peptides (A6b, A7b and A8b) which were not recovered from LBa. However, although LBa did not produce any peptide spots which were equivalent to A6b or A8b the significance of A7b is not clear since it was obtained from the peptides which remained at the origin, during electrophoresis at pH 6.5. Neither A5a nor A5b and A7b, which each remained at the origin in the first dimension, were well resolved. These peptides were recovered as elongated smears along the axis and may not reflect real differences between the proteins rather than the peptides adhering to the paper during electrophoresis.

Fig. 3.19a shows the two dimensional map of the basic peptides from LBa and LBb and Fig. 3.19b shows a diagramatic representation

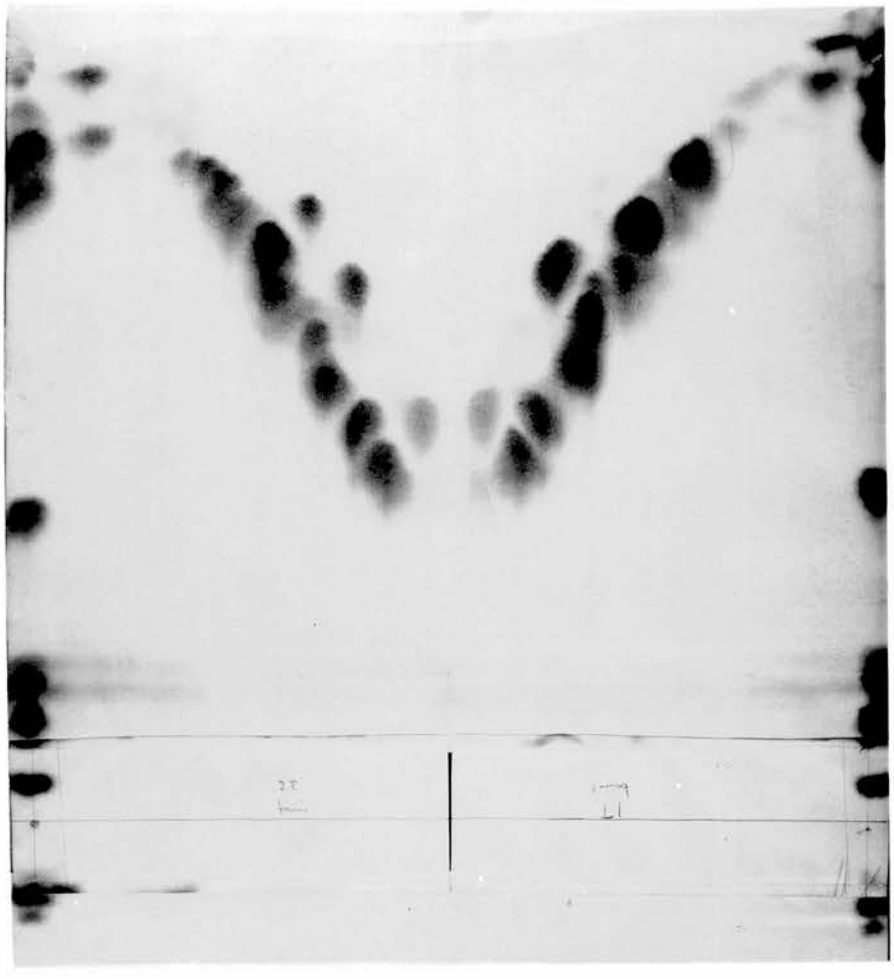


Figure 3.19a

Figure 3.19a Two dimensional "map" of the basic peptides from tryptic digests of LBa and LBb. The tryptic digests of portions (1.5 mg of protein) of LBa and LBb were prepared as described. The peptides were separated by high-voltage paper electrophoresis at pH 6.5 in the first dimension and the basic peptides were cut out of the paper as described. These peptides were separated in the second dimension by high-voltage paper electrophoresis at pH 3.5. The peptides from LBa are on the right hand half of the "map" and the peptides from LBb are on the left hand half of the "map" as indicated (1T and 2T respectively). A diagramatic representation of the "map" is shown in Fig. 3.19b.

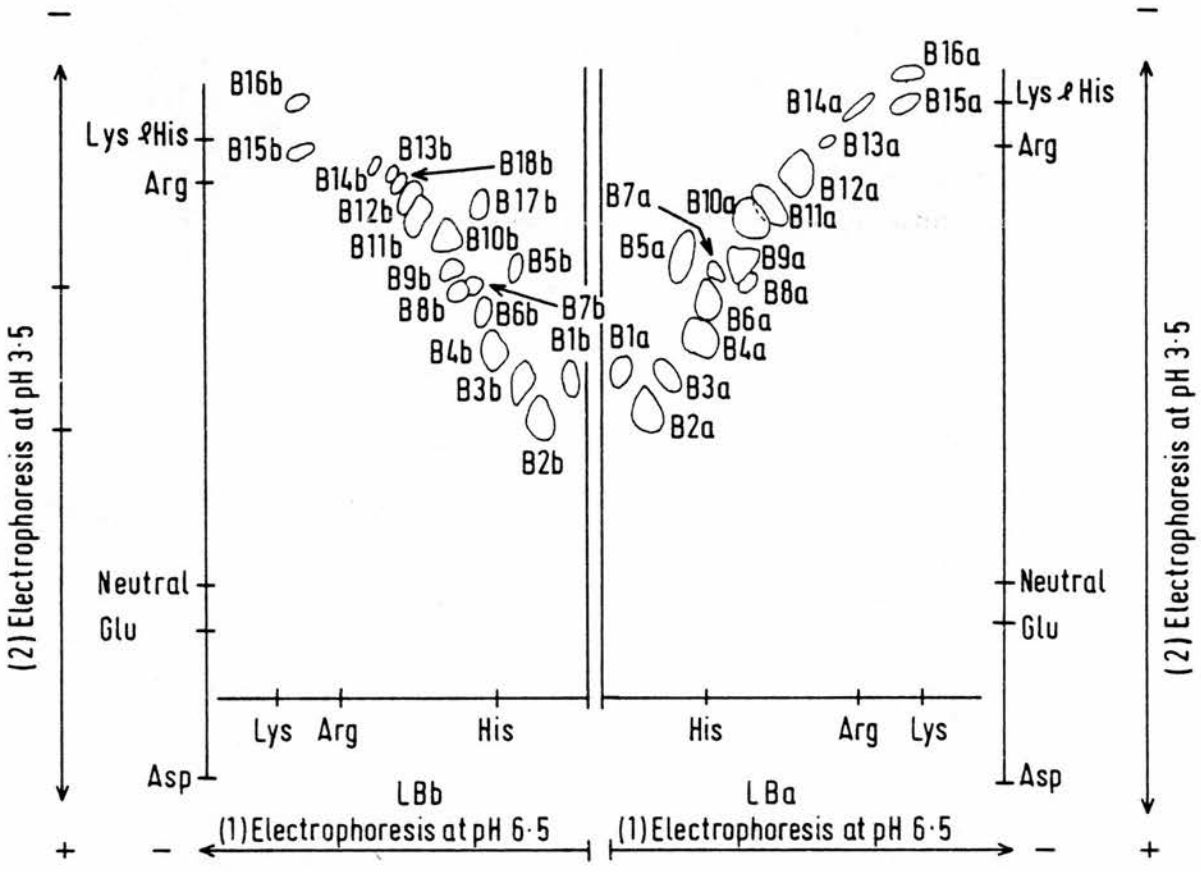


Figure 3.19b Diagramatic representation of the two dimensional "map" of the basic peptides from tryptic digests of LBa and LBb. The basic peptides from LBa and LBb were separated as described in Fig. 3.19a. Definition of the symbols is given in the text.

of the same 'map'. The main bulk of peptides run diagonally across the electrophoresis paper forming a "V"-shaped two dimensional map. Although these are not well separated, differences in the patterns produced by these peptides were observed. A tentative identification of the peptides was made and 16 spots from LBa and 18 spots from LBb were provisionally designated Bla -B16a and Blb-B18b respectively. A marked difference between LBa and LBb was observed in the peptides which lay outside the main "V"-shape.

Two peptide spots were obtained from LBa (Bla and B5a) which migrated relatively further in the second dimension than in the first (when compared with the other peptides in the digest) and were therefore not included in the general "V"-shaped peptide mass. However LBb contained an additional peptide, giving a total of 3 peptide spots (Blb, B5b and B17b), which lay outside this "V"-shaped pattern. There also appeared to be an additional peptide (B18b) in LBb which was sandwiched between Bllb and B12b which was not observed between Bl la and B12a. Whilst it appears from the "map" that both B17b and B18b are additional peptides which are not found in LBa it is possible that both B5a and Blla each contain two unresolved peptides which, due to the substitution of an amino acid, have been resolved in LBb to produce the additional peptides. In either event,

however, these peptides reveal structural differences between the two proteins.

Three dimensional peptide "maps" of the neutral peptides from LBa and LBb

The neutral peptides, obtained from LBa and LBb after electrophoresis at pH 6.5, were also subjected to electrophoresis at pH 3.5 in the second dimension, as were the acidic and basic peptides (see Fig. 2.8). However, since the first dimension resulted in the neutral peptides running as a single band (Fig. 3.17) the second dimension produced "maps" of LBa and LBb which comprised only single columns of peptides. No differences in these neutral peptides of LBa and LBb were observed but since the various peptides were poorly resolved after the second electrophoretic run further separation of the neutral peptides was achieved by descending chromatography in a third dimension (Figs. 3.20a and b). Fig. 3.20a shows the three dimensional "map" of the neutral peptides from LBa and LBb and Fig. 3.20b shows a diagramatic representation of the same "map". These show that at least 10 peptide spots (N1a-N10a and N1b-N10b) appear to be common to both proteins. LBb also appeared to contain two peptides (N11b and N12b) which were not observed in LBa.

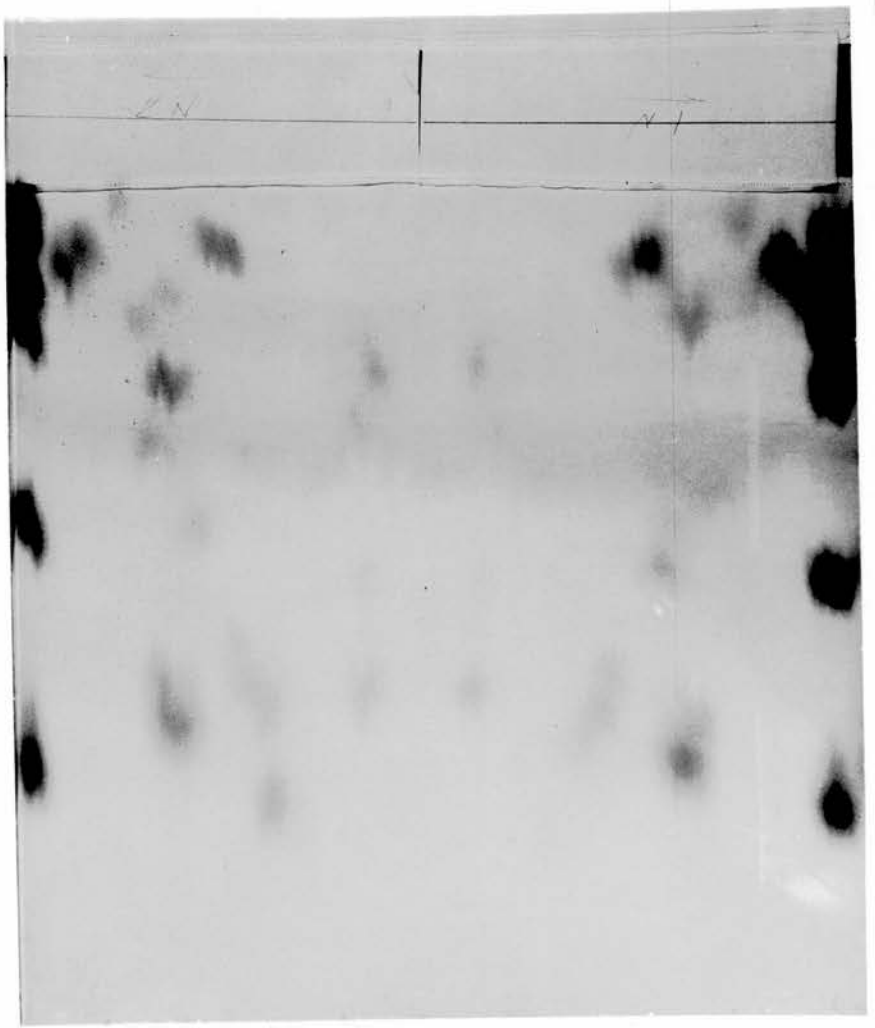


Figure 3.20a

Figure 3.20a Tryptic "map" of the neutral peptides from LBa and LBb. The tryptic digests of portions (1.5 mg of protein) of LBa and LBb were prepared. The single spot of neutral peptides obtained from LBa and LBb after high-voltage paper electrophoresis at pH 6.5 (3.17) were separated in a second dimension by high-voltage paper electrophoresis at pH 3.5. These were further resolved by descending chromatography using butan-1-ol/acetic acid/water/pyridine (15: 3:12:10, by vol.) for 16 h in a third dimension. The peptides from LBa are on the right hand half of the "map" and the peptides from LBb are on the left hand half of the "map" as indicated (1N and 2N respectively). A diagramatic representation of the "map" is shown in Fig. 3.20b.

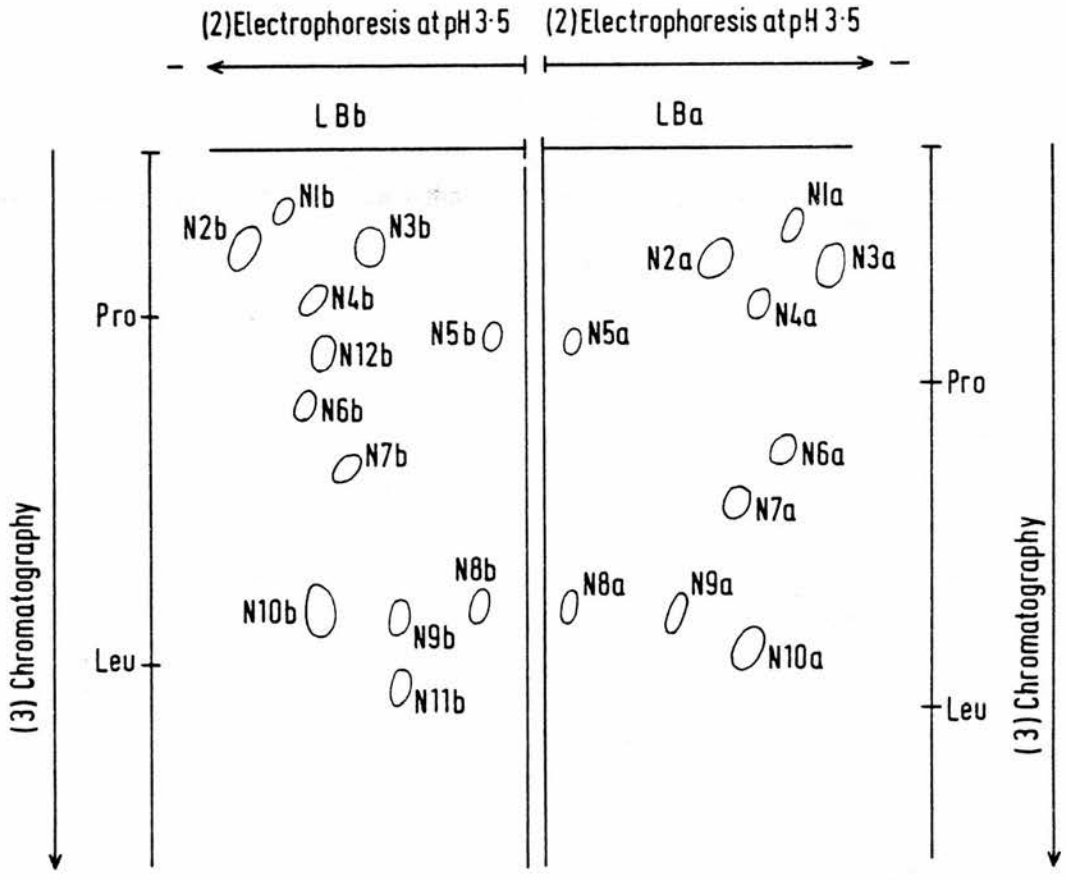


Figure 3.20b Diagramatic representation of the tryptic "map" of the neutral peptides from LBa and LBb. The neutral peptides from LBa and LBb were separated as described in Fig. 3.20a. Definition of the symbols is given in the text.

The results from the three peptide "maps" shown in Figs. 3.18-3.20 suggest that digestion of LBa with trypsin produced 31 peptides and digestion of LBb with trypsin produced 38 peptides. If it is assumed that LBa (YaYa) comprises two identical subunits then although only 31 peptide spots were observed each LBa molecule would produce 62 peptides. This would indicate that each LBa dimer contains a total of 60 tryptic cleavage sites. Since the specificity of trypsin is limited to peptide bonds associated with the carboxyl groups of lysine and arginine this suggests that LBa contains at least 60 arginine + lysine residues per molecule. This is in agreement with the data in Table 3.4 which shows that LBa (YaYa) contains 38 lysine residues/mol and 22 arginine residues/mol. However Table 3.4 also indicates that LBb (YaYc) contains 3 extra lysine residues/mol and 3 extra arginine residues/mol which suggests that tryptic digests of LBb should produce 6 more peptides than LBa. LBb in actual fact produced 7 extra peptides which is in good agreement with what would be expected from the amino acid compositions. The similarity of the peptide "maps" from LBa and LBb suggests that these two proteins possess a great deal of homology. Since LBb produced 7 extra peptides, which were not recovered from LBa, LBb probably possesses an additional sequence of amino acids. From the subunit compositions (Fig. 3.13) it was calculated that this postulated sequence would comprise 26 amino

acids. This additional sequence does not result in the gross amino acid compositions of LBa and LBb appearing significantly different. This sequence therefore probably comprises amino acids whose percentage composition is approximately the same as that of LBa and contains 7 tryptic cleavage sites.

DISCUSSION

Hepatic transport of organic anions and ligandin

An important function of the liver is the removal of both endogenous and exogenous anions from the blood and their excretion into bile. Various anionic dyes have been used clinically to test this ability of the liver; most commonly used are bromosulphophthalein (Kaplowitz et al., 1973) and indocyanine green (Kitazima & Shibata, 1975). Because of its association with jaundice, bilirubin clearance by the liver has been extensively studied and, to a large extent, present understanding of hepatic anion transport has been shaped by investigations into bilirubin transport. Bernstein et al. (1966) showed that when radioactive bilirubin is injected intravenously into rats, 60% of the injected bilirubin is found in the liver supernatant and 40% in the liver particulate fraction. Gel-exclusion chromatography of a mixture of rat liver cytosol and bilirubin demonstrated that 80% of the bilirubin in supernatant is bound to a protein of mol. wt. 45 000 (Levi et al., 1969b; Arias et al., 1976). This protein was called Y protein and since it also bound bromosulphophthalein and indocyanine green it was proposed that it was involved in the intrahepatic transport of various anions. Phylogenetic studies (Levine et al., 1971) and investigations into neonatal jaundice (Levi et al., 1969a, 1970) supported this hypothesis. Although the involvement of Y protein in anion transport was not clearly defined,

it was none-the-less generally assumed that it served as a general carrier protein which transported anions from the sinusoidal membrane to the canalicular membrane.

Litwack et al. (1971) reported that the Y protein, described by Arias and his co-workers, was the same protein as a carcinogen-binding protein, which had been isolated by Ketterer et al. (1967), and a cortisol-metabolite-binding protein, which had been purified by Morey & Litwack (1969); the three protein preparations possessed similar physicochemical and immuno-chemical properties. The three groups renamed their respective proteins ligandin. The name ligandin emphasised the widely held belief that this protein was responsible for the intrahepatic transport of a large number of anions.

In addition to its anion-binding properties ligandin was subsequently shown to possess enzyme activity (Kaplowitz et al., 1973) and Habig et al. (1974b) reported that in the rat liver ligandin, prepared by the method of Arias and his co-workers, is identical to glutathione S-transferase B. There are at least 7 glutathione S-transferases in rat liver cytosol and Ketley et al. (1975) have shown that transferases C, B, A and AA were all able to bind bilirubin and indocyanine green. These workers suggested that although binding was not specific, ligands should possess a hydrophobic moiety for binding to take place. Further, Ketley et al. (1975) suggested that since the term ligandin was used to describe a

protein with a wide range of binding affinities, all the glutathione S-transferases could therefore be called ligandins.

The reason for the large number of similar glutathione S-transferases in rat liver cytosol is not clear. It is not known whether each of the ionically distinct enzymes is the product of a separate gene or whether several transferases are coded for by a single gene. It appears likely that at least several of these enzymes are coded for by a single gene since transferases A and C have similar amino acid compositions and antisera raised against either enzyme cross-reacts with the other transferase (Habig et al., 1974b). This possibility is supported by the observation that although there are 7 transferases they each comprise 2 of only 3 possible monomers (Bass et al., 1977a). An analogy may exist between the situation in human liver, where 5 ionically distinct transferases were purified which were postulated to have arisen as a result of an in vivo deamidation of a single gene product, (Habig et al., 1976a).

The major group of anions transported by the liver are the bile acids. In man and rat the liver transports 30 g and 200 mg of bile acids and bile salts per day respectively. Since the bile acids possess a hydrophobic moiety, the perhydrocyclopentanophenanthrene ring structure, it is likely that certain glutathione S-transferases would bind this group

of compounds. Strange et al. (1977b) have purified two lithocholic acid -binding proteins from rat liver cytosol. Although neither protein was identified, both possessed glutathione S-transferase activity. These two proteins may be involved in bile acid transport across the hepatocyte.

Aims of the study

The aims of this thesis were to; (1), determine whether any of the transferases bound the quantitatively important primary bile acid, cholic acid; (2), determine whether the proteins which bound cholic acid and lithocholic acid possessed a common subunit (Ya, Yb or Yc); (3), identify the two purified lithocholic acid-binding proteins and (4), determine whether these two proteins could be the product of a single gene or whether they are coded separately and therefore represent isoenzymes.

Bile acid binding by glutathione S-transferases

The experiments described show that at least 7 peaks of glutathione S-transferase activity in hepatic cytosol can be resolved by CM-Sephadex chromatography; of these 6 were able to catalyse the conjugation of GSH with 1-chloro-2,4-dinitrobenzene. These 6 were designated

peaks 1, 2, 3, 4(i), 4(ii) and 5 according to their order of elution from CM-Sephadex. The cholic acid-binding activity of these 6 enzyme-containing peaks was examined by an equilibrium chromatographic method which showed that peaks 1, 3, 4(i) and 5 bound the bile acid. Although only ligandin has previously been reported to bind cholic acid (Tipping et al., 1976), these experiments show that at least 4 transferases bind this bile acid. The lithocholic acid-binding activity of the glutathione S-transferases was examined by a non-equilibrium chromatographic method which showed that peaks 1, 3 and 4(i) bound lithocholic acid. These experiments show that 3 of the enzyme-containing peaks bind both bile acids. However, although these results suggest that peak 5 possesses only cholic acid-binding activity the cholic acid and lithocholic acid-binding data are not directly comparable since the lithocholic acid binding was studied under non-equilibrium conditions which would fail to demonstrate binding in instances where the protein: ligand interaction was weak and the complex dissociated during chromatography.

Since certain transferases may be coded for by a single gene the various enzyme peaks were examined by electrophoresis to determine whether certain functions of the various transferase peaks could be ascribed to a particular monomer. Discontinuous SDS/ polyacrylamide gel electrophoresis indicated that cholic acid-binding

was associated with the Ya and Yc monomers. The enzyme-containing peaks which bound lithocholic acid all possessed the Ya monomer and the peaks which catalysed the conjugation of GSH with 1,2-dichloro-4-nitrobenzene (peaks 1, 2 and 4(ii) all possessed the Yb band.

Identification of the lithocholic acid-binding proteins LBa and LBb

Peaks 3 and 4(i) contain the two lithocholic acid-binding proteins described by Strange et al. (1977b). Both these peaks comprise proteins which could be ligandin on the basis of substrate specificity, phenobarbitone induction, subunit composition and anion-binding. To identify these proteins, glutathione S-transferase B and ligandin were prepared. Glutathione S-transferase B was purified by the method of Habig et al. (1974b, 1976a) and ligandin by the procedure described by Arias et al. (1976) and Bass et al. (1977a). Examination of these two proteins by CM-Sephadex chromatography showed that both ligandin and glutathione S-transferase B eluted separately as single peaks of protein and enzyme activity at volumes equivalent to peak 3 and peak 4(i) respectively. This indicates that ligandin and glutathione S-transferase B, prepared by published procedures, are not identical proteins, as reported by Habig et al. (1974b), but are separate proteins. Ligandin and glutathione S-transferase B both bind lithocholic acid and appear to be identical to LBa and LBb respectively.

Identification of the glutathione S-transferases

The glutathione S-transferases have previously been classified by their order of elution from CM-cellulose (Habig et al., 1974b; Jakoby et al., 1976a, b). A comparison between the CM-cellulose data and the CM-Sephadex elution profile suggests that transferases D and E elute in peak 1, transferase C in peak 2, ligandin in peak 3, transferase B in peak 4(i), transferase A in peak 4(ii) and transferase AA in peak 5 (Table 4.1).

The presence of transferase C in peak 2 and transferase A in peak 4(ii) is supported by the substantial GSH:1,2-dichloro-4-nitrobenzene conjugating activity found in these two peaks (Habig et al., 1974b). The cholic acid-binding experiments therefore showed that ligandin and glutathione S-transferase B, as well as glutathione S-transferase AA and one or more of the transferases in peak 1 can bind cholic acid. Ligandin, glutathione S-transferase B and one or more of the transferases in peak 1 can also bind lithocholic acid.

The identity of the transferase which eluted from CM-Sephadex between peaks 3 and 4(i) is not known. However, since it was only able to catalyse the conjugation of GSH with 1,2-dichloro-4-nitrobenzene it is unlikely to be either transferase E, D, C, B, A or AA as Jakoby

Table 4.1 Proposed identification of the transferase peaks

peak	elution volume (ml) from CM-Sephadex	glutathione S-transferase(s) contained in the peak
1	15-40	D, E
2	43-85	C
3	91-115	"ligandin" (LBa)
4(i)	130-154	B (LBb)
4(ii)	155-180	A
5	197-241	AA

et al. (1976a, b) have shown that these are all able to catalyse the conjugation of GSH with 1-chloro-2,4-dinitrobenzene. This group of workers have reported that during the preparation of these transferases (Table 2.2) an enzyme which was able to catalyse the conjugation of GSH with menaphthyl sulphate was discarded during DEAE-cellulose chromatography (Pabst et al., 1973). This enzyme was designated transferase M (Habig et al., 1974b) and has been described by Gillham (1971, 1973). Although it is not known whether transferase M can conjugate GSH with 1-chloro 2,4-dinitrobenzene, this enzyme may account for the GSH:1,2-dichloro-4-dinitrobenzene conjugating activity observed between peaks 3 and 4(i). These results suggest that although 7 ionically distinct glutathione S-transferases can be separated by ion-exchange chromatography there are a total of at least 8 transferases in rat liver cytosol.

The use of the term ligandin

The name ligandin was originally used to describe three apparently homogeneous protein preparations which were considered to be one protein (Litwack et al., 1971). The subunit composition of the 3 original preparations of ligandin is not known, although initially it was thought that ligandin comprised two identical monomers (Litwack et al., 1971). However, the subsequent use of discontinuous SDS/ polyacrylamide gel electrophoresis has shown different preparations

of ligandin to contain either YaYa (Bass et al., 1977a) or YaYc dimers (Bhargava et al., 1978) or even a mixture of these (Carne et al., 1979). Since Bhargava et al. (1978) essentially used the purification procedure described by Arias et al. (1976) it is surprising that their ligandin comprised a Ya Yc dimer; however the pH of the buffer used in their ion-exchange chromatography steps was reduced (from pH8.8 to pH 8.6) and purification was carried out in the presence of phenylmethylsulphonyl fluoride. The YaYa and YaYc dimers are very similar and this change in the pH of the eluting buffer may have been sufficient to result in purification of a YaYc dimer rather than the YaYa dimer. The use of the term ligandin in the absence of subunit compositions is therefore not definitive. The term ligandin should perhaps be abandoned and the proteins redefined according to their subunit composition as either YaYa protein or YaYc protein. In this thesis, ligandin is defined as YaYa protein (or LBa) since the preparation of ligandin, by the method of Arias et al. (1976), gave this protein. Although the use of the Arias preparation to define ligandin may appear arbitary, the work of Arias and his colleagues has played a central part in the description and identification of ligandin in the literature; Arias was a co-author with Litwack et al. (1971) when the term ligandin was coined and he was also a co-author with Habig et al. (1974b) when ligandin was reported to be identical to glutathione S-transferase B.

Ligandin, a microheterogeneous protein?

It has been proposed by Ketterer and his colleagues that ligandin is a protein which exhibits "microheterogeneity". This suggestion is based on the finding that apparently pure preparations of ligandin can be resolved into two proteins. Ketterer et al. (1976b) reported that these two proteins had identical amino acid compositions and yielded similar peptide "maps". Carne et al. (1979) have subsequently shown that the two proteins comprised YaYa and YaYc dimers. Ketterer and his colleagues therefore refer to ligandin as YaYa, YaYc or a mixture of the two proteins (Carne et al., 1979). However, the majority of the experiments which this group have carried out with ligandin, since they resolved the two proteins, have been performed using the YaYa protein (Carne et al., 1979). It is not clear what Ketterer and his co-workers mean by "microheterogeneity". They may mean that the 2 proteins are interconvertable; presumably the separate chromatography of either pure YaYa or YaYc protein on CM-Sephadex would result in the elution of 2 enzyme-containing peaks with YaYa and YaYc composition. The elution of a homogeneous preparation of either YaYa or YaYc protein from CM-Sephadex as 2 protein-containing peaks could also possibly occur as a purification artifact. However, ligandin and glutathione S-transferase B eluted

as single peaks from CM-Sephadex. These results suggest that ligandin and glutathione S-transferase B are separate proteins and that they can be prepared to a degree of purity such that they elute from CM-Sephadex as single peaks of protein. Further, the subunit composition of these two proteins was unchanged after storage for 5 months at -10^{o}C suggesting that the two proteins are not interchangeable during storage.

Other workers have described ligandin preparations which do not demonstrate this type of "microhetergeneity". In the present study and in that of Bass et al. (1977a) ligandin, prepared by the method of Arias et al. (1976), was shown by discontinuous SDS/ polyacrylamide gel electrophoresis to contain predominantly the Ya monomer; indicating that the dimeric protein comprises a YaYa dimer. Although small amounts of the Yc monomer were detected in both studies there was no support for the suggestion that the presence of the Yc monomer indicates "microheterogeniety" rather than an impurity in an incompletely purified protein preparation. Alternatively, Ketterer and his colleagues may have used the term "microheterogeneity" to describe a putative carbohydrate modification since many glycoproteins exhibit heterogeneity. This inherent variation is due to differences in the carbohydrate content, rather than the amino acid sequence, of these proteins. Therefore,

whilst a particular glycoprotein will have a unique amino acid sequence it may have different carbohydrate chains added to it, after synthesis, giving rise to different "forms" of a single gene product. This variation results because carbohydrate side chains do not have their structure defined by a template in a way comparable with the genetic coding of a polypeptide sequence. Instead, they are formed by the stepwise addition of sugar residues catalysed by specific glycosyl transferases (Marshall, 1974). The specificity of this process is dependent on the sequential availability of activated monosaccharides and individual transferases. For example, α_1-antitrypsin is referred to as a microheterogeneous glycoprotein since it contains varying amounts of sialic acid (Jeppsson et al., 1978; Carrell & Owen, 1979). This variation gives rise to charge differences between different "forms" of α_1-antitrypsin which only become apparent on electrophoresis at a pH near the isoelectric point of the protein. However, since there is no evidence in the literature to suggest that either YaYa or YaYc protein is a glycoprotein, the existence of a carbohydrate-based"microheterogeneity" to explain the recovery of both YaYa and YaYc proteins in certain ligandin preparations is purely speculative.

Use of terms LBa and LBb

In this thesis the two purified lithocholic acid-binding proteins

which eluted in peaks 3 and 4(i) are referred to as LBa and LBb respectively, rather than ligandin and glutathione S-transferase B. Using this nomenclature it appears that ligandin purified by the method of Arias et al. (1976) contains LBa, ligandin purified by the method of Carne et al. (1979) contains both LBa and LBb and glutathione S-transferase B prepared by the method of Habig et al. (1974b, 1976a) contains LBb. These results suggest that "ligandin" prepared by particular groups of workers either contains two proteins (and is therefore only partially purified) or a single protein, in which case the preparations made by different groups may not comprise the same protein.

LBa and LBb; are they genetically distinct?

The relationship between LBa and LBb is not clear. It is unlikely that they arise as a result of non-specific degradation since they can be prepared separately from each other and yield sharply defined monomer bands. Since these 2 proteins are functionally similar they may represent different "forms" of a single protein. The occurrence of multiple molecular "forms" of a protein can arise for a number of reasons; first, different "forms" can arise at synthesis. This usually results from the transcription of at least two structural genes which

code for functionally similar proteins. These proteins therefore are separately translated and do not share a common synthetic pathway. Since these proteins are functionally similar, but genetically distinct, they represent isoenzymes. Second, differences may be introduced after synthesis (post-ribosomal). In this instance a single structural gene is transcribed and the resulting protein species, which is formed after translation, is subsequently modified to produce a mixture of proteins which may include the original precursor protein. As these proteins are not genetically distinct, but share a common synthetic pathway, they do not represent isoenzymes. Since both LBa and LBb are physically similar these two transferases were examined to determine whether they could be coded for by the same gene.

A comparison between amino acid compositions is the simplest way to determine whether two functionally related proteins are isoenzymes or not. This procedure however is unlikely to detect significant differences between proteins which although distinct are evolutionarily closely related. The amino acid compositions of LBa and LBb were therefore determined and the amino acid recoveries from the two proteins (Table 3.3) are very similar. This is in agreement with the findings of Carne et al. (1979) who were unable to find any statistical differences between the amino acid compositions

Amino Acid	Residues/mol		
	Habig et al (glutathione S-transferase β)	Ketterer et al (β-ABP: ligandin)	Kirsch et al (γ protein: ligandin)
Lys	36	40	34
His	6	6	5
Arg	22	24	21
Asx	37	40	36
Thr	11	14	12
Ser	18	18	15
Glx	46	44	42
Pro	20	20	19
Gly	21	20	19
Ala	31	30	28
½ Cys	4	6	4
Val	25	18	16
Met	8	14	15
Ile	18	20	18
Leu	50	50	46
Tyr	13	16	15
Phe	17	18	17
Trp	9	2	7
Total	392	400	369

Table 4.2 The amino acid compositions which have been published for glutathione S-transferase B (Habig et al., 1974b) and for "ligandin" (Ketterer et al., 1975; Kirsch et al., 1975). The total number of amino acids which each group calculated to be present is indicated.

of these two proteins. The amino acid compositions presented in Table 3.4 are in good agreement with the data which Habig et al. (1974b) have reported for glutathione S-transferase B and which Ketterer et al. (1975) and Arias and his workers (Kirsch et al., 1975) have reported for "ligandin" (Table 4.2). The only significant difference between the amino acid compositions calculated for LBa, LBb and the data reported by the other groups of workers was in the recovery of tryptophan. However, since this is a residue which is destroyed during hydrolysis these differences may only represent methodological rather than real differences. The similar amino acid compositions and the fact that both LBa and LBb were induced in phenobarbitone-treated rats suggests that these two transferases may have arisen as the result of a post-synthetic modification of a single gene product.

Multiple protein "forms"; covalent and non-covalent modifications of a single gene product

The multiple molecular "forms" of proteins, which are presumed to have been synthesised from an identical genetic code and therefore arise as the result of a post-synthetic modification, can be divided into two groups. First, the multiple "forms" may possess non-covalent

differences in their structure or secondly, they may possess covalent differences in their structure. The second type of post-synthetic modification would appear to be the more likely explanation for the differences between LBa and LBb since non-covalent modifications, such as the binding of various molecules (e.g. coenzymes, prosthetic groups, fatty acids, hormones, nucleic acids or membrane fragments), the non-specific aggregation of subunits or the formation of "stable" conformational variants,are unlikely to account for the different subunit compositions of the two proteins because the SDS present denatures the protein preventing binding and minimising aggregation. Peptide "mapping" using "Cleveland gels" also revealed structural differences between the two proteins and further supported the hypothesis that if LBa and LBb represented different "forms" of a single gene product then they arose from covalent modifications.

This second category includes a large number of different post-synthetic modifications such as; a) deamidation, b) oxidation of methionine or cysteine, c) reduction of cystine, d) mixed disulphides, e) decarboxylation, f) modification of prosthetic groups, g) cleavage of peptide bonds, h) differences in carbohydrate content, i) phosphorylation or sulphation. Although each of these possibilities was not thoroughly explored certain modifications appeared unlikely to account for the electrophoretic differences which were observed

between LBa and LBb. In particular it is unlikely that deamidation, decarboxylation, phosphorylation or sulphation would account for the difference in size (mol. wt. 3 000) between the Ya and Yc subunits. Nor does it appear likely that the differences between LBa and LBb result from the formation of mixed disulphides or different oxidation states of equivalent methionine or cysteine residues since no differences were observed in the subunit composition when the concentration of 2-mercaptoethanol was varied between 0-5% (v/v) during preparation of the two proteins for SDS/polyacrylamide gel electrophoresis. Further, the amino acid analyses presented in Table 3.3 did not suggest the presence of any carbohydrate groups (i.e. glucosamine or galactosamine) and so it was thought unlikely that a carbohydrate-modification could account for the difference between the two proteins. Several groups have determined the amino acid composition of their "ligandin" preparations (LBa and/or LBb) (Table 4.2) and no data has been published suggesting the presence of either a prosthetic group, sialic acid residues or other carbohydrate groups which may account for the difference between LBa and LBb. It was therefore thought, on the basis of the limited amount of data available, that the most likely explanation for the differences between the two binding proteins is that LBb contains an additional sequence of amino acids (approx. 26 residues) which is not found in LBa. Two and three dimensional peptide "maps" were therefore constructed using

high-voltage paper electrophoresis. These showed that the tryptic digests of LBa produced 31 different peptides suggesting that the YaYa dimer yields 62 peptides and contains 60 tryptic cleavage sites. LBb produced an additional 7 peptides suggesting that the YaYc dimer yields 69 peptides and contains 67 tryptic cleavage sites. Although none of the peptides were sequenced, both proteins produced peptides which possessed similar mobilities during electrophoresis and chromatography, suggesting that the peptides obtained from LBa are also present in LBb. The similar peptide "maps" obtained from LBa and LBb suggests that the two proteins possess a large amount of sequence homology and that the Ya monomeric band of the two proteins is identical. If the difference between the two proteins resides in a hypothetical carbohydrate group the same number of peptides should have been obtained from LBa and LBb. The data therefore, supports the hypothesis that the difference between LBa and LBb is due to the Yc subunit in LBb containing an additional amino acid sequence which contains 7 tryptic cleavage sites. However, the Ya and Yc monomers have not been dissociated and separately analysed and therefore the existence of the putative additional amino acid sequence in the Yc subunit has not been exhaustively investigated. It is not known whether this putative extra amino acid sequence contained in Yc is located at the "N" or "C" terminus or is situated elsewhere in the monomer, as an inserted sequence. Further, in the

absence of extensive sequence data the possibility that Ya and Yc are coded for by two closely related structural genes (which have diverged from a common ancestral gene) cannot be excluded but the phenobarbitone induction and the two and three dimensional peptide "maps" are consistent with the hypothesis that these two subunits, which are found in LBa and LBb, are the product of a single gene.

A product-precursor relationship may exist between LBa and LBb similar to that described for penicillin amido-β-lactamyhydrolase. A comparison between the DNA sequence of the gene coding for this protein and the amino acid sequence of the active enzyme demonstrated that pencillin amido-β-lactamhydrolase is synthesised as a 286 amino acid precursor (mol. wt. 28 900) from which 23N terminal amino acids are removed to produce the mature enzyme (mol. wt. approx. 27 000) (Ambler & Scott, 1978; Sutcliffe, 1978). An analagous situation may exist between the Ya and Yc monomers of the two lithocholic acid-binding proteins. These two proteins may be coded for by a single gene and synthesised as a YcYc precursor (mol. wt. 50 000) which is subsequently converted into either YaYc (mol. wt. 47 000) or YaYa (mol. wt. 44 000) protein by the successive cleavage and removal of approx. 26 terminal amino acids. The subunit compositions indicate that peak 5 (transferase AA) comprises a YcYc dimer. This may be the putative precursor protein.

Are several glutathione S-transferases the product of a single gene?

Jakoby et al. (1978) reported that in the rat liver the glutathione S-transferases have similar physical properties and overlapping substrate specificities. Jakoby and his colleagues have purified 5 of these enzymes (transferases E, C, B, A and AA) and shown that the amino acid compositions of transferases C and A and B and AA are similar (Table 4.3) (Habig et al., 1974b, 1976a). However, significant differences exist between the alanine and valine content of these 2 groups of transferases (Habig et al., 1974b, 1976a). The compositions suggest that transferases B and AA may be the product of a single gene and transferases C and A may also be coded for by a single but separate gene. Habig et al. (1974b) have reported that both transferases C and A cross react with antisera raised against each other, supporting the hypothesis that these 2 enzymes are the product of a single gene. It is not clear which, if either, of these 2 transferases (A and C) represents the precursor, but the subunit compositions, which indicates that both these enzymes comprise YbYb dimers, suggests that the postulated post-synthetic modification involves minor structural changes such as deamidation, decarboxylation , oxidation of methionine or cysteine or reduction of cystine. Although the possibility of a non-covalent modification of transferase A and/or C cannot be excluded from this list of possible post-synthetic modifications it appears

Moles of amino acid/45000g transferase				
Transferase	AA	A	B	C
Amino Acid				
Lys	34	34	36	35
His	7	6	6	6
Arg	26	21	22	21
Asx	41	45	37	44
Thr	8	13	11	12
Ser	12	20	18	17
Glx	47	42	46	41
Pro	25	22	20	22
Gly	23	19	21	21
Ala	33	20	31	20
$\frac{1}{2}$Cys	2	6	4	6
Val	31	11	25	12
Met	10	10	8	10
Ile	14	22	18	19
Leu	49	45	50	45
Tyr	19	23	13	22
Phe	15	20	17	22
Trp	2	6	9	6
	Total 398	385	392	381

Table 4.3 The amino acid compositions which have been published for glutathione S-transferase C, B, A and AA (Habig et al., 1974b, 1976a). The total number of amino acids calculated for each transferase is indicated.

unlikely, since the enzymes were thoroughly dialysed before chromatography. The amino acid composition of transferase E has not been determined but since it is immunologically distinct from transferases A, B and C (Habig et al., 1974b) it may be coded for separately from these enzymes. The work of Jakoby and his colleagues suggests that in the rat there are at least 3 genes which code for the glutathione S-transferases but that their products have similar amino acid compositions. The subunit compositions indicate that these genes code for proteins of 25 000, 23 500 or 22 000 mol. wt. Certain of the Ya, Yb, Yc bands are likely to contain more than one gene product since together transferases A, B and C possess all three monomer bands but transferase E, which the immunological study of Habig et al.(1974b) indicated was coded separately, probably comprises Yb and/or Yc monomers.

In the human liver 5 ionically distinct glutathione S-transferases have been isolated (Habig et al., 1976b). These proteins had different amino acid compositions and were immuno-chemically distinct from the rat liver transferases. Since these enzymes had apparently identical amino acid compositions and were immunologically indistinguishable from each other, it was proposed that these are coded for by a single gene. However, since the subunit compositions have not been determined

it is not known whether the postulated post-synthetic modification is similar to the LBa:LBb or the transferase A:transferase C situation in the rat liver.

Identity of the 17 000 mol. wt. bile acid-binding protein

The identity of the low mol. wt. (approx. 17 000) cholic acid-binding component (Fig. 3.5a(a)) is not clear and the results do not indicate whether this binding species is identical to the low mol. wt. lithocholic acid-binding component (Fig. 3.6). However, it appears likely that the low mol. wt. cholic acid-binding component is identical to the aminoazode-binding protein A (mol. wt. 14 000) which Ketterer et al. (1976a) have shown also binds cholic acid. Further, since Ketterer et al. (1976a) showed that this protein bound a large number of steroids (including deoxycholic acid and taurodeoxycholic acid), it is likely that this protein also binds lithocholic acid. Several other groups of workers have also studied low mol. wt. binding proteins; for example, Z protein (Levi et al., 1969a; Mishkin et al., 1972; Mishkin & Torcotte, 1974a, b; Kamisaka et al., 1975; Warner & Neims, 1975), fatty acid-binding protein (Ockner et al., 1972) and squalene and sterol carrier protein (Grabowski et al., 1976). Since the various preparations all bind a number of common ligands it has been suggested that a general non-specific low mol. wt. binding

protein may exist which has binding properties similar to "ligandin" (LBa) (Ketterer et al., 1976a). These preparations may therefore all be able to bind cholic acid and lithocholic acid.

Physiological significance of bile acid-binding by cytosolic proteins

The physiological importance of bile acid-binding by the glutathione S-transferases is not clear. The various physiological functions which have been ascribed to the glutathione S-transferases have been discussed. These functions can be divided into three groups based on the three types of activity which these proteins possess. First, they have enzyme activity and catalyse the conjugation of GSH to various electrophilic compounds. Secondly, they can bind non-covalently a number of hydrophobic non-substrate ligands. Thirdly, it has been shown that certain transferases can bind covalently an aminoazo-dye metabolite and 1-chloro-2,4-dinitrobenzene. In the most recent review article on the glutathione S-transferases Jakoby (1978) considered the physiological importance of the non-covalent binding activity of the transferases. He proposed that on a quantitative basis the major ligand for the transferases was bilirubin and therefore, in this context, one of the major functions of the transferases was to keep bilirubin in solution inside the hepatocyte in the same way that albumin solubilises bilirubin in the plasma. Further Jakoby (1978)

suggested that since at least transferase E has been identified in association with hepatic plasma membranes, the transferases could participate at the sinusoidal surface of the hepatocyte, in mediating the influx of anions into the cell and that they may be responsible for a facilitated diffusion process.

Recently, Arias and his co-workers investigated the physiological role of "ligandin" (LBa) in bilirubin transport by the liver (Wolkoff et al., 1979). They studied the kinetics of bilirubin uptake in isolated perfused rat livers from normal, phenobarbitone and thyroidectomized animals; the concentration of "ligandin" was increased 25% and 100% in the thyroidectomized and phenobarbitone treated rats respectively. These experiments showed that the net uptake of bilirubin by the livers of animals which possessed increased levels of ligandin was significantly greater than the uptake in control animals. Their work supports the hypothesis that "ligandin" is involved in the uptake of bilirubin but offers no data about the role of the other transferases. More specifically, Wolkoff et al. (1979) constructed a compartmental model for the hepatic transport of bilirubin and they proposed that the physiological role of "ligandin" is to control the efflux of bilirubin from the hepatocyte back to the plasma rather than to control the influx of bilirubin from plasma to the hepatocyte.

The hepatic transport of bilirubin has received much attention since failure to excrete bilirubin results in jaundice. However, the size of the bile acid pool is at least 10 times that of bilirubin and as bile acids must traverse the liver at least 5-10 times per day the liver transports 50-100 times more bile acid than bilirubin per day. The quantitatively most important function of the glutathione S-transferases therefore may be to assist bile acid transport. It is not clear what role these enzymes play in bile acid transport. Strange et al. (1979b) have calculated that the bile acids cross the hepatocyte in free solution; they are probably not transported across the liver bound to carrier proteins since the bile acid transit time across the liver appears to be too rapid for the bile acids to diffuse across the hepatocyte bound to protein. The transferases may be involved in the hepatic transport of bile acids in a non-carrier manner. Certain transferases may influence the uptake of bile acids by the liver either by controlling influx, as proposed by Jakoby (1978) for transferase E, or by controlling efflux, as proposed by Wolkoff et al. (1979) for "ligandin". These enzymes may be either an integral part of the bile acid-binding sites in liver surface membranes which were described by Accatino & Simon (1976) or may directly interact with those sites. Alternatively the bile acid-binding activity of the transferases may indirectly assist bile acid transport. Strange et al. (1979b) showed that bile acids partition into subcellular

organelles and it is possible that the transferases keep bile acids in the cytosol and so restrict their partitioning into membrane lipid, thereby promoting their rapid excretion into bile. This cytosolic binding (storage) capacity may be important in dealing with the transiently high concentrations of bile acids which are transported as a bolus across the liver following reabsorption from the gut after a meal. In this case the transferases would keep the bile acids in solution thereby helping to prevent precipitation of the less soluble bile acids (the mono- and di-hydroxy bile acids) and by restricting the partitioning of the bile acids into the subcellular organelles prevent possible disruption of membranes through the detergent activity of the bile acids.

The 17 000 mol.wt. bile acid-binding component(s) may have similar physiological functions in the hepatocyte as those which have been proposed for the bile acid-binding glutathione S-transferases. However, if the bile acid-binding component(s) is identical to the squalene and sterol carrier protein, which binds several bile acid precursors including 7 α-hydroxy-4-cholesten-3-one, then the possibility exists that cholic acid and lithocholic acid could competitively inhibit the binding of bile acid precursors to the carrier protein. As this protein is postulated to carry the intermediates in the conversion of cholesterol to the bile acids to their relevant enzymes in the synthetic pathway, it is evident that through

competitive inhibition between the bile acids and their precursors, bile acids could modify the rate of their own synthesis. Whilst such a mechanism would not be of major importance in the regulation of bile acid synthesis, since the cholesterol 7 α-hydroxylase step is the rate limiting step in the synthetic pathway, this possiblity merits investigation.

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PUBLICATIONS

The following papers were published as a result of the work carried out for this thesis:-

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Partial Purification of Two Lithocholic Acid-Binding Proteins from Rat Liver 100000*g* Supernatants

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1. The partial purification of two lithocholic acid-binding proteins from liver 100000*g* supernatants is described. 2. Gel-filtration, $(NH_4)_2SO_4$ fractionation, $Ca_3(PO_4)_2$ fractionation and ion-exchange chromatography were used. 3. Both proteins exhibited glutathione *S*-transferase activity; one may be the non-specific anion-binding protein ligandin. 4. Glutathione *S*-transferase activity of one of the binding proteins was inhibited by lithocholic acid.

The removal of anions from plasma and their excretion into bile is an important function of the liver. Ligandin, a non-specific anion-binding protein present in hepatic 100000*g* supernatants, may be an important component of the uptake of many different anions from plasma (Levi *et al.*, 1969; Litwack *et al.*, 1971). Kaplowitz *et al.* (1973) showed that ligandin has glutathione *S*-transferase activity (EC 2.5.1.18), and Habig *et al.* (1974*a*,*b*) showed that purified ligandin had the same amino acid composition and enzymic activities as glutathione *S*-transferase B.

Bile acid transport is one aspect of liver function which, although quantitatively important, is poorly understood. In the rat (total bile acid pool about 40 μmol) approx. 400 μmol of bile acid is removed from the portal vein and is transported across the liver each day. The uptake of sodium taurocholate from the extracellular fluid into the liver follows Michaelis–Menten kinetics both in the intact dog liver (Glasinovic *et al.*, 1975) and in isolated rat hepatocytes (Schwarz *et al.*, 1975). This suggests that bile acid transport is a carrier-mediated process. Proteins having binding sites for cholic acid, glycocholic acid, chenodeoxycholic acid and lithocholic acid were demonstrated in 100000*g* supernatants from rat livers by an equilibrium-dialysis technique (Strange *et al.*, 1976, 1977). The large values for both the dissociation constants of binding of these bile acids (approx. 1 μM) and the concentration of binding sites (approx. 1 μmol/g of supernatant protein) suggested that these binding proteins might be acting as carrier proteins in the transport of bile acids across the hepatocyte.

We now describe (1) the partial purification of two lithocholic acid-binding components, (2) their examination for glutathione *S*-transferase activity and (3) the effect of bile acids on the glutathione *S*-transferase activity of the two lithocholic acid-binding components.

Materials and Methods

Chemicals

Lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid), chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid), deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid), cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) and the glycine conjugate of cholic acid were purchased from Maybridge Chemical Co., Tintagel, Cornwall, U.K., and were purified as described by Strange *et al.* (1977). [24-^{14}C]Lithocholic acid (59 Ci/mol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and shown to be 99% pure by t.l.c. Calcium phosphate gel was obtained from BDH Chemicals, Poole, Dorset, U.K. Sephadex G-75, DEAE-Sephadex A-50 and CM-Sephadex C-50 were from Pharmacia Fine Chemicals Ltd., London W5 5SS, U.K. Ethacrynic acid [2,3-dichloro-4-(2-methylenebutyryl)phenoxyacetic acid] was generously given by Merck, Sharp and Dohme, Hoddesdon, Herts., U.K.

Analytical methods

Enzyme assays were performed as described by Habig *et al.* (1974*b*) except that a temperature of 37°C was used. The conjugation of glutathione with 1,2-dichloro-4-nitrobenzene and 1-chloro-2,4-dinitrobenzene was studied at 340nm at pH7.5 and 6.5 respectively. Conjugation with ethacrynic acid was studied at 270nm at pH7.5. Reaction rates were corrected for small rates (less than 5% of total rate) of non-enzyme-catalysed conjugation of glutathione with 1-chloro-2,4-dinitrobenzene and ethacrynic acid.

To identify the lithocholic acid-binding components radioactive lithocholic acid (16nmol; 1 μCi) was added to portions (2ml) of the partially purified

preparations described below. The mixtures were eluted from a column (30cm×2cm; void volume 50ml) of Sephadex G-75 at 4°C by using sodium/potassium phosphate buffer (20mM; pH7.4) containing NaCl (100mM) (Strange *et al.*, 1976, 1977). This buffer is referred to as phosphate/NaCl buffer. The flow rate was 22ml/h and the fraction volume was 3.5ml. Radioactive lithocholic acid was eluted with proteins of mol.wt. approx. 40000 and the [^{14}C]lithocholic acid-binding activity was expressed as the ratio between the peak radioactivity (d.p.s./ml) and the protein concentration (mg/ml) in the fraction.

All buffers were prepared at 20°C. Radioactive counting and protein assays were performed as described by Strange *et al.* (1976). Agarose-gel electrophoresis was performed in sodium barbiturate/barbituric acid buffer (50mM; pH8.6) on Universal agarose plates obtained from Corning-Eel (Evans Electroselenium Ltd., Halstead, Essex, U.K.). Polyacrylamide-gel electrophoresis was performed at room temperature (20°C) on discs of 10% gel prepared from acrylamide and *NN'*-methylenebisacrylamide (30:1, w/w) with *NNN'N'*-tetramethylethylenediamine as catalyst. Na^+ concentrations in column fractions were measured by flame photometry with an IL 343 photometer [Instrumentation Laboratory (U.K.) Ltd., Altrincham, Cheshire, U.K.].

Purification of lithocholic acid-binding components

Solution 1. Male Wistar rats (300–350g) were anaesthetized with diethyl ether and their livers were perfused *in situ* with the phosphate/NaCl buffer to which sucrose was added to a final concentration of 250mM. The livers were homogenized and 100000*g* supernatants were prepared as described by Strange *et al.* (1976). Portions (40ml; approx. 40mg of protein/ml) of supernatant were transferred to a column (5.5cm×24cm) of Sephadex G-75 which was equilibrated and eluted with the phosphate/NaCl buffer at 4°C. The flow rate was 60ml/h, the fraction volume was 10ml and the void volume was 160ml. Fractions eluted between 250 and 350ml contained proteins of mol.wt. 30000–60000. They were collected and combined (solution 1).

Solution 2. Solid $(NH_4)_2SO_4$ was added at room temperature with stirring to solution 1 until 55% saturation was obtained (Dixon & Webb, 1964). The solution was left without stirring for 30min then centrifuged (2500*g*, 15min, 20°C) and the precipitate discarded. More $(NH_4)_2SO_4$ was added to the supernatant until 85% saturation was reached, and the solution was left for 30min then centrifuged (50000*g*, 25min, 10°C) and the supernatant discarded. The precipitate was redissolved in sodium/potassium phosphate buffer (20mM; pH7.4; 10ml) and the solution was dialysed at 4°C for 15h against four changes (each of 2 litres) of the same buffer, giving solution 2.

Solution 3. Calcium phosphate gel was washed with sodium/potassium phosphate buffer (20mM; pH7.4) and added to solution 2 (1mg of gel/mg of protein) at room temperature. The solution was stirred for 1h, centrifuged (2500*g*, 15min, 20°C) and the precipitate discarded. The supernatant was dialysed against four changes (each of 2 litres) of Tris/HCl buffer (20mM; pH8.1) at 4°C for 15h, giving solution 3.

Solution 4. Solution 3 was added to a column (0.9cm×15cm) of DEAE-Sephadex A-50 equilibrated and eluted at 4°C with Tris/HCl buffer (20mM; pH8.1). The flow rate was 6.5ml/h and the fraction volume was 1.5ml. A NaCl gradient was established after 30ml. To obtain the gradient a solution of NaCl (500mM) in Tris/HCl buffer (20mM; pH8.1) was used. This solution was added (6.5ml/h), with mixing, to 40ml of Tris/HCl buffer (20mM; pH8.1) contained in the eluent reservoir. Eluent was pumped (6.5ml/h) from this reservoir to the DEAE-Sephadex column. Fractions eluted between 3.0 and 18.0ml were combined and dialysed against four changes (each of 2 litres) of sodium/potassium phosphate buffer (10mM; pH7.4) at 4°C for 20h giving solution 4. Fractions eluted between 37.5 and 45.0ml were also combined and dialysed against sodium/potassium phosphate buffer (10mM; pH7.4).

Solution 4 was mixed with 50μl of [^{14}C]lithocholic acid (16nmol; 1μCi) and was added to a column (0.9cm×15cm) of CM-Sephadex C-50, equilibrated and eluted with sodium/potassium phosphate buffer (10mM; pH7.4). After 27ml had been collected a NaCl gradient was established. NaCl was added to phosphate buffer (10mM; pH7.4) to give a final concentration of 50mM. This solution was added (6.5ml/h), with mixing, to 50ml of phosphate buffer (10mM; pH7.4) in the eluent reservoir. Eluent was pumped (6.5ml/h) from the reservoir to the CM-Sephadex column. The fraction volume was 1.5ml. The fractions eluted between 9.0 and 30.0ml, 31.5 and 39ml (peak 1) and 45.0 and 58.5ml (peak 2) were separately combined and the three solutions were examined for the presence of lithocholic acid-binding components.

Peak 1 and peak 2 were concentrated by dialysis at 1°C against poly(ethylene glycol) (mol.wt. 25000) and were run in agarose-gel and polyacrylamide-gel electrophoresis systems.

Bile acid inhibition of glutathione S-transferase activity

The effects of cholic acid, glycocholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid on glutathione *S*-transferase activity in peak 1 and peak 2 from the CM-Sephadex column were studied by using reduced glutathione and 1-chloro-2,4-dinitrobenzene as substrates. Portions (5μl; 1.5μg of protein) of peak 1 and peak 2 were added to

reaction mixtures containing sodium/potassium phosphate buffer (1.3ml; 100mM; pH7.45), the bile acid (0–300μM) under study and 1-chloro-2,4-dinitrobenzene (1mM). Since lithocholic acid is only slightly soluble in aqueous solution concentrations up to 40μM were used. The reaction was initiated by adding either 0.5 or 1.0mM-glutathione. Since binding of lithocholic acid to supernatant proteins had been studied only at pH7.4 the effects of bile acids on glutathione *S*-transferase activity were studied at this pH. Initial experiments confirmed that the reaction rate was not altered by changing the pH of the reaction mixture from 6.5 to 7.45. The non-enzymic rate, however, was increased to 20% of the total reaction rate in the absence of lithocholic acid. It was subtracted from the total measured reaction rate in all cases. Assays were performed in quadruplicate.

Results

Purification of lithocholic acid-binding components

The protein contents, [^{14}C]lithocholic acid-binding activities and specific enzyme activities for 100000*g* supernatant from rat liver and for each of the partially purified preparations from it are shown in Table 1.

At each stage of the purification procedure the discarded solutions were also tested for [^{14}C]lithocholic acid-binding activity and glutathione *S*-transferase activity. Except for low [^{14}C]lithocholic acid-binding activity (less than 350d.p.s./mg of protein) and glutathione *S*-transferase activity (less than 10 A_{340} units change/min per mg of protein) found in both the redissolved precipitate formed by adding $(NH_4)_2SO_4$ to 55% saturation to solution 1 and in a sodium/potassium phosphate buffer (500mM; pH7.4) wash of the discarded calcium phosphate gel, none was found.

When solution 3 was added to a DEAE-Sephadex A-50 column, a broad peak of protein was eluted at 3.0–18.0ml which was partly resolved into two peaks of glutathione *S*-transferase activity (Fig. 1). These fractions were combined and shown to contain lithocholic acid-binding activity. A second group of protein-containing fractions was eluted with the NaCl gradient at 36.0–60.0ml. Some of these showed glutathione *S*-transferase activity, but combined fractions 37.5–45.0ml did not contain [^{14}C]-lithocholic acid-binding activity.

Solution 4 was applied to a CM-Sephadex C-50 column (Fig. 2). Some protein was eluted between 9.0 and 30.0ml before the start of the NaCl gradient. These fractions contained little glutathione *S*-transferase activity or [^{14}C]lithocholic acid-binding activity. Two peaks of protein (peak 1 and peak 2) were eluted with the NaCl gradient; both contained [^{14}C]lithocholic acid-binding activity and glutathione *S*-transferase activity with 1-chloro-2,4-dinitrobenzene and ethacrynic acid as substrates, but were unable to catalyse the conjugation of glutathione with 1,2-dichloro-4-nitrobenzene. A similar elution pattern was obtained when solution 4 was added without lithocholic acid to the CM-Sephadex column.

On agarose-gel and polyacrylamide-gel electrophoresis, samples of both peak 1 and peak 2 showed single bands of protein.

Bile acid inhibition of glutathione S-transferase activity

Cholic acid, glycocholic acid, chenodeoxycholic acid and deoxycholic acid had no effect on glutathione *S*-transferase activity in either peak 1 or peak 2 from the CM-Sephadex column. Lithocholic acid had no effect on enzyme activity in peak 2, but did inhibit activity in peak 1. Straight lines were obtained by plotting the reciprocal of the maximum velocity

Table 1. *Purification of the lithocholic acid-binding proteins*

Supernatant fractions (100000*g*) were prepared from liver homogenates and two lithocholic acid-binding proteins were isolated by using $(NH_4)_2SO_4$ and $Ca_3(PO_4)_2$-gel fractionation and ion-exchange chromatography (see the Materials and Methods section). At each stage of the purification procedure the preparations were examined for lithocholic acid-binding activity and glutathione *S*-transferase activity.

	Protein (mg)	Lithocholic acid binding (d.p.s./mg)	Conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (change in A_{340}/min per mg of protein)
100000*g* supernatant	1600	1200	27
Sephadex G-75	500	2000	50
$(NH_4)_2SO_4$ fractionation	120	4000	70
Calcium phosphate gel	50	6000	100
DEAE-Sephadex	10	10000	200
CM-Sephadex			
Peak 1	5.5	13000	250
Peak 2	3.5	8000	230

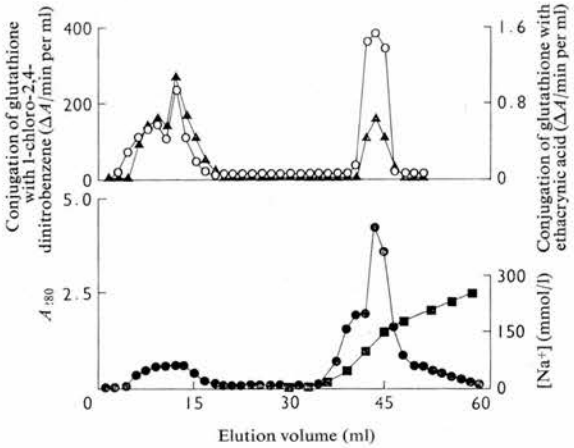

Fig. 1. *Elution pattern of solution* 3 *of the partially purified lithocholic acid-binding proteins from DEAE-Sephadex*
Solution 3 of the partially purified lithocholic acid-binding proteins, isolated from rat liver 100000*g* supernatants by gel-exclusion chromatography, $(NH_4)_2SO_4$ and $Ca_3(PO_4)_2$ gel fractionation (see the Materials and Methods section) was eluted from a DEAE-Sephadex A-50 column with Tris/HCl buffer (20mM; pH8.1). Fractions (1.5ml) were collected and the A_{280} (●), Na^+ concentration (■) and glutathione *S*-transferase activity with ethacrynic acid (▲) and 1-chloro-2,4-dinitrobenzene (○) as substrates were measured.

against the concentration of lithocholic acid. At glutathione concentrations of 0.5 and 1.0mM the intercepts (±S.E., $n = 5$) were 18.5 ± 3.5 μM and 20.4 ± 3.8 μM respectively. The confidence interval for the difference between the two estimates of the intercept is −1.03 to +1.41. Since this interval includes 0, the intercepts are not significantly different, suggesting that the inhibition is non-competitive

Discussion

The experiments described show the presence of at least two proteins in hepatic 100000*g* supernatants able to bind lithocholic acid. Although exhaustive criteria of protein purity were not applied, agarose- and polyacrylamide-gel electrophoresis of the two protein-containing solutions obtained from the CM-Sephadex column demonstrated the presence of only a single protein band in each solution. Both these lithocholic acid-binding components had glutathione *S*-transferase activity. The system used to detect lithocholic acid binding is a non-equilibrium one, and was used in preference to an equilibrium system (such as equilibrium dialysis), since it was known to be less likely to detect non-specific binding of lithocholic acid (Strange *et al.*, 1977).

Ligandin is one of a group of several enzymes that have glutathione *S*-transferase activity (Habig *et al.*, 1974*b*). These workers have determined the relative

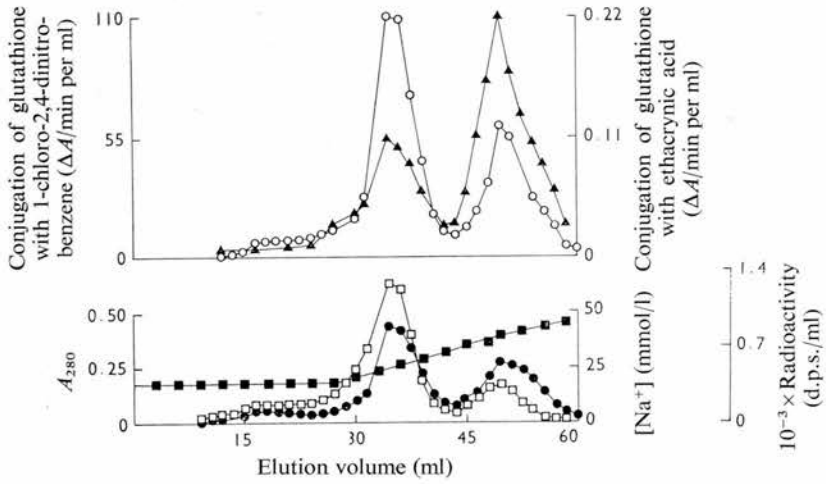

Fig. 2. *Elution pattern of solution* 4 *of the partially purified lithocholic acid-binding proteins from CM-Sephadex*
Fractions eluted between 3.0 and 18.0ml from the DEAE-Sephadex column were collected, combined and after dialysis against sodium/potassium phosphate buffer (10mM; pH7.4) were mixed with [^{14}C]lithocholic acid (16nmol; 1 μCi) and eluted from a column of CM-Sephadex C-50. Fractions (1.5ml) were collected and the A_{280} (●), [^{14}C]-lithocholic acid (□), Na^+ concentration (■) and glutathione *S*-transferase activity with ethacrynic acid (▲) and 1-chloro-2,4-dinitrobenzene (○) as substrates were measured.

specific activities of purified glutathione *S*-transferases A, B, C and E for various substrates, but, since other glutathione *S*-transferases are known to exist although not yet purified, identification of the protein solutions from the CM-Sephadex column is difficult. Habig *et al.* (1974*b*) showed that the ratio of glutathione–1-chloro-2,4-dinitrobenzene-conjugating activity to glutathione–ethacrynic acid-conjugating activity of ligandin was 45:1. Peak 1 and peak 2 from the CM-Sephadex column have ratios of 1000:1 and 250:1 respectively. Since peak 2 exhibited the highest activity for conjugating ethacrynic acid and glutathione, but had virtually no activity for conjugating glutathione and 1,2-dichloro-4-nitrobenzene, it is suggested that it is ligandin. The difference between the ratios obtained in these experiments and those of Habig *et al.* (1974*a*,*b*) may be a result of the use of lithocholic acid binding as a primary marker during the purification procedure; no attempt was made to ensure retention of enzyme activity. Glutathione *S*-transferase activity of the two lithocholic acid-binding proteins was not affected by cholic acid, glycocholic acid, chenodeoxycholic acid or deoxycholic acid in concentrations up to 300 μM. Okishio & Nair (1966) have shown that the concentration of cholic acid, the predominant bile acid in rat liver 100000*g* supernatants, is approx. 100 μM. Although lithocholic acid in concentrations up to 60 μM had no effect on enzyme activity in the protein provisionally identified as ligandin (peak 2), inhibition of glutathione *S*-transferase activity of peak 1 did occur. Since the concentration of lithocholic acid in the liver is unknown the relevance of this finding is not clear.

Both of the lithocholic acid-binding proteins described here have glutathione *S*-transferase activity and one of these may be the non-specific anion-binding protein ligandin. Although purified ligandin can bind cholic acid and its taurine and glycine conjugates (Tipping *et al.*, 1976) it is not known whether hepatic transport of bile acids is mediated by a mechanism similar to that of the transport of bromosulphthalein and bilirubin. O'Maille *et al.* (1966) showed two effects when they studied the excretion of sodium taurocholate and bromosulphthalein into bile. Although infusions of sodium taurocholate to anaesthetized dogs increased the maximum rate of bromosulphthalein excretion into bile the maximum excretion rate of sodium taurocholate was decreased by bromosulphthalein infusion. They concluded that the increased excretion of bromosulphthalein was secondary to the increased bile flow stimulated by infusion of sodium taurocholate, and further that the inhibition of sodium taurocholate excretion suggested a common pathway for the hepatic transport of bile acids and bromosulphthalein. Since lithocholic acid binding to proteins in 100000*g* supernatants is inhibited by other bile acids (Strange *et al.*, 1977), ligandin (or other glutathione *S*-transferase enzymes) may act as a non-specific bile acid-binding protein involved in the transport of bile acids as well as other anions.

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Identification of Two Lithocholic Acid-Binding Proteins

SEPARATION OF LIGANDIN FROM GLUTATHIONE *S*-TRANSFERASE B

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1. Two lithocholic acid-binding proteins in rat liver cytosol, previously shown to have glutathione *S*-transferase activity, were resolved by CM-Sephadex chromatography. 2. Phenobarbitone administration resulted in induction of both binding proteins. 3. The two proteins had distinct subunit compositions indicating that they are dimers with mol.wts. 44000 and 47000. 4. The two lithocholic acid-binding proteins were identified by comparing their elution volumes from CM-Sephadex with those of purified ligandin and glutathione *S*-transferase B prepared by published procedures. Ligandin and glutathione *S*-transferase B were eluted separately, as single peaks of enzyme activity, at volumes equivalent to the two lithocholic acid-binding proteins. 5. Peptide 'mapping' revealed structural differences between the two proteins.

Intravenous injection of unconjugated bilirubin into rats results in rapid hepatic clearance of the anion from the blood, its passage across the hepatocyte and its excretion into bile. Subcellular fractionation showed that most of the bilirubin in these livers was in cytosol (Brown *et al.*, 1964). Further, gel-exclusion chromatography of cytosol from similarly treated animals or of hepatic cytosol mixed with bilirubin *in vitro* resulted in three peaks of protein-bound bilirubin (Levi *et al.*, 1969*b*). These peaks were called X (eluted in the void volume), Y (mol.wt. approx. 45000) and Z (mol.wt. approx. 12000). The binding component in the Y fraction was shown to be a protein and was called ligandin because of its ability to bind, non-covalently, a variety of organic anions (Litwack *et al.*, 1971). Circumstantial evidence obtained from phylogenetic studies and investigations into neonatal jaundice led to the hypothesis that ligandin is involved in the hepatic transport of many different anions (Levine *et al.*, 1971; Levi *et al.*, 1969*a*, 1970).

Kaplowitz *et al.* (1973) showed that ligandin from rat liver possessed glutathione *S*-transferase activity, and Habig *et al.* (1974*a*) reported that ligandin was identical with glutathione *S*-transferase B, one of a group of at least seven enzymes that catalyse the conjugation of glutathione to a number of electrophilic compounds (Jakoby *et al.*, 1976*a*). Ligandin can therefore be defined by its enzymic activity, the ability to conjugate glutathione with different substrates, including 1-chloro-2,4-dinitrobenzene and ethacrynic acid, and by its ability to bind non-substrate anions.

Preparation of purified ligandin has been described by several groups using different purification techniques (Morey & Litwack, 1969; Habig *et al.*, 1974*b*; Kamisaka *et al.*, 1975; Tipping *et al.*, 1976). Present evidence shows ligandin to be a basic protein (pI 8.7–9.0), which comprises 4.0–4.5% of the total protein in rat liver cytosol; it has a mol.wt. of 46000 and consists of two subunits, Ya (mol.wt. 22000) and Yc (mol.wt. 25000). However, there is now doubt about whether the different purified preparations of ligandin contain only one protein or indeed if they comprise the same protein. These doubts arise partly because of the wide range of pI values (8.4–9.8) obtained by different groups (Litwack *et al.*, 1971; Jakoby *et al.*, 1976*b*) and also because these preparations exhibit properties compatible either with their containing contaminating glutathione *S*-transferases or that preparations of ligandin, originally pure, are modified during storage to a mixture of ligandin and other proteins (Habig *et al.*, 1974*b*; Ketterer *et al.*, 1976; Listowsky *et al.*, 1976). For example Carne *et al.* (1979) have demonstrated that ligandin can be further resolved by ion-exchange chromatography into dimeric proteins comprising YaYa and YaYc monomers.

We have isolated two lithocholic acid-binding proteins from the Y fraction of rat liver cytosol (Strange *et al.*, 1977). Although neither protein has been identified, both had glutathione *S*-transferase activity and substrate specificities compatible with their being ligandin.

We now describe experiments to identify these

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

two proteins. Their monomeric composition, peptide 'maps' and the effect of phenobarbitone on their concentrations have been investigated: phenobarbitone has been reported to increase the synthesis of ligandin (Reyes *et al.*, 1971; Fleischner *et al.*, 1972; Arias *et al.*, 1976). Further, glutathione *S*-transferase B was prepared by the procedure of Habig *et al.* (1976*a*) and ligandin by that described by Arias *et al.* (1976), and these two purified proteins have been compared with the bile acid-binding proteins.

Materials and Methods

Chemicals

[24-^{14}C]Lithocholic acid (59 Ci/mol) was from The Radiochemical Centre, Amersham, Bucks., U.K., and was shown to be 99% pure by t.l.c. (Hamilton & Muldrey, 1961) before use. Acrylamide, *NN'*-methylenebisacrylamide, *NNN'N'*-tetramethylethylenediamine and 1-chloro-2,4-dinitrobenzene of analytical grade were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and 1,2-dichloro-4-nitrobenzene was from Eastman Kodak Co., Rochester, NY, U.S.A., and was twice recrystallized from ethanol before use. GSH, rat albumin, ovalbumin, α-chymotrypsinogen, α-chymotrypsin, ribonuclease A, TEAE (triethylaminoethyl)-cellulose and Coomassie Brilliant Blue G were from Sigma, Poole, Dorset, U.K. CM-Sephadex C-50 and QAE (quaternary aminoethyl)-Sephadex A-50 were from Pharmacia Fine Chemicals, London W5 5SS, U.K. CM-cellulose (Whatman CM52) and DEAE-cellulose (Whatman DE52) were purchased from Whatman, Maidstone, Kent, U.K., and Bio-Gel A-0.5m (200–400 mesh) was from Bio-Rad Laboratories, Bromley, Kent, U.K. Poly(ethylene glycol) (mol.wt. 25000) was from Union Carbide, Southampton, Hants., U.K., and Spectrapor dialysis membrane (mol.wt. cut-off of 12000–14000) was from Spectrapor, Spectrum Medical Industries, Los Angeles, CA, U.S.A. Bromosulphophthalein was from Hynson, Westcott and Dunning, Baltimore, MD, U.S.A.

Analytical methods

Glutathione *S*-transferase activity was measured at 37°C by following the conjugation of GSH with either 1,2-dichloro-4-nitrobenzene or 1-chloro-2,4-dinitrobenzene at 340nm (Habig *et al.*, 1974*b*). Bromosulphophthalein concentrations were measured by the A_{580} after addition of NaOH (100μl; 10M) to 250μl portions of column fractions. Protein concentrations in samples eluted from columns were calculated from the A_{280} values. Radioactivity and Na^+ concentrations were measured as described by Strange *et al.* (1977).

Animals

Male Wistar rats (230–290g) fed *ad libitum* were used. Phenobarbitone-treated rats were given subcutaneous injections of sodium phenobarbitone in sterile water (0.5ml; approx. 100mg/kg body wt.) for 7 successive days and were killed on the eighth day. Phenobarbitone administration resulted in a 30–50% increase in liver weight compared with untreated animals.

Buffers

The compositions of the buffers used, and the temperatures at which they were prepared, were; buffer A, 10mM-sodium phosphate, pH7.4 (20°C); buffer B, 10mM-Tris/HCl, pH8.1 (4°C); buffer C, 10mM-sodium phosphate, pH6.7 (20°C); and buffer D, 10mM-Tris/HCl, pH8.8 (4°C).

Preparation of cytosol

Rats were anaesthetized with ether and the livers perfused *in situ* through a portal-vein cannula with approx. 20ml of ice-cold buffer A containing sucrose (250mM), until free of blood. The liver was removed, homogenized in 20ml of the perfusion buffer and the homogenate centrifuged (30min, 4°C, 18000*g*). The supernatant was decanted off, re-centrifuged (120min, 4°C, 100000*g*) and after removal of the lipid layer the clear supernatant was stored on ice.

Separation of the glutathione S-transferases in cytosol

Cytosol from two rats was dialysed (4°C, 9h) against two changes, each of 2 litres, of buffer A. The dialysed solution (12ml; approx. 325mg of protein) was eluted (4°C, 16ml/h) from a column of CM-Sephadex (2.2cm×15cm) which was equilibrated with buffer A. The fraction volume was 2.7ml. After 70ml had been eluted the glutathione *S*-transferases, which were retained by the ion-exchanger, were eluted with a continuous 0–80mM-NaCl gradient in buffer A (Strange *et al.*, 1977).

Discontinuous polyacrylamide-gel electrophoresis

This was performed in the presence of 0.1% SDS by using vertical slab gels (0.075cm×12cm×20cm) (Laemmli, 1970) in an RGA/500 electrophoresis apparatus obtained from Raven Scientific Ltd., Haverhill, Suffolk, U.K. Samples were prepared for electrophoresis by heating at 85°C for 10min in an aqueous solution containing SDS (1%, w/v), 2-mercaptoethanol (0.1%, v/v), Bromophenol Blue (0.002%, w/v) and sucrose (10%, w/v) as described by Maizel (1971). Portions (40μl) of these mixtures were run through the stacking gel [(3%, w/v) polyacrylamide in Tris/HCl buffer (0.125M, pH6.8)] at 6W and through the resolving gel [12.5% (w/v) polyacrylamide in Tris/HCl buffer (0.375M, pH8.9)] at 2–4W. The gels were stained (2h, 20°C) in a 0.2%

(w/v) solution of Coomassie Brilliant Blue in water/methanol/acetic acid (50:50:7, by vol.) and destained in water/methanol/acetic acid (88:5:7, by vol.). After slicing, the gels were scanned at 580nm by using a Vitatron TLD 100 flying-spot densitometer from Fisons Scientific Apparatus, Loughborough, Leics., U.K.

The Ya, Yb and Yc monomers present in the Y fraction (Bass *et al.*, 1977) were identified as follows. Cytosol (3ml, 40mg of protein) was eluted (4°C, 22ml/h) from a column (2.5cm×38cm) of Bio-Gel A-0.5m. The fraction volume was 3.7ml. The void volume (Dextran Blue) was 75ml and the salt volume (Na^+) was 195ml. The three fractions which contained the maximum glutathione *S*-transferase activities (eluted between 127 and 138ml) were combined and examined by discontinuous polyacrylamide-gel electrophoresis. Densitometry of the electrophoretic pattern demonstrated that 85% of the total protein present was recovered in three bands (Ya, Yb and Yc).

Peptide 'mapping' of lithocholic acid-binding proteins by limited proteolytic digestion in the presence of SDS

The method used was that described by Cleveland *et al.* (1977). The two purified lithocholic acid-binding proteins (approx. 1mg of protein/ml) were each heated to 95°C for 2min in the presence of 0.2% SDS and 1mM-EDTA. These mixtures were cooled (37°C, 10min) and proteolytic digestion was carried out at 37°C by addition of various amounts of α-chymotrypsin (10μl, containing 14, 1.4, 0.14, 0.014 or 0.0014μg of protein) to portions (50μl, approx. 50μg of protein) of the purified lithocholic acid-binding proteins. After 45min, 2-mercaptoethanol, SDS, sucrose and Bromophenol Blue were added to final concentrations of 1, 2, 10 and 0.002% respectively and the digestions were terminated by heating (95°C, 10min). Portions (50μl) of the digest, which contained 25μg of digested lithocholic acid-binding protein and 7–0.0007μg of either proteolytic enzyme, were analysed by discontinuous SDS/polyacrylamide-gel electrophoresis on slabs (0.075cm×12cm×20cm) by using a 3% stacking gel and 16.5% polyacrylamide resolving gel as described above. As a control, chymotrypsin was incubated alone at the highest concentration used for digestion (140μg/ml).

Preparation of lithocholic acid-binding proteins 1 *and* 2

These were prepared by the method of Strange *et al.* (1977).

Preparation of glutathione S-transferase B

This was prepared as described by Jakoby *et al.* (1976*a*). Livers from two rats were frozen in a beaker placed in a mixture of solid CO_2 and ethanol (approx. −72°C). After 30min the frozen livers were removed, added to 40ml of water (4°C) and crushed with a pestle. This preparation was blended for 30s in a Sunbeam liquidizer model PB-P (Sunbeam Corp., Maribyrnong, Victoria, Australia) and the resulting solution was centrifuged (1h, 10000*g*). The lipid layer was removed and 16ml of the supernatant applied to a DEAE-cellulose column (2.2cm×15cm) which was equilibrated and eluted (22ml/h, 4°C) with buffer B. The enzyme-containing fractions eluted between 29 and 65ml were combined. Solid $(NH_4)_2SO_4$ was added with stirring at 20°C until 90% saturation was reached (Dixon & Webb, 1964). After standing (30min, 20°C) the solution was centrifuged (10000*g*, 45min, 20°C). The supernatant was discarded, the precipitate redissolved in 10ml of buffer C and the solution dialysed (16h, 4°C) against 2 litres of the same buffer. The dialysed solution was applied to a CM-cellulose column (2.2cm×15cm) equilibrated and eluted (22ml/h, 4°C) with buffer C. The fraction volume was 3.7ml. After 120ml of eluate had been collected a continuous 0–80mM-NaCl gradient in the same buffer was initiated (Habig *et al.*, 1974*b*). Five peaks of enzyme activity, able to catalyse the conjugation of GSH with 1-chloro-2,4-dinitrobenzene, were eluted at Na^+ concentrations of 15, 37, 50, 59 and 70mM. Only the enzyme-containing peaks eluted at Na^+ concentrations of 37 and 59mM were able to catalyse the conjugation of GSH and 1,2-dichloro-4-nitrobenzene, indicating that these peaks contain transferases C and A respectively (Habig *et al.*, 1974*b*). The enzyme-containing peak eluted between these two transferases at 50mM-Na^+ concentration is glutathione *S*-transferase B (Habig *et al.*, 1974*b*).

Preparation of ligandin

Ligandin was prepared essentially by the method of Arias *et al.* (1976). After perfusion *in situ* with approx. 20ml of ice-cold buffer A, 100g of liver was homogenized in 300ml of buffer A containing 250mM-sucrose. The homogenate was centrifuged (100000*g*, 90min, 4°C) and the resulting supernatant was dialysed against 5 litres of buffer D (16h, 4°C). The dialysed material (approx. 80ml) was eluted (25ml/h) from a column (2.5cm×100cm) of TEAE-cellulose equilibrated with buffer D. The first protein peak, eluted between 350 and 500ml, was combined, concentrated to 10ml by dialysis against poly(ethylene glycol) and redialysed (16h, 4°C) against 2 litres of buffer A. The resulting solution was incubated (60min, 4°C) with 20mg of bromosulphophthalein and eluted (24ml/h) with buffer A from a column (2.5cm×100cm) of Sephadex G-100 (Kirsch *et al.*, 1975). The six fractions (elution volume 265–300ml, approx. mol.wt. 45000) containing the maximum amount of protein-bound bromosulphophthalein were combined and concentrated to approx. 5ml by dialysis against poly(ethylene glycol).

After dialysis (16h, 4°C) against 2 litres of buffer D this solution was eluted (30ml/h) from a column (1.5cm × 60cm) of QAE-Sephadex A-50 equilibrated with buffer D. Fractions eluted between 30 and 60ml contained ligandin (Bass *et al.*, 1977).

Results

Elution of the glutathione S-transferases from CM-Sephadex

CM-Sephadex resolved rat liver cytosol into five peaks of glutathione *S*-transferase activity (measured by the conjugation of 1-chloro-2,4-dinitrobenzene and GSH) (Fig. 1). These peaks were designated 1–5 by their elution volumes: peak 1, 15–40ml; peak 2, 43–85ml; peak 3, 91–120ml; peak 4, 130–180ml; and peak 5, 197–241ml. Enzyme activity in peak 4 included a shoulder, indicating the presence of at least two enzymes.

Material in peaks 1, 2 and part of peak 4 (elution volume 155–180ml) was able to catalyse the conjugation of GSH with 1,2-dichloro-4-nitrobenzene. A small peak, containing 1,2-dichloro-4-nitrobenzene–GSH-conjugating activity, was eluted between peaks 3 and 4 (elution volume 115–130ml). Enzyme activity in peak 4 was partially resolved by CM-Sephadex chromatography, since the initial fractions (elution volume 130–154ml) were unable to catalyse the conjugation of GSH with 1,2-dichloro-4-nitrobenzene, whereas the later fractions (elution volume 155–180ml) were able to catalyse the conjugation of GSH with both 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene.

The combined elution profile of the 1-chloro-2,4-dinitrobenzene–GSH- and 1,2-dichloro-4-nitrobenzene–GSH-conjugating activities from CM-Sephadex demonstrated the presence of at least seven glutathione *S*-transferases in hepatic cytosol, six of which were able to catalyse the conjugation of 1-chloro-2,4-dinitrobenzene and GSH.

To identify the lithocholic acid-binding proteins, cytosol (12ml; approx. 325mg of protein) was incubated (60min, 4°C) with [^{14}C]lithocholic acid (1μCi; 17nmol) and the mixture eluted from CM-Sephadex. Two peaks of radiolabelled lithocholic acid were eluted with the NaCl gradient at Na^+ concentrations of 40mM and 57mM, corresponding to the elution volumes of peak 3 (91–120ml) and the initial fractions of peak 4 (elution volume 130–154ml). Peak 3 contained 55% and the first part of peak 4 contained 30% of the added radioactivity.

Jakoby *et al.* (1976*a*) have prepared six of the glutathione *S*-transferases and termed them E and D (eluted together) and C, B, A and AA by their order of elution from CM-cellulose. Transferases B, A and AA were eluted with a NaCl gradient. On the basis of elution volume and substrate specificities, either of the two lithocholic acid-binding proteins could be ligandin.

Effect of phenobarbitone on the glutathione S-transferases

Phenobarbitone administration has been reported to increase the concentration of hepatic ligandin (Arias *et al.*, 1976), and the effect of this agent on the elution profile of the glutathione *S*-transferases from CM-Sephadex was studied (Fig. 2). A comparison of the elution profiles obtained from normal rats

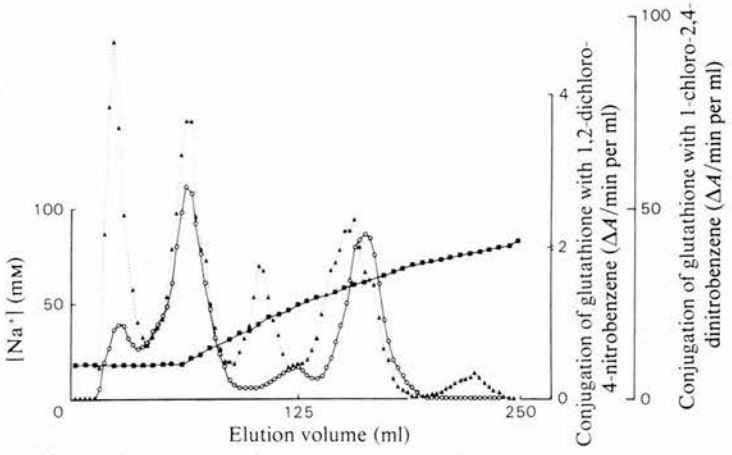

Fig. 1. *Elution pattern from CM-Sephadex of cytosol from normal rats*

Rat liver cytosol (approx. 325mg of protein) from normal rats was dialysed against buffer A and eluted from a column (2.2cm × 15cm) of CM-Sephadex C-50. Fractions (2.7ml) were collected and the Na^+ concentrations (■) determined. Glutathione *S*-transferase activity was also measured with 1-chloro-2,4-dinitrobenzene (▲) or 1,2-dichloro-4-nitrobenzene (○) as substrate.

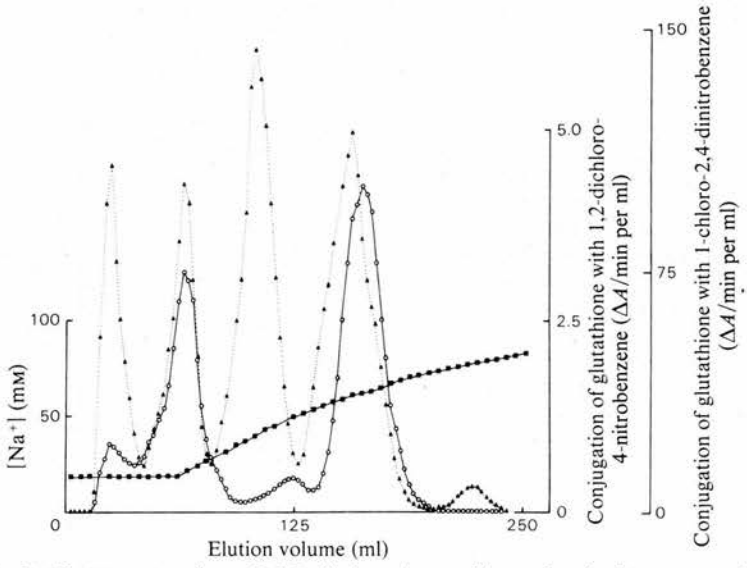

Fig. 2. *Elution pattern from CM-Sephadex of cytosol from phenobarbitone-treated rats*

Rat liver cytosol (approx. 325 mg of protein) from phenobarbitone-treated rats was dialysed against buffer A and eluted from a column (2.2 cm × 15 cm) of CM-Sephadex C-50. Fractions (2.7 ml) were collected and the Na^+ concentrations (■) determined. Glutathione *S*-transferase activity was also measured with 1-chloro-2,4-dinitrobenzene (▲) or 1,2-dichloro-4-nitrobenzene (○) as substrates.

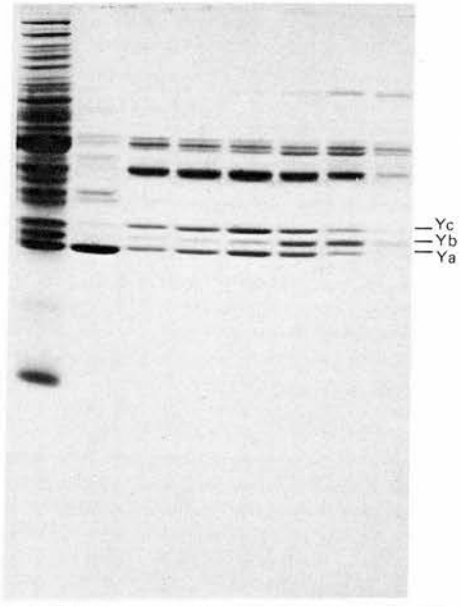

Fig. 3. *SDS/polyacrylamide-gel electrophoresis of peaks 3 and 4*

Peaks 3 and 4 were prepared by CM-Sephadex chromatography of hepatic cytosol (approx. 325 mg of protein) from normal rats. Fractions (2.7 ml) were collected and assayed for glutathione *S*-transferase activity. The three fractions (elution volume 99–107 ml) that were eluted in peak 3 and had maximum enzyme activity were combined, and fractions that were eluted at 136, 141, 147, 157, 165 and 173 ml across peak 4 were collected, and 15 μl (approx. 10 μg of protein) was removed from each fraction and prepared for electrophoresis. The samples were applied from left to right as follows: whole hepatic cytosol, the three combined fractions (eluted at 99–107 ml) from peak 3, and fractions eluted at 136, 141, 147, 157, 165 and 173 ml. The origin is at the top of the gel and samples were run down towards the anode. The Ya (mol.wt. 22000), Yb (mol.wt. 23500) and Yc (mol.wt. 25000) bands are indicated.

(Fig. 1) and from phenobarbitone-treated rats (Fig. 2) shows that the elution volumes of the glutathione *S*-transferase peaks were not changed by phenobarbitone treatment. Transferase activity in peaks 1 and 2, measured by using either 1-chloro-2,4-dinitrobenzene or 1,2-dichloro-4-nitrobenzene, was unaffected by phenobarbitone treatment. Transferase activity in peak 3 was increased approx. 3.5-fold when measured with 1-chloro-2,4-dinitrobenzene. Transferase activity in peak 4 towards both substrates was increased approx. 2-fold. Transferase activity in peak 5, measured with 1-chloro-2,4-dinitrobenzene and GSH, was similar in both groups of animals. The enzyme activities in both the first

and second parts of peak 4 appear to be increased by phenobarbitone treatment.

Effects of phenobarbitone on the subunit composition of the glutathione S-transferases

To identify the Ya, Yb and Yc bands, the Y fraction was prepared. Discontinuous SDS/polyacrylamide-gel electrophresis of a portion (approx. 20μg of protein) of this preparation demonstrated that 85% of the total protein was recovered in three bands, each containing approximately equal amounts of protein. The mol.wts. of these bands were calculated to be 22000, 23500 and 25000 by comparing their electrophoretic mobilities against rat albumin (mol.wt. 68000), ovalbumin (mol.wt. 45000), α-chymotrypsinogen (mol.wt. 25000) and ribonuclease A (mol.wt. 12700).

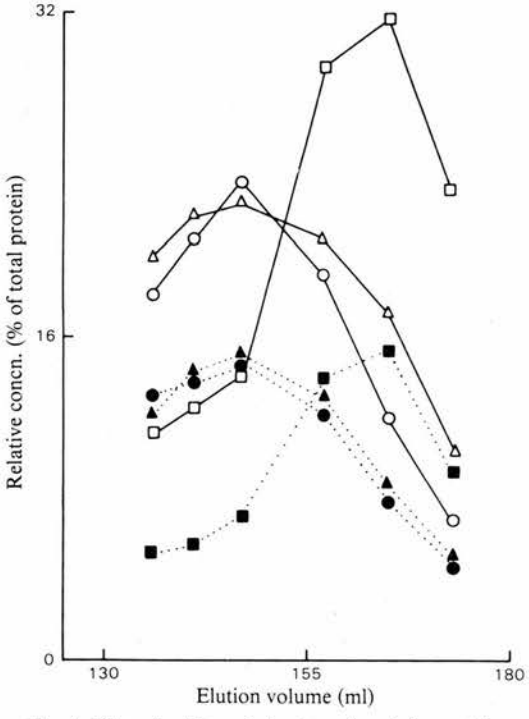

Fig. 4. *Phenobarbitone induction of peak-4 proteins*
Peak 4 was prepared by CM-Sephadex chromatography of whole cytosol. Electrophoresis of samples (20μl) from fractions that were eluted at 136, 141, 147, 157, 165 and 173ml from both phenobarbitone-treated and untreated rats was performed as described for Fig. 3. The relative concentrations in the peak-4 fractions of Ya (▲), Yb (■) and Yc (●) subunits in untreated rats and Ya (△), Yb (□) and Yc (○) subunits in phenobarbitone-treated rats were determined by densitometry and expressed as a percentage of the total protein in each fraction.

Equal portions of cytosol (approx. 325mg of protein) from control and phenobarbitone-treated rats were chromatographed on columns of CM-Sephadex. Electrophoresis of portions (15μl, approx. 10μg of protein) of fractions of peak 3 obtained from control animals indicated that 80% of the protein

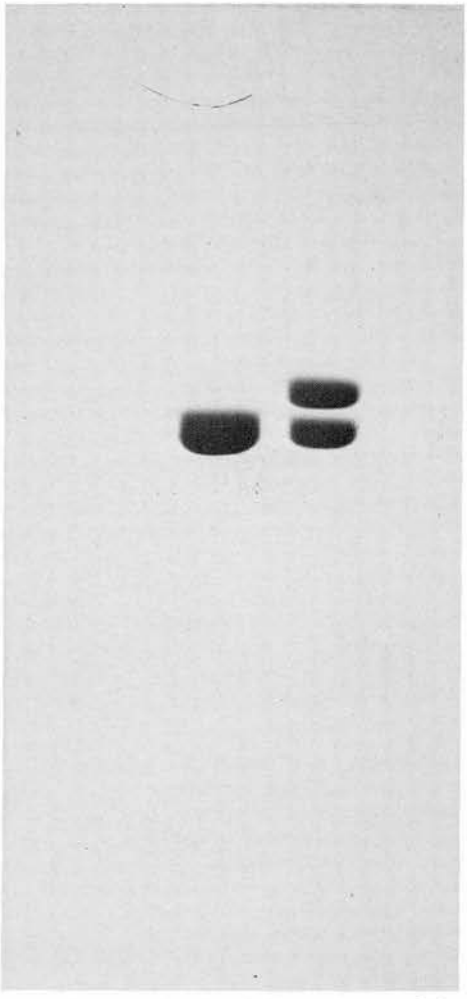

Fig. 5. *SDS/polyacrylamide-gel electrophoresis of the two lithocholic acid-binding proteins*
The two lithocholic acid-binding proteins were purified from CM-Sephadex by the method of Strange *et al.* (1977) and prepared for discontinuous SDS/polyacrylamide-gel electrophoresis as described by Maizel (1971). Portions of each protein (15μg) were applied to the gels (12.5% polyacrylamide resolving gel) and were run from the cathode (top) to the anode (bottom). Purified protein which was eluted from CM-Sephadex at 40mM-Na^+ was applied to the left-hand side of the gel and protein eluted at 57mM-Na^+ was applied to the right-hand side of the gel.

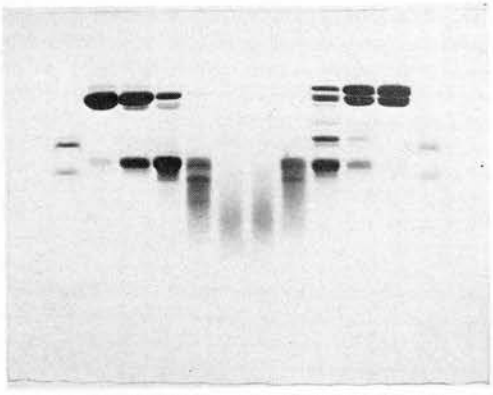

Fig. 6. *Partial proteolytic digestion of the lithocholic acid-binding proteins*

Portions (50 μg) of the lithocholic acid-binding proteins were digested with various amounts of α-chymotrypsin as described in the Materials and Methods section. After 45 min digestion, the reaction mixtures were applied to discontinuous SDS/polyacrylamide gels and run from the cathode (top) to the anode (bottom). The samples applied were: (1) chymotrypsin (7 μg); (2)–(6) lithocholic acid binding-protein (YaYa) (25 μg) and 0.0007 μg, 0.007 μg, 0.07 μg, 0.7 μg or 7 μg of chymotrypsin respectively; (7)–(11) lithocholic acid-binding protein (YaYc) (25 μg) and 7 μg, 0.7 μg 0.07 μg, 0.007 μg or 0.0007 μg of chymotrypsin respectively; (12) chymotrypsin (7 μg). Chymotrypsin treated in the same way as the lithocholic acid-binding proteins was dissociated into its subunits (mol.wts. approx. 17000 and 11000; Schroeder, 1968) and was used as a molecular-weight marker (channels 1 and 12).

migrated with the Ya band, indicating that the YaYa dimer is responsible for the enzyme activity of peak 3 (Fig. 3). Phenobarbitone treatment resulted in a 3-fold increase in the amount of the Ya monomer. Only small amounts of the Yb and Yc monomers were found in control animals, and these were not changed by phenobarbitone treatment.

For normal rats, electrophoresis of portions (15 μl, approx. 15 μg of protein) of fractions from peak 4 (136, 141, 147, 157, 165 and 173 ml) (Fig. 3) demonstrated a change in the Ya, Yb, Yc subunit composition (Fig. 4). The initial fractions contained primarily Ya and Yc subunits, in equal concentrations, whereas the later fractions contained predominantly Yb subunit. The Yb content changed from approx. 16% (elution volume 136 ml) to 55% (elution volume 173 ml) of the total YaYbYc protein; this suggested that a YaYc dimer is responsible for the enzyme and lithocholic acid-binding activities in the initial fractions of peak 4, and a YbYb dimer for the enzyme activity in the later fractions. After phenobarbitone treatment the concentrations of the Ya, Yb and Yc bands were each increased approx. 2-fold in all the fractions across peak 4 (Fig. 4). The concentrations of other bands were unchanged. Phenobarbitone treatment therefore appears to induce both of the two partially resolved glutathione *S*-transferases in peak 4.

Peak 3 and the initial fractions of peak 4, which contain the two lithocholic acid-binding proteins, are both induced by phenobarbitone treatment and could therefore be ligandin. Ligandin has been described as a YaYc dimer (Daniel *et al.*, 1977; Bhargava *et al.*, 1978), which is consistent with its

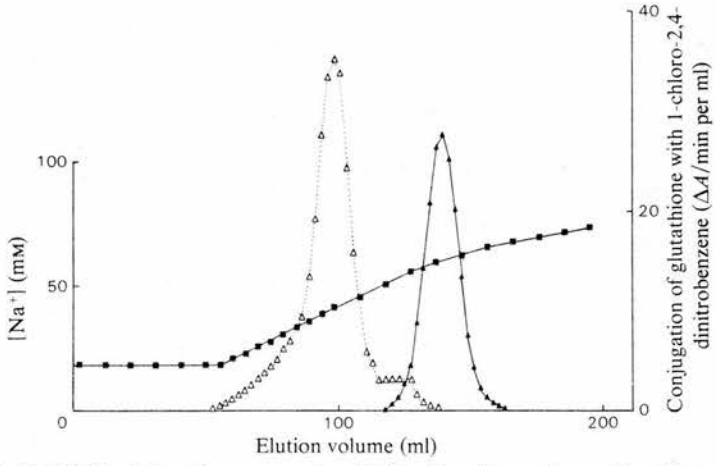

Fig. 7. *CM-Sephadex chromatography of glutathione S-transferase B and ligandin*

Glutathione S-transferase B and ligandin were prepared and 5 ml (approx. 1 mg) of each preparation was eluted (16.0 ml/h) from CM-Sephadex C-50. Fractions (2.67 ml) were collected and the Na^+ concentrations (■) determined. The results from the separate chromatography of the two protein preparations are combined. GSH–1-chloro-2,4-dinitrobenzene-conjugating activities in the eluate from chromatographed glutathione *S*-transferase B (▲) and ligandin (△) were measured.

being eluted in peak 4. However, Bass *et al.* (1977) have shown that their preparation of ligandin contained predominantly YaYa protein, which is consistent with it being eluted in peak 3, and Carne *et al.* (1979) prepared ligandin which could be resolved into YaYa protein and a YaYc protein. The identity of the two bile acid-binding proteins was therefore established by preparing ligandin and glutathione *S*-transferase B and eluting the two purified proteins from CM-Sephadex.

Peptide 'mapping' of lithocholic acid-binding proteins

Before proteolytic digestion, portions of the two binding proteins were examined by discontinuous SDS/polyacrylamide-gel electrophoresis. Fig. 5 shows that the protein eluted in peak 3 from CM-Sephadex migrated as a single band, which was identified as the Ya monomer, and that eluted in the initial fractions of peak 4 migrated as two bands, identified as the Ya and Yc monomers.

Limited proteolysis of these two proteins with chymotrypsin followed by SDS/polyacrylamide-gel electrophoresis revealed two major digestion products, which were common to both proteins (Fig. 6). Comparison of the mobility of these products with that of the subunits of chymotrypsin showed them to have mol.wts. about 10000 and 13000. Fig. 6, however, also revealed digestion products of mol.wts. about 19000 and 17000, which are found in the protein from the initial fractions of peak 4 (glutathione *S*-transferase B), but not in the other protein.

Elution of ligandin and glutathione S-transferase B from CM-Sephadex

Chromatography of ligandin from CM-Sephadex resulted in elution of a peak (85–120ml) of protein and enzyme activity (measured with 1-chloro-2,4-dinitrobenzene and GSH) at a Na^+ concentration of 40mM (Fig. 7). Glutathione *S*-transferase B was eluted (130–155ml) from CM-Sephadex as a single peak of protein and enzyme activity (measured with 1-chloro-2,4-dinitrobenzene and GSH) at a Na^+ concentration of 57mM (Fig. 7). The glutathione *S*-transferase activity of ligandin and glutathione *S*-transferase B with 1,2-dichloro-4-nitrobenzene was less than 0.3% of that with 1-chloro-2,4-dinitrobenzene. When either ligandin or glutathione *S*-transferase B was incubated (4°C, 60min) with [^{14}C]lithocholic acid (1μCi; 17nmol) before elution from CM-Sephadex, single peaks of radioactivity were eluted with the protein peaks, indicating that both ligandin and glutathione *S*-transferase B bind lithocholic acid.

Discussion

Our experiments show that seven glutathione *S*-transferases in liver cytosol can be resolved by CM-Sephadex chromatography: of these six were able to catalyse the conjugation of GSH with 1-chloro-2,4-dinitrobenzene. Both peak 3 and the initial fractions of peak 4, which contain the two previously described lithocholic acid-binding proteins, contain proteins that could be ligandin on the basis of substrate specificity, phenobarbitone induction, subunit composition and anion binding.

To identify these two protein peaks, ligandin and glutathione *S*-transferase B were purified and examined by CM-Sephadex chromatography. The two proteins were eluted separately as single peaks of protein and enzyme activity at volumes equivalent to peak 3 and the initial fractions of peak 4 respectively. Neither protein exhibited the 'microheterogeneity' described by Ketterer *et al.* (1976) or Carne *et al.* (1979). These results demonstrate that ligandin, prepared by the procedure described by Arias *et al.* (1976) and Bass *et al.* (1977), and glutathione *S*-transferase B, purified by the method of Habig *et al.* (1976*a*), are separate proteins and that they can be prepared to a degree of purity such that they are eluted from CM-Sephadex as single peaks of protein.

The suggestion that ligandin is a protein which exhibits 'microheterogeneity' is based on the finding that apparently pure preparations of ligandin can be resolved into two similar but separate proteins that have similar amino acid compositions and are composed of either YaYa or YaYc monomers (Carne *et al.*, 1979). Other workers, however, have described ligandin preparations that do not demonstrate 'microheterogeneity'. In the present study and in that of Bass *et al.* (1977), ligandin, prepared by the method of Arias *et al.* (1976), was shown by discontinuous SDS/polyacrylamide-gel electrophoresis to contain predominantly the Ya monomer, indicating that the dimeric protein comprises a YaYa dimer. Since Bhargava *et al.* (1978) also used the purification procedure described by Arias *et al.* (1976), it is surprising that their ligandin was a YaYc dimer. The procedure of Bhargarva *et al.* (1978) was, however, slightly different from that originally described by Arias *et al.* (1976); the pH of the buffer used in their ion-exchange-chromatography steps was lower and the purification was carried out in the presence of phenylmethanesulphonyl fluoride. The YaYa and YaYc dimers are very similar, and this change in the pH of the eluting buffer may have been sufficient to result in purification of a YaYc dimer rather than the YaYa dimer.

The name ligandin was originally used to describe three apparently homogeneous protein preparations which, on the basis of molecular-weight studies, amino acid compositions and immunological cross-reactivities, were considered to be one protein (Litwack *et al.*, 1971). The subunit composition of these preparations of ligandin is not known, and, since subsequent preparations of this protein were

found to contain either YaYa or YaYc dimers or even a mixture of these proteins, it is not possible to define the subunit composition of ligandin on the basis of the original preparation. Our preparation of ligandin contains Ya monomers. Our experiments also indicate that the YaYc dimer can be prepared to a degree of purity such that it is eluted from CM-Sephadex as a single peak of enzyme activity; it also demonstrated no evidence of 'microheterogeneity'. This protein, prepared by the procedure of Habig *et al.* (1976*a*), is glutathione *S*-transferase B.

The finding that ligandin and glutathione *S*-transferase B are separate proteins is in contrast with the work of Habig *et al.* (1974*a*), who reported that ligandin, prepared by the method of Arias *et al.* (1976), and glutathione *S*-transferase B were identical; the two proteins were immunologically indistinguishable, were induced by phenobarbitone and had identical amino acid compositions and similar substrate specificities. The subunit composition of this preparation of ligandin is not known; it may have been a YaYc dimer and therefore been identical with glutathione *S*-transferase B. However, even if this preparation was a YaYa dimer, it is not surprising that the two proteins were thought to be identical. These two proteins are similar (Carne *et al.*, 1979), and it is not surprising that antiserum raised against one protein cross-reacts with the other or that their substrate specificities are similar. However, we have previously reported that lithocholic acid inhibits the enzyme activity of the protein now known to be ligandin, but not that of glutathione *S*-transferase B (Strange *et al.*, 1977). Further, although their amino acid compositions have been reported to be identical, we have now shown differences in their peptide 'maps'. We confirm that both proteins are induced by phenobarbitone.

A variety of glutathione *S*-transferases have been described in both human and rat liver. Five ionically distinct glutathione *S*-transferases have been isolated from human liver; all have the same amino acid composition, suggesting that they are coded for by a single gene and that the different enzymes arise as a result of deamidation *in vivo* or some other post-synthetic modification (Habig *et al.*, 1976*b*). In rat liver seven glutathione *S*-transferases, having similar physical properties and overlapping substrate specificities, have been reported; it is not clear how many genes code for these enzymes. Although not all the glutathione *S*-transferases in rat liver have been purified, the amino acid compositions of at least some of the enzymes (transferases C, B, A and AA) are similar, indicating that some of these enzymes may be coded for by the same gene (Habig *et al.*, 1974*b*, 1976*a*). However, the enzymes in peak 3 and peak 4 may be coded separately, since they are the only ones that are induced by phenobarbitone. The number of enzymes actually transcribed may be therefore less than the seven so far found. This would account for the presence of only three separate monomer bands (Ya, Yb and Yc) in the Y fraction. The relationship between the two lithocholic acid-binding proteins is unclear. It is unlikely that they arise as a result of non-specific degradation, since they can be prepared separately from each other. Possibly a product–precursor relationship exists between the two proteins. They may be coded for by a single gene and synthesized as a YcYc precursor (mol.wt. 50000), which is subsequently converted into either YaYc (mol.wt. 47000) or YaYa (mol.wt. 44000) protein by the cleavage and removal of approx. 25 terminal amino acids.

Our results suggest that the two previously described lithocholic acid-binding proteins are, in fact, ligandin and glutathione *S*-transferase B. These two proteins are both induced by phenobarbitone and have similar substrate specificities, but are eluted differently from CM-Sephadex and possess different subunit compositions. It is not clear whether the two proteins are coded for by the same gene and represent alternative forms of the same protein, or by different genes and therefore represent isoenzymes.

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Cholic Acid Binding by Glutathione *S*-Transferases from Rat Liver Cytosol

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Cholic acid-binding activity in cytosol from rat livers appears to be mainly associated with enzymes having glutathione *S*-transferase activity; at least four of the enzymes in this group can bind the bile acid. Examination of the subunit compositions of different glutathione *S*-transferases indicated that cholic acid binding and the ability to conjugate reduced glutathione with 1,2-dichloro-4-nitrobenzene may be ascribed to different subunits.

The removal of bile acids from the blood and their secretion into bile is an important aspect of hepatic function. Transport across the hepatocyte from the sinusoidal membrane to the canalicular membrane may involve cytosolic bile acid-binding components whose molecular weight (about 45000) and lack of specificity towards different bile acids (Strange *et al.*, 1977*a*) suggested the involvement of ligandin, a non-specific anion-binding protein (Litwack *et al.*, 1971). Although the identity of ligandin may now be in doubt (Hayes *et al.*, 1979), it is one of at least seven enzymes found in rat liver cytosol which have glutathione *S*-transferase activity.

The glutathione *S*-transferases comprise a group of enzymes with overlapping substrate specificities, some of which can also bind non-substrate ligands (Jakoby, 1978). They have molecular weights of about 46000 and comprise two of three possible subunits: Ya (mol.wt. 22000), Yb (mol.wt. 23500) and Yc (mol.wt. 25000) (Bass *et al.*, 1977). Ligandin has been described as a YaYa dimer (Bass *et al.*, 1977; Hayes *et al.*, 1979), a YaYc dimer (Listowsky *et al.*, 1976) or as a mixture of two proteins comprising YaYa and YaYc dimers (Carne *et al.*, 1979). In the present study, we have defined ligandin (prepared by the method of Arias *et al.*, 1976) as a YaYa dimer.

We have purified two lithocholic acid-binding proteins from rat liver cytosol (Strange *et al.*, 1977*b*; Hayes *et al.*, 1979); both proteins possessed glutathione *S*-transferase activity, and they were identified as ligandin and glutathione *S*-transferase B (Hayes *et al.*, 1979). We now describe experiments firstly to determine if these and other glutathione *S*-transferases can bind the quantitatively more important primary bile acid, cholic acid, and secondly to compare the subunit composition of the enzymes that bind cholic acid with those that do not.

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

Materials and Methods

Chemicals

Cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) was from Maybridge Chemical Co., Tintagel, Cornwall, U.K., and [2,4-^{3}H]cholic acid (14Ci/mmol) was from New England Nuclear, Dreieichenhain, West Germany. They were shown to be 99% pure by t.l.c. (Hamilton & Muldrey, 1961). Bio-Gel A-0.5m (200–400mesh) was from Bio-Rad Laboratories, Bromley, Kent, U.K.

Analytical methods

Glutathione *S*-transferase activity was measured at 37°C by following the conjugation of glutathione with either 1,2-dichloro-4-nitrobenzene or 1-chloro-2,4-dinitrobenzene at 340nm (Habig *et al.*, 1974). Radioactivity counting, determination of Na^+ and protein concentrations were performed as described by Strange *et al.* (1977*b*).

Buffers

The compositions of the buffers used, and the temperatures at which they were prepared, were: buffer A, 10mM-sodium phosphate, pH7.4 (20°C); buffer B, 20mM-sodium phosphate/100mM-NaCl, pH7.4 (20°C); and buffer C, 20mM-Tris/HCl, pH8.6 (4°C).

Experimental and Results

Separation of glutathione S-transferases in cytosol and examination of their cholic acid-binding activity

Cytosol prepared from two rat livers (Strange *et al.*, 1977*a*) was dialysed (4°C, 9h) against two changes, each of 2 litres, of buffer A and eluted from a CM-

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Table 1. *Elution characteristics, subunit compositions and cholic acid binding by glutathione S-transferases from rat liver cytosol*
The glutathione *S*-transferases in rat liver cytosol were separated by a combination of CM-Sephadex and DEAE-Sephadex chromatography. Their monomer composition was determined by SDS/polyacrylamide-gel electrophoresis and their ability to bind cholic acid by an equilibrium chromatographic method.

Peak	Elution vol. from CM-Sephadex (ml)	Elution vol. from DEAE-Sephadex (ml)	Percentage subunit composition Ya	Yb	Yc	Cholic acid binding
1	15–40		5	25	70	Yes
2	43–85		5	95	0	No
3	91–115		90	5	5	Yes
4	130–180					
4(i)	130–154	20–53	50	0	50	Yes
4(ii)	155–180	127–153	0	100	0	No
5	197–241		0	0	100	Yes

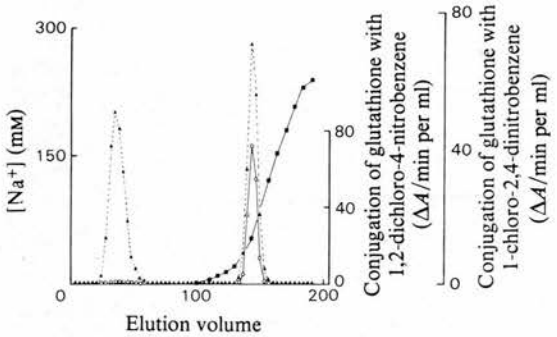

Fig. 1. *DEAE-Sephadex chromatography of peak* 4
Cytosol was eluted from a CM-Sephadex column and peak 4 (elution vol. 130–180ml) was combined and concentrated. After dialysis against buffer C, peak 4 was eluted from a DEAE-Sephadex column. Fractions (3.4ml) were collected and the glutathione *S*-transferase activity with 1-chloro-2,4-dinitrobenzene (▲) and 1,2-dichloro-4-nitrobenzene (○) and the Na^+ concentration (■) were measured.

Sephadex column (2.2cm×15cm) (Hayes *et al.*, 1979). Five peaks with GSH–1-chloro-2,4-dinitrobenzene-conjugating activity were eluted and designated 1–5 by their elution volumes (Table 1) (Hayes *et al.*, 1979). They were each combined and peaks 2–5 were concentrated, to about 5ml, by dialysis at 4°C against poly(ethylene glycol). Peaks 1, 2, 3 and 5 were dialysed against two changes, each of 2 litres, of buffer B (4°C, 15h).

After concentration the glutathione *S*-transferases in peak 4 were dialysed against two changes, each of 2 litres, of buffer C. This solution was applied to a DEAE-Sephadex A-50 column (2.2cm×15cm), which was equilibrated and eluted (20ml/h, 4°C) with buffer C. A NaCl gradient (0–500mM) in buffer C was established after 95ml of the buffer had passed through (Strange *et al.*, 1977*b*). Fractions (3.3ml) were collected and analysed for glutathione *S*-transferase activity by measuring the conjugation of GSH with either 1-chloro-2,4-dinitrobenzene or 1,2-dichloro-4-nitrobenzene. Peak 4 was resolved into two peaks of enzyme activity (Fig. 1); these were designated peak 4(i) and peak 4(ii) (Table 1). Both peaks were concentrated to about 5ml by dialysis at 4°C against poly(ethylene glycol) and then dialysed against two changes, each of 2 litres, of buffer B (4°C, 15h).

The glutathione *S*-transferases have previously been classified by their order of elution from CM-cellulose (Habig *et al.*, 1974; Jakoby *et al.*, 1976), which suggests that transferases D, E and M are eluted in peak 1, transferase C is eluted in peak 2, ligandin and glutathione *S*-transferase B are eluted in peaks 3 and 4(i) respectively (Hayes *et al.*, 1979), transferase A is eluted in peak 4(ii) and transferase AA in peak 5. The presence of transferase C in peak 2 and transferase A in peak 4(ii) is supported by the substantial GSH–1,2-dichloro-4-nitrobenzene-conjugating activity found in these two peaks (Habig *et al.*, 1974).

The binding of cholic acid by whole cytosol and the separate transferase peaks was studied by using an equilibrium chromatographic method (Wood & Cooper, 1970). A Bio-Gel A-0.5m column (2.5cm×38cm) was equilibrated and eluted at 4°C with buffer B containing [^{3}H]cholic acid (10nM; 150d.p.s./ml). The flow rate was 20.5ml/h and the fraction volume was 3.4ml. The elution volume of Blue Dextran was 75ml and of [^{3}H]cholic acid 200ml. Portions (3ml) of cytosol and glutathione *S*-transferase peaks 1, 2, 3, 4(i), 4(ii) or 5, containing approx. 80, 100, 15, 15, 15 or 15mg of protein respectively, were diluted with an equal volume of buffer B containing [^{3}H]cholic acid (20nM; 300d.p.s./ml). After incubation (60min, 4°C), this mixture was eluted from the Bio-Gel column with buffer B containing [^{3}H]cholic acid (10nM; 150d.p.s./ml). The elution of cytosol from the Bio-Gel column showed that the cholic acid-

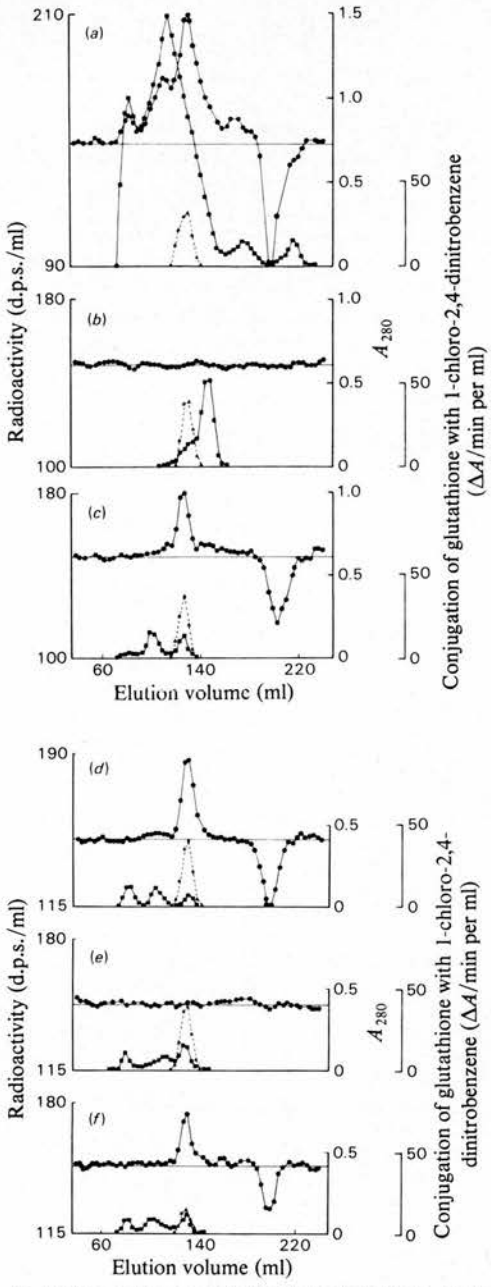

Fig. 2. *Elution patterns of individual glutathione S-transferase peaks from Bio-Gel equilibrated with [^{3}H]cholic acid*

The transferase peaks [1, 2, 3, 4(i), 4(ii) and 5] obtained after ion-exchange chromatography were concentrated and dialysed against buffer B. These were eluted from a Bio-Gel A-0.5m column equilibrated with [^{3}H]cholic acid as follows: peak 1, (*a*); peak 2, (*b*); peak 3, (*c*); peak 4(i), (*d*); peak 4(ii), (*e*); and peak 5, (*f*). Fractions (3.4ml) were collected and the A_{280} (■), the radioactivity (●) and glutathione *S*-transferase activity with 1-chloro-2,4-dinitrobenzene (▲) were measured. The solid horizontal line represents the radioactivity in the elution buffer.

binding activity was eluted as a single peak, which was associated with glutathione *S*-transferase activity. The elution of peaks 1–5 from the Bio-Gel column also showed that cholic acid binding by peaks 1, 3, 4(i) and 5 was associated with enzyme activity (Fig. 2). Cholic acid binding by peak 1 was not restricted to enzyme-containing fractions, but was also found in fractions with the same elution volume as rat albumin (110ml). Peak 1 also contained a component of mol.wt. about 15000 which bound cholic acid. This may be the lithocholic acid-binding protein previously described by Strange *et al.* (1977*a*), which was shown to be eluted from CM-Sephadex in peak 1 (J. D. Hayes, R. C. Strange & I. W. Percy-Robb, unpublished work). Ketterer *et al.* (1976) have also described a binding component of mol.wt. 14000 which binds cholic acid. No cholic acid binding was demonstrated in peaks 2 or 4(ii), despite the recovery of substantial enzyme activity.

Subunit composition of the glutathione S-transferases

Before discontinuous SDS/polyacrylamide-gel electrophoresis, the glutathione *S*-transferases were partially purified. Two portions of cytosol (each 5ml; approx. 180mg of protein) were eluted (22ml/h) with buffer B from the Bio-Gel column (2.5cm × 38cm). The enzyme-containing solutions, eluted between 120 and 145ml, were combined. Examination of a small portion of this mixture by SDS/polyacrylamide-gel electrophoresis showed that about 85% of the protein in this mixture migrated with the Ya, Yb or Yc monomer bands (Bass *et al.*, 1977; Hayes *et al.*, 1979). $(NH_4)_2SO_4$ was added to the combined enzyme-containing eluate, and the precipitate formed between 55 and 85% saturation was collected, redissolved in 12ml of buffer A and dialysed against two changes, each of 2 litres, of the same buffer (4°C, 15h) (Dixon & Webb, 1964; Strange *et al.*, 1977*b*). The dialysed solution was eluted from CM-Sephadex and five peaks of glutathione *S*-transferase activity were obtained. Peak 4 was rechromatographed on DEAE-Sephadex as described above. Portions (10μg of protein) of each peak were examined by discontinuous SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970; Hayes *et al.*, 1979). The gels were scanned at 580nm as previously described (Hayes *et al.*, 1979) and the percentage monomer composition of each peak was determined (Table 1). Peak 1 probably comprises a mixture of transferases, since it contains mainly unequal amounts of the Yb and Yc monomers. Peak 2 appears to comprise a YbYb dimer. Peaks 3 and 4(i) have previously been

purified and shown to comprise YaYa and YaYc dimers respectively (Hayes *et al.*, 1979). Peak 4(ii) comprised a YbYb dimer and peak 5 a YcYc dimer.

Discussion

We have previously described two lithocholic acid-binding proteins in rat liver cytosol which possessed glutathione *S*-transferase activity (Strange *et al.*, 1977*b*). These two enzymes were eluted in peaks 3 and 4(i) and have been identified as ligandin (prepared by the method of Arias *et al.*, 1976) and glutathione *S*-transferase B respectively (Hayes *et al.*, 1979). Ligandin has variously been described as a YaYa dimer (Bass *et al.*, 1977; Hayes *et al.*, 1979), a YaYc dimer (Listowsky *et al.*, 1976) or as a mixture of two proteins comprising YaYa and YaYc dimers (Carne *et al.*, 1979). Since the subunit composition of the original ligandin preparations (Litwack *et al.*, 1971) is unknown, the term ligandin should perhaps be abandoned and the proteins redefined according to their subunit composition as YaYa or YaYc protein.

The experiments now described show that the YaYa protein and YaYc protein as well as glutathione *S*-transferase AA and one or more of the transferases in peak 1 can bind cholic acid. SDS/polyacrylamide-gel electrophoresis indicated that cholic acid binding was associated with either the Ya or the Yc monomer. The proteins that catalysed the conjugation of GSH with 1,2-dichloro-4-nitrobenzene [in peaks 1, 2 and 4(ii)] all possessed the Yb band.

Although at least seven ionically distinct glutathione *S*-transferases exist in rat liver, SDS/polyacrylamide-gel electrophoresis has demonstrated that they each comprise two of three known monomers (Ya, Yb, Yc). Habig *et al.* (1974, 1976) have shown that transferases C and A, and B and AA, have similar amino acid compositions: significant differences exist between the alanine and valine contents of these two groups of transferases. The compositions suggest that transferases B and AA may be the product of a single gene, and transferases C and A may also be coded for by a single but separate gene. Habig *et al.* (1974) have reported that both transferases C and A cross-react with antisera raised against each other, which supports the hypothesis that these two enzymes are the product of a single gene. The elution position and substrate specificity of peaks 2 and 4(ii) suggested that they contained transferase C and transferase A respectively. Both of these enzymes comprise YbYb dimers and therefore they may be formed by post-synthetic modification involving minor structural changes. Peak 4(i) has been shown to contain transferase B (YaYc protein) (Hayes *et al.*, 1979), and the elution position and substrate specificity of peak 5 suggested that it contained transferase AA. Both of these transferases possess a Yc monomer. We have previously shown (Hayes *et al.*, 1979) that ligandin (YaYa protein) and glutathione *S*-transferase B (YaYc protein) are separate proteins, and we postulated that they may be synthesized as a YcYc protein, which is subsequently converted into YaYc or YaYa protein by the specific removal of 26 terminal amino acids. Our subunit data show that transferase AA comprises a YcYc dimer. This may represent the putative precursor protein.

Listowsky *et al.* (1976) have described a preparation of glutathione *S*-transferase A which had the same monomer composition (YaYc) as glutathione *S*-transferase B. Our results do not agree with this finding, since only peak 4(i) has this composition and this peak has been identified as glutathione *S*-transferase B (Hayes *et al.*, 1979). Further, this peak possesses no GSH–1,2-dichloro-4-nitrobenzene-conjugating activity, which indicates that it does not contain transferase A (Fig. 1).

The physiological importance of bile acid binding by the transferases is not clear. It appears unlikely that the bile acids are transported across the hepatocyte bound to glutathione *S*-transferases, since the observed bile acid transit times across the liver appear to be too rapid for the bile acids to diffuse across the hepatocyte bound to protein (Strange *et al.*, 1979). The transferases may keep bile acids in cytosol, and so restrict their partitioning into membrane lipid, thereby promoting their rapid clearance into bile.

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