THE ENTEROHEPATIC CIRCULATION OF XENOBIOTICS IN THE RAT

BY

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ABSTRACT

The enterohepatic circulation of morphine, lysergic acid diethylamide (LSD), diphenylacetic acid and phenolphthalein has been investigated, the latter in detail as a model compound for investigating the enterohepatic circulation of xenobiotics. Variations in the parameters characteristic of enterohepatic circulation were observed between the compounds studied such as the extent of biliary excretion, gut luminal hydrolysis, absorption from the gut, intestinal mucosal conjugation, biliary re-excretion and loss from enterohepatic circulation to the faeces, systemic blood and urine. These variations could be related in part to certain physicochemical properties of the individual compounds and their metabolites, in particular their relative lipid solubilities.

The role of the gut microflora and the conjugation capacity of the intestinal mucosa in the enterohepatic circulation of these compounds was investigated. Gut microfloral hydrolysis of the biliary metabolites (glucuronic acid conjugates) of these compounds was found to be a prerequisite to their extensive absorption from the gut and subsequent enterohepatic circulation. Intestinal mucosal conjugation with glucuronic acid was found to be considerable (the equivalent of 30 - 50% of an oral dose) with morphine (and also phenol and 1-naphthol), but shown to be minimal (1 - 2%) for phenolphthalein and not detectable with diphenylacetic acid. Intestinal mucosal conjugation with sulphate was not detected for most of the compounds investigated and when present (with phenol and 1-naphthol), the rate of sulphoconjugation was estimated to be approximately 10 times lower than the corresponding rate of conjugation with glucuronic acid. All the compounds were shown to be systemically available as their glucuronic acid conjugates from enterohepatic circulation, but significant changes in their systemic blood levels attributable to enterohepatic circulation were observed only with phenolphthalein, seen as a secondary peak in the systemic blood level vs. time profile for this compound.

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CHAPTER ONE

INTRODUCTION

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Characteristics of Enterohepatic Circulation

The cycle of events whereby a compound or its metabolites are excreted in the bile, absorbed from the gastrointestinal tract and returned via the portal circulation to the liver is termed an enterohepatic cycle. Re-excretion of the compound or its metabolites in the bile and subsequent recycling between the liver and gastrointestinal tract is known as enterohepatic circulation, (EHC). Compounds can remain in enterohepatic circulation until such time as they are lost either to the faeces, as a result of their incomplete absorption from the gastrointestinal tract, or to the peripheral circulation resulting from inadequate hepatic extraction and may ultimately appear in the urine, (see Fig. 1.1). An alternative route of EHC is that following intestinal absorption, compounds enter the lymphatic system rather than the portal circulation and are transported in this system, entering the systemic blood via the thoracic duct and returning to the liver via the hepatic artery. The lymphatic route would allow entry to tissue compartments which may be inaccessible to compounds restricted to the portal route of EHC as a result of hepatic extraction. The lymphatic route is known to be important in the EHC of cholesterol, certain hormones and some vitamins, but thought to be relatively unimportant for drugs (De Marco and Levine,1969; Sieber et al., 1974).

The term enterohepatic circulation was first used to describe the cycling of bile acids between the liver and gut of the dog, (Tappeiner, 1884). EHC of several endogenous compounds has been reported, notably the major components of the bile including bile acids and salts, (Hofmann, 1977), bilirubin (Lester et al., 1965) and cholesterol (Siperstein et al., 1952 a, b). Other endogenous compounds known to enter EHC include many steroid hormones, (Taylor,1971 a), vitamin A and B_{12} (Zachman and Olson, 1964, Grasbeck et al., 1958). Many exogenous compounds are known to enter EHC and will be discussed later. The enterohepatic cycle can conveniently be divided into three phases: a) biliary excretion, b) gastrointestinal absorption and c) hepatic extraction. The metabolism of a compound may be a pre-requisite for its EHC and can be associated with both the hepatic and gastro-intestinal phases of EHC.

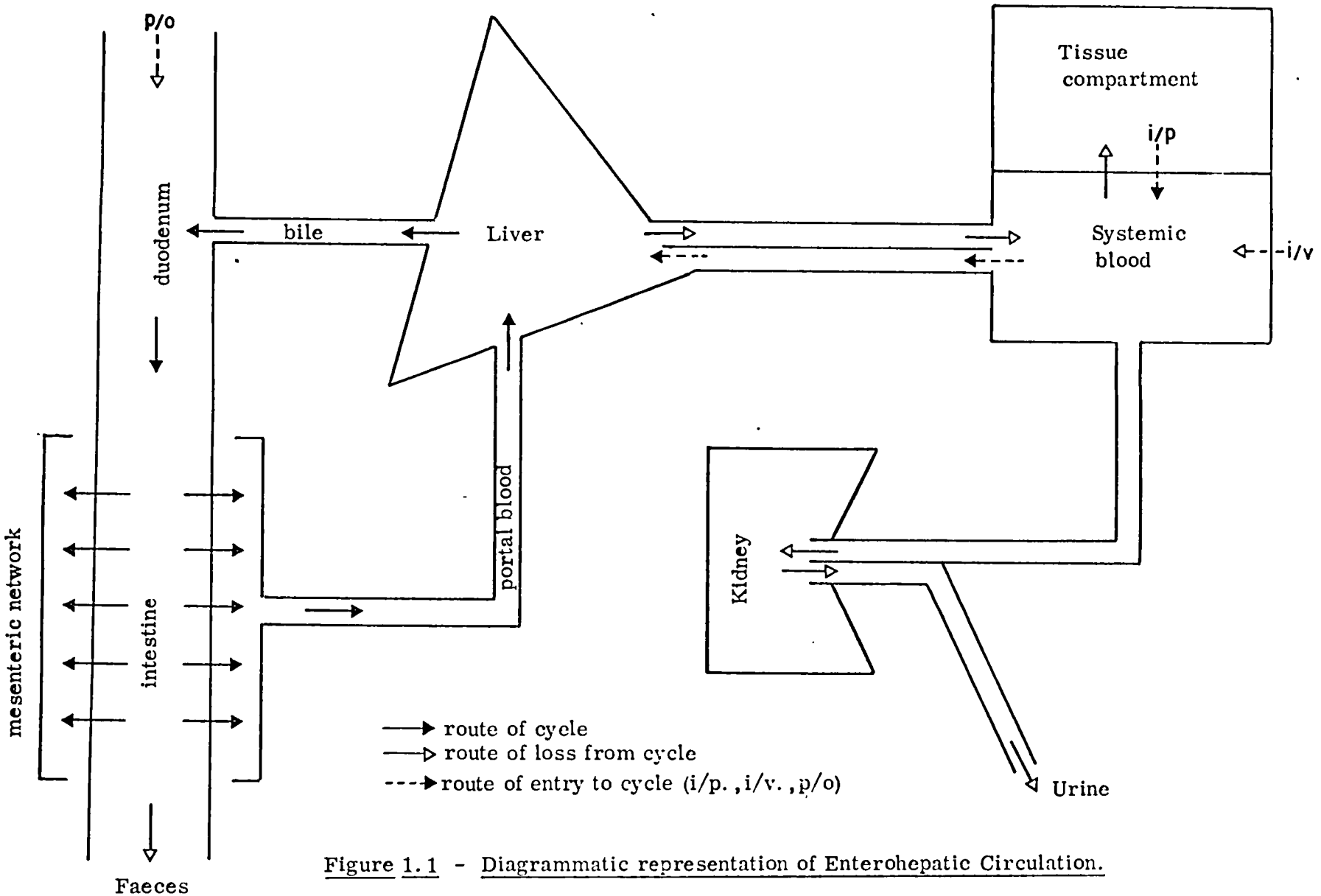


Figure 1.1 - Diagrammatic representation of Enterohepatic Circulation.

Metabolism of xenobiotics

Williams (1959) proposed that the sequence of chemical reactions resulting in the metabolism of a foreign compound could be divided into two phases. In the first phase (Phase I), a compound is subject to oxidative, reductive or hydrolytic reactions or a combination of these reactions, culminating in the introduction of OH, CO_2H, NH_2 or SH groups in the molecule that may provide suitable sites for the second phase (Phase II) reactions. The phase II reactions are synthetic in nature and are referred to as conjugations. Compounds can undergo both phases of metabolism but some may be involved in one phase only. In general, the overall effect of the biphasic metabolism is a) an increased polarity, allowing the compound to be more readily excreted in the urine and bile, b) a decrease in toxicity and c) a reduction or loss of pharmacological response to the drug. Reactions associated with phase I metabolism may proceed spontaneously or catalysed by enzymes, whereas phase II reactions are usually enzyme catalysed. Enzymes involved in both phase I and phase II reactions occur mainly in the liver and to a lesser extent in the tissues of the gastrointestinal tract, kidneys, lungs, adrenal glands and the blood. Many of these enzymes are located within the lipoprotein environment of the endoplasmic reticulum of cells, others occuring in the mitochondria and cytoplasm. Recent studies in which the enzyme activity of hepatic endoplasmic reticulum was measured following its partial solubilisation by chaotropic agents, (Vainio, 1973) suggest that certain phase I enzymes are located superficially in relation to those phase II enzymes responsible for glucuronidation reactions.

Phase II reactions

Although the phase I reactions are important in providing sites for subsequent phase II conjugations, it is the phase II reactions which are perhaps more relevant to EHC, in that they provide the increase in polarity and molecular weight which favours the biliary excretion of compounds. In order that a compound may undergo conjugation, it must possess a suitable acceptor site such as an OH, NH_2, COOH or SH group or have been provided with such a group as a result of phase I reactions. The conjugation reaction involves the addition of a conjugating agent, which is an endogenous compound derived from carbohydrate or protein sources, to a compound, termed the

second substrate at its acceptor site. The most common conjugating agents, with respect to biliary excretion and intestinal wall metabolism are glucuronic acid, glutathione, sulphate and amino acids. The first step in conjugation reactions is the activation of either the conjugating agent or the second substrate by their forming a complex with nucleotides specific for the particular conjugating agent involved. In the second step, the activated complex may then react with the second substrate or the conjugating agent to form the conjugate; the process summarised as follows:-

Type 1

second substrate + nucleotide ⟶ activated second substrate .... (1)

activated second substrate + conjugating agent ⟶ conjugate + nucleotide .... (2)

Type 2

conjugating agent + nucleotide ⟶ activated conjugating agent .... (1)

activated conjugating agent + second substrate ⟶ conjugate + nucleotide .... (2)

Conjugations following a Type 1 pattern include reactions involving amino acids as the conjugating agents, occuring in the mitochondria of the liver and kidney. Examples of Type 2 reactions are the conjugations with glucuronic acid and sulphate which occur in the endoplasmic reticulum or cytoplasm of the liver and several other tissues.

Conjugation with glucuronic acid

The prevalence of glucuronic acid conjugation in mammals may result from the ease with which glucuronic acid can be synthesised from carbohydrates and its ability to combine with a wide range of second substrate acceptor sites. Alternative conjugation pathways to glucuronidation appear to be limited both in the range of acceptor sites with which they may react to form conjugates and availability of substrates and complementary enzymes, (Smith and Williams, 1966). Dutton and Storey (1951) showed that liver homogenates would only yield glucuronidation rates comparable with intact cells on the addition of boiled liver extract, the 'active ingredient' of which was found to be the nucleotide uridine diphosphate (UDP) glucuronic acid, (Caputto et al., 1950), the structure of which is shown in Fig. 1.2.

The formation of UDP-glucuronic acid was shown to occur via a three step reaction as outlined below, (Strominger et al., 1957)

Glycogen $\xrightarrow{H_3PO_4}$ α-glucose-1-phosphate (1)

α-glucose-1-phosphate + UTP $\longrightarrow$ UDP-glucose + PP (2)

UDP-glucose $\xrightarrow{NAD,\ O_2}$ UDP-glucuronic acid (3)

Having formed UDP-glucuronic acid, a reaction could be demonstrated involving glycosyl transfer from UDP-glucuronic acid to the acceptor site on the second substrate, the reaction catalysed by an enzyme UDP-glucuronyl-transferase, an example shown in Fig.1.2.

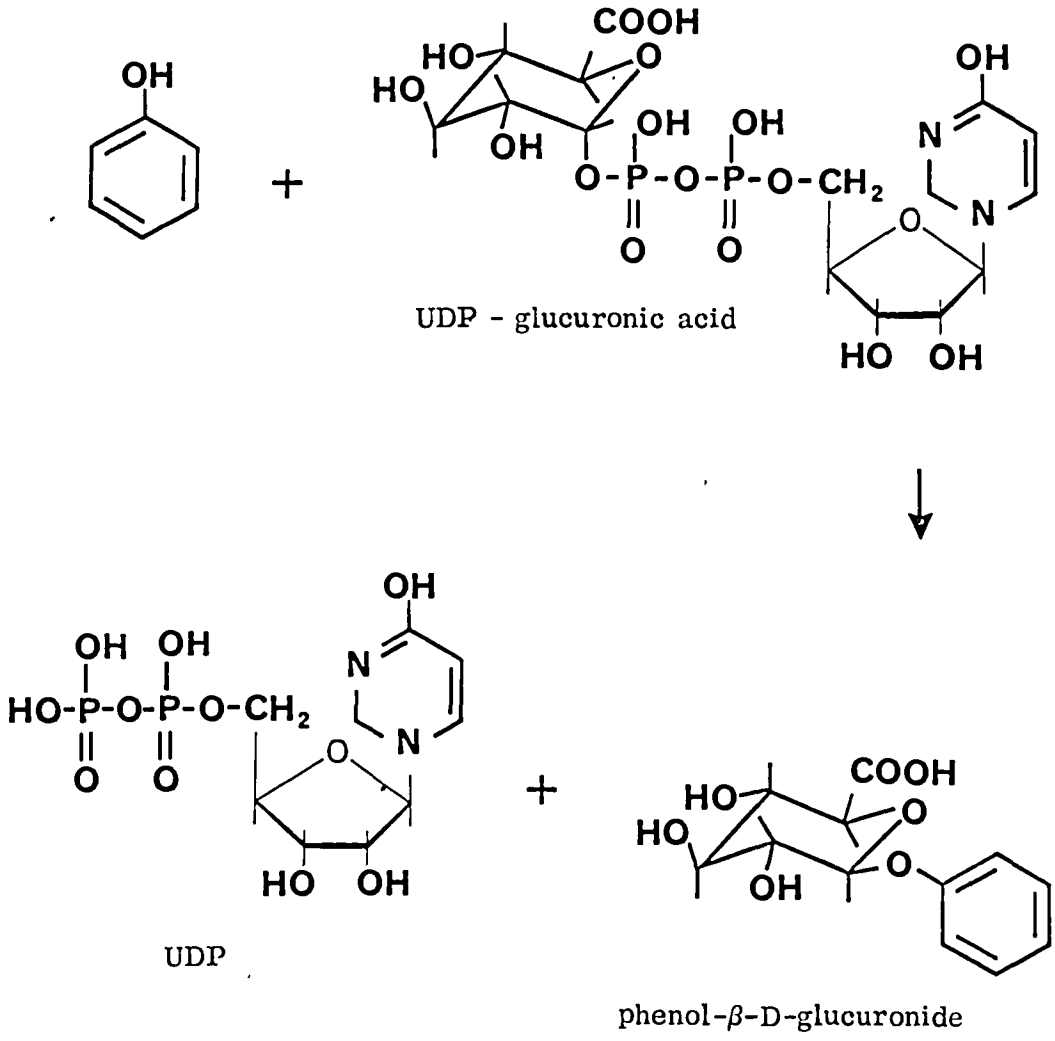


Fig 1.2 Formation of phenol β-d-glucuronide

UDP-glucuronyl transferase appears to be widely distributed throughout the tissues of the body and has been reported to occur in the liver, kidney, intestine, lung, thymus, adrenal glands and spleen, (Dutton, 1977).

The stability of the glucuronide depends primarily on the nature of the linkage between the glucuronic acid group and the acceptor site on the second substrate, which varies according to the particular acceptor site involved. Smith (1973) has listed 14 of the most frequently observed acceptor sites, (Table 1.1).

Table 1.1 Common acceptor groupings in glucuronidation reactions.

A) Hydroxyl - Phenolic	C) Amino and imino - Aromatic
Enolic	Carbamate
Alcoholic - primary	Sulphonamide
secondary	Heterocyclic
tertiary	D) Sulphydryl - Thiol
Hydroxylamine	Carbodithiolic
B) Carboxyl - Aromatic	
Aliphatic	

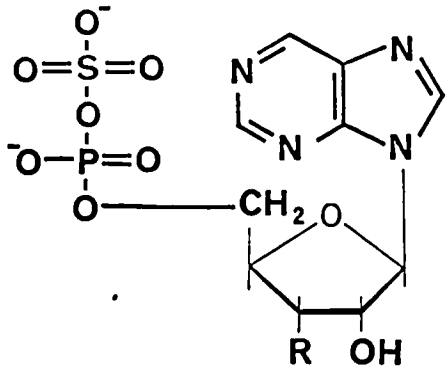
A species difference in ability to form glucuronic acid conjugates with a variety of second substrates has been observed, (Hirom et al., 1977). The amount of glucuronide conjugate formed with benzoic acid was shown to be 18% in the dog, 22% in the ferret, but not detected in the cat, (Bridges et al., 1970). An apparent inability of the cat to form glucuronides has been observed for many compounds including several phenols which were conjugated with glucuronic acid only to the extent of 1 - 3% of the dose, (Capel et al., 1972, 1974). The inability of the cat to form glucuronides has been suggested to be a deficiency in the enzyme UDP-glucuronyl transferase (Hirom et al., 1977) as this species does not appear to lack hepatic UDP-glucose dehydrogenase or UDP-glucuronic acid, (Dutton,1966). This low capacity for glucuronide formation does not seem to apply to all compounds as shown by phenolphthalein where 30 - 40% of a dose is conjugated with glucuronic acid in the cat (Abou-El - Makarem,1967, Capel et al., 1974). The ability of the cat to form glucuronides with certain compounds and not with others cannot be explained solely on the basis of a deficiency of UDP-glucuronyl transferase. With the

recent identification of several distinct forms of UDP-glucuronyl transferase (Zakim et al., 1973, Vessey et al., 1973 and Del Villar, 1975), it seems possible that the cat may be deficient only in certain forms of the enzyme, which could explain why this species is able to form glucuronides with certain compounds and only small amounts with others, (Hirom et al., 1977).

Conjugation with sulphate

In comparison with the occurence of glucuronidation, sulphoconjugation occurs less frequently in mammals, but for certain compounds, such as steroids, (Taylor, 1971a) and phenols, (Dodgson, 1977) it is the major route of conjugation.

Sulphoconjugates may be formed with the following acceptor groups: -OH, NH_2 and SH resulting in C-O-S, N-S, N-O-S, P-O-S and S-S linkages respectively, where C-O-S appears to predominate. The pathway for sulphoconjugation involves initial formation of two 'active' intermediates, adenine-5'-phosphosulphate (APS) and 3'-phospho-adenosine 5'-phosphosulphate (PAPS), the former being the precursor of the latter, their structures outlined below.



R=OH (APS)

$R=HO_2PO$ (PAPS)

The final step in the pathway is the transfer of the sulphate from PAPS to the acceptor site of the second substrate; the completed pathway equated as follows:

$$ATP + SO_4 \rightleftharpoons APS + Pi \quad (1)$$

$$APS + ATP \longrightarrow PAPS + ADP \quad (2)$$

PAPS + acceptor ——→ PAP + sulphated acceptor (3)

The three enzymes catalysing steps (1) - (3) as outlined above are ATP-sulphate adenylyl transferase (1), ATP-adenylyl sulphate 3'-phospho transferase (2), and sulphotransferases (3).

Conjugation with glutathione

Glutathione is a tripeptide of the amino acids, glutaric acid, glycine and cysteine that can combine via a thio-ester linkage to form a glutathione conjugate with a wide range of second substrates (Boyland and Chasseaud, 1969 (a) and Chasseaud, 1973). The reaction is catalysed by the enzyme glutathione S-transferase which has been shown to exist in several forms, (Chasseaud, 1973). The particular enzyme involved in a reaction depends on the nature of the second substrate, examples being glutathione S-alkyltransferase, (Johnson, 1966), glutathione S-epoxidetransferase(Boyland and Williams, 1965) and glutathione S-aralkyltransferase, (Boyland and Chasseaud, 1969 (b)). More recently, the purification and isolation of these enzymes has given rise to a new nomenclature based on their elution pattern from carboxymethyl cellulose and are now classified as glutathione S-transferases AA, A, B, C, D, E and M, (Ketley et al., 1975). These enzymes are characterised by their broad and overlapping second substrate specificities.

The mechanism of action of glutathione S-transferase has been suggested to be the result of a conformational change in the enzyme induced by the binding of glutathione to the enzyme, this change allowing the binding of the second substrate, (Gillham, 1973). The glutathione moiety is thought to be attached to its binding site on the enzyme in ionized form as the thiolate ion. This nucleophile is then able to react with any electrophile present in the second substrate which is bound at a site adjacent to the glutathione binding site as shown in Fig. 1.3. Glutathione conjugates have only been identified in the bile, but their metabolites, the mercapturic acids, occur in the urine. Metabolites of glutathione conjugates formed by removal of amino acid residues of the tripeptide moiety such as cysteinylglycine and cysteine of N-acetyl cysteine conjugates can also occur in the bile.

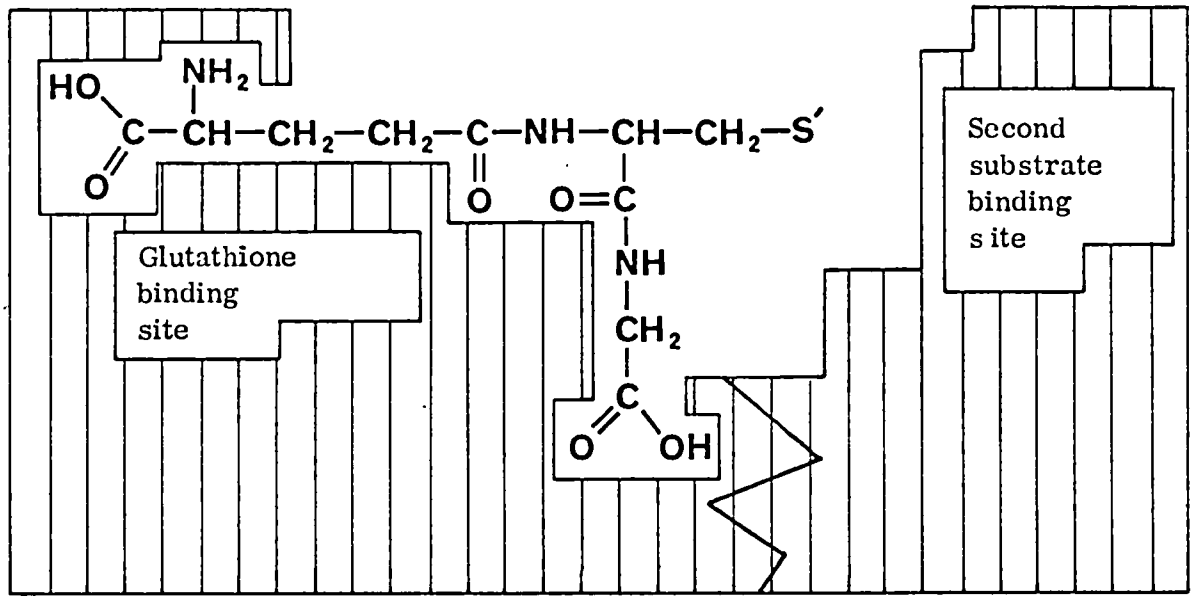


Fig. 1.3 Diagrammatic representation of the binding sites on glutathione S-transferase

Hepatic events contributing to EHC

The two phases of EHC occuring in the liver are biliary excretion and hepatic extraction or uptake. Hepatic metabolism, as stated earlier is often a pre-requisite for the EHC of a compound where the metabolically induced structural changes in compounds serve to promote their biliary excretion. If the processes of hepatic uptake, hepatic metabolism and biliary excretion are considered as belonging to separate compartments within the liver, the hepatic events of EHC may be represented by a simple compartmental model as shown below in Fig 1.4. Compounds metabolised by the liver pass through all three compartments between the blood and bile while compounds excreted in the bile unchanged may not pass through the compartment responsible for hepatic metabolism. There is evidence to suggest that certain compounds such as creatinine and erythritol pass directly from the blood to bile without entering the hepatocyte by an extra-cellular route, (Forker, 1967).

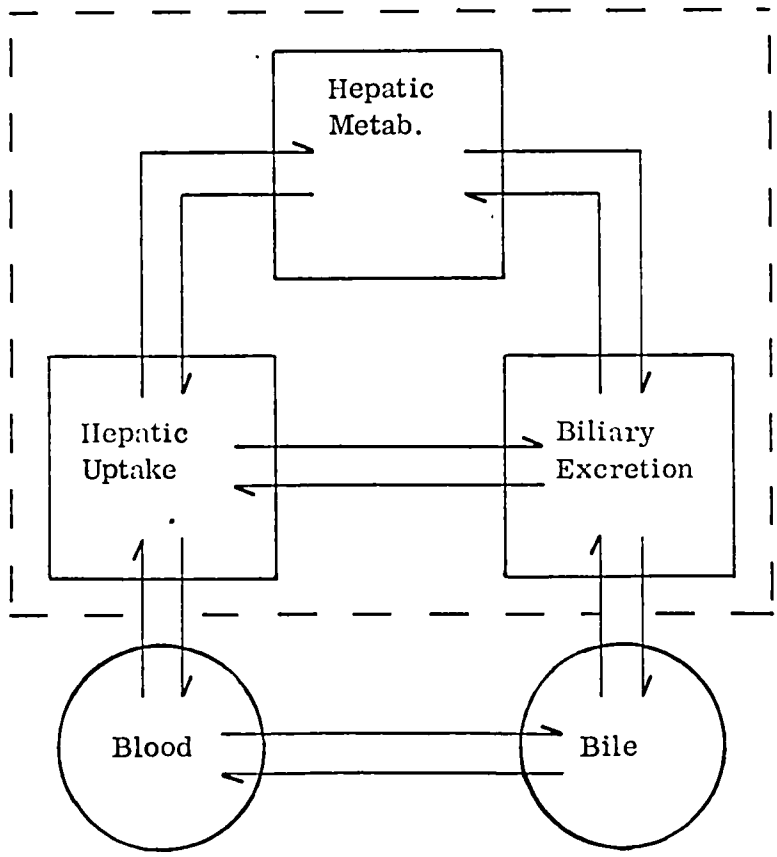


Fig 1.4 Diagrammatic representation of the hepatic events concerned with EHC

Anatomy of the liver

The liver is a wedge shaped organ arbitarily divided into four lobes and weighing about 1.5Kg in adult man. It's blood supply consists of the portal vein which provides about 80% of the hepatic circulation and the hepatic artery providing the remaining 20%. The portal vein carries the majority of blood draining from the gut in such a way that compounds absorbed from the gut into the blood stream via the portal vein, must first 'pass' the liver before entering the general circulation. The portal vein enters the liver at the porta hepatis where it repeatedly divides within the liver into smaller branches, finally entering an anastomosing network of sinusoids. The hepatic artery divides in a similar manner and hence the sinusoids contain mixed arterial and venous (portal) blood which percolates past the hepatocytes before finally entering the central veins. The central veins are branches of the hepatic vein which provides the venous return from the entire organ, draining into the inferior venae cava.

The earliest anatomical descriptions of the cellular organisation of the liver were derived from observations made in the pig (Kiernan, 1833). Octagonal tracts termed 'lobules' were seen to be separated from one another by septa of connective tissue. Within each lobule were rows of hepatocytes and sinusoids radiating from a central efferent vein. At the periphery of the lobule were areas containing branches of the hepatic artery, portal vein, bile ductules and lymphatics, (see Fig. 1.5a). Observations made in the liver under certain pathological conditions showed that only part of a lobule or adjacent portions of two neighbouring lobules were effected, giving rise to the suggestion that smaller liver units exist termed 'acini', (Rapport et al., 1954). The acinus is an area of tissue supplied by a primary afferent branch of the portal vein and hepatic artery, (see Fig. 1.5 b). Blood enters the sinusoids from the hepatic arteriole and portal venule, percolating along plates of hepatocytes to enter the central vein, (see Fig. 1.5 c). The hepatocytes secrete bile into the intra-cellular network of canaliculi which themselves drain into the biliary ductules.

Anatomy and physiology of the gall bladder

The gall bladder is a pear shaped organ located close to or between the liver lobes, it's neck narrowing to form the cystic duct which joins with the common bile duct. Composed mainly of muscle and connective tissue, the gall bladder functions as a storage organ for hepatic bile, it's contraction releasing bile into the duodenum via the bile duct, this process being under

Figure 1.5 (a) Hepatic lobule

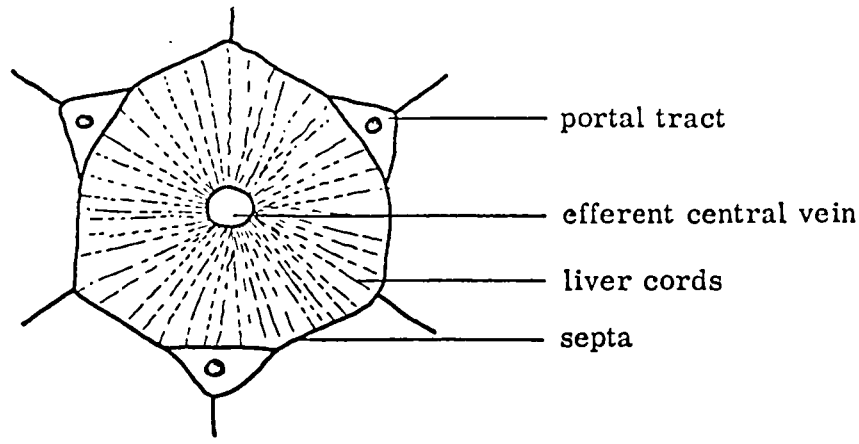


Figure 1.5 (b) Liver achinus

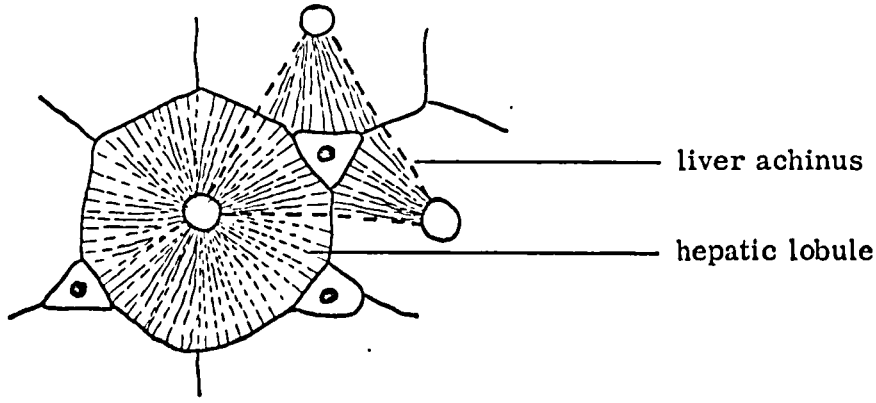


Figure 1.5 (c) Liver achinus-histological section.

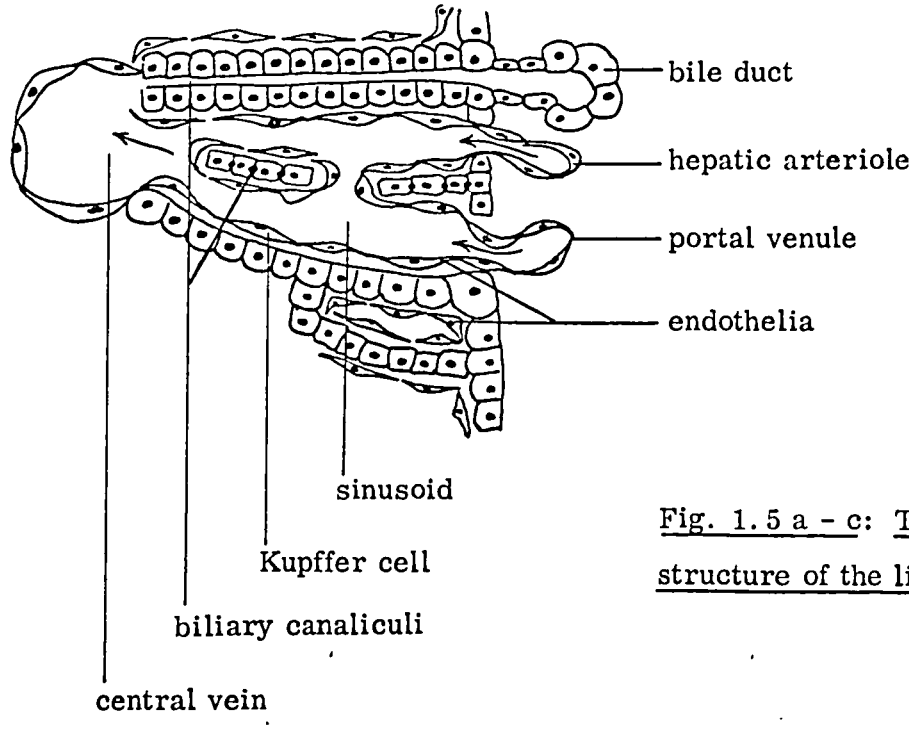


Fig. 1.5 a - c: The micro-structure of the liver.

hormonal control. Studies of bile release from the gall bladder show that the bulk is released intermittantly in response to the ingestion of food, although there appears to be a smaller continuous release from the organ even under conditions of starvation, (Hepner, 1975). Bile concentrating effects of the gall bladder are illustrated in Table 1.2 where it can be seen that most components of the bile in gall bladder bile exist at higher concentrations than those found in hepatic bile due to a re-absorption of water from the bile by the organ. Certain components present in the bile have been shown to be re-absorbed by the gall bladder, notably bicarbonate, chloride, sodium and potassium ions, so as to maintain the isotonicity of gall bladder bile with plasma, (Diamond, 1965). The primary re-absorption process, resulting in the difference in concentration of bile components between the bile of the gall bladder and that of hepatic bile, is thought to be the reabsorption of water and sodium chloride in isotonic proportions, (Diamond, 1965). The process by which solutes are absorbed may be a sodium chloride pump whereas the mechanism of water reabsorption is unclear, but thought to be co-diffusion, local diffusion or a double-membrane effect, (Diamond, 1965).

Bile composition

The composition of the bile varies widely with factors such as species differences and modification by the gall bladder, (see Table 1.2). Bile consists primarily of water (97 - 98%) while solids account for the remaining 2 - 3%. Bile acids and lipids make up 53 - 71% and 23 - 29% respectively of the total solids. The remaining solids include bilirubin and other bile pigments, inorganic salts and proteins.

a) Bile acids and their salts

In man the primary bile acids cholic and chenodeoxycholic acids are synthesised in the liver along a 15 step pathway from cholesterol (Danielsson and Sjovall, 1975). The control of bile acid synthesis appears to be regulated homeostatically by bile acids. However it follows that as cholesterol is the precursor of the bile acids, the supply of cholesterol may be the rate limiting step in bile acid synthesis, (Mitropoulus et al., 1973). Bile acids synthesised in the liver from cholesterol are conjugated in the liver with taurine and glycine, their conjugates forming salts with biliary metallic cations at biliary

Table 1.2 Composition of the bile

Constituent	Hepatic Bile	Gall Bladder Bile
Water	97 - 78%	84%
Bile Acids	1.2 - 1.7%	2.3 - 7.7%
Conjugated Bile Acids	0.96 - 1.2%	1.8 - 6.2%
Free Bile Acids	0.28 - 0.52%	20%
Cholic Acid	0.39 - 0.63%	1.2 - 3.3 %
Deoxycholic Acid	0.85 - 0.88%	1.1 - 4.3%
Bilirubin	17 - 71 mg/100ml	50 - 1000 mg/100ml
Cholesterol	86 - 176 mg/100 ml	100 - 900 mg/100 ml
Protein	180 mg/100 ml	450 mg/100 ml
Fatty Acids	0.02 - 0.14%	80-1, 600 mg/100 ml
Lecithin	250 mg/100ml	350 mg/100 ml
Carbohydrates	61 mg/100 ml	239 mg/100 ml
Reducing Sugars	30 mg/100 ml	80 mg/100 ml
Bases	80 - 90 meq/l	280 - 300 meq/l
Bicarbonate	20 - 25 meq/l	8 - 12 meq/l
Calcium	8 - 10 meq/l	25 - 28 meq/l
Chloride	90 - 100 meq/l	16 - 19 meq/l
Phosphorus	14.8 mg/100 ml	140 mg/100 ml

from - Documenta Giegy Scientific Tables (1970)

pH to form bile salts. The proportion of glycine to taurine conjugated with bile acids has been shown to vary with species (Haslewood, 1967). In man and rat, conjugates of both amino acids are formed whereas in the rabbit only glycine conjugates occur. In clinical conditions of cholestasis, cholic acid and chenodeoxycholic acids have been shown to form sulphate esters which are excreted in the urine, (Stiehl et al., 1976).

Metabolism of the primary bile acids by the gut flora of the colon and caecum give rise to the secondary bile acids. Deconjugation of the primary bile salts followed by the removal of the 7α-hydroxyl group from cholic and chenodeoxycholic acids gives rise to the major secondary bile acids deoxycholic and lithocholic acids respectively, (Bergstrom and Danielsson, 1965). The contribution of the secondary bile acids to the bile composition depends upon the amount formed in the gut and their ability to undergo EHC. Deoxycholic acid is the major bile acid in the rabbit gall bladder bile and constitutes 20% of the bile acids in human bile whereas in rat, deoxycholic acid reaching the liver is reconverted to cholic acid so is virtually absent in bile. The absence of lithocholic acid in the bile of many species may be explained by its poor intestinal absorption following binding to water insoluble residues and gut bacteria in the gut lumen, (Gustafson and Norman, 1962). The faecal bile acids are a complex mixture of gut microbial metabolites of the primary and secondary bile acids. The composition of the faecal bile acids will depend on the variety of gut flora present in the gut lumen. Their contribution to bile composition is small as relatively small amounts of faecal bile acids are formed in the gut lumen and are poorly absorbed from the gut.

b) Biliary lipids

Lipids occuring in the bile consist mainly of phospholipids and cholesterol with traces on cholesterol esters, glycerides and free fatty acids. The major bile phospholipids are phosphatidyl cholines (lecithins) with smaller amounts of lysophosphatidyl cholines, phosphatidyl ethanolamines and traces of sphigmomyelins, Lecithins account for over 80% of the total phospholipids in human bile, (Hauton, 1976). A difference between the fatty acid composition of lecithins found in the liver and those occuring in the bile, (Zilversmit and Van Handel, 1958) has suggested that there is a specific pool in the liver from which biliary

lecithins are derived, (Nilsson and Schersten, 1970). Other workers believe that hepatic and biliary lecithins are derived from the same pool and that the different molecular species found in the bile result from selective steps in their biliary excretion, (Curstedt, 1974). A study of the distribution of CDP-cholicdiglyceride transferase, an enzyme involved in lecithin synthesis, indicated that the endoplasmic reticulum rather than the canalicular membrane, was the most likely site for the synthesis of biliary lethicins, (Gregory et al., 1975). Regulation of phospholipid synthesis is to some degree governed by bile acids but the extent to which they are involved is not known, (Gregory, et al., 1975). Cholesterol constitutes about 4% of the total bile solids and although insoluble in water, is present in the bile as a water soluble complex consisting of bile salts, non esterified cholesterol and phospholipids, (see Fig. 1.6). Carey and Small (1970) investigated the nature of the bile lipid complex using synthetic mixed micelles of lecithin, cholesterol and phospholipids, but recent evidence suggests that the models are too simple to be compatible with bile lipid complexes and that protein may form an integral part of the complex, (Hauton et al., 1965, Hauton, 1976). Biliary lipid complexes in detergent rich media have molecular weights of approx. 100,000, but increasing the detergent concentration leads to aggregation of molecules to form vessicles of 200 - 400 Å, (Carey and Small, 1970). The origin of the biliary lipid complex is thought to result from the physiological detergent solubilisation of the liver endoplasmic reticulum in the vacinity of the bile canaliculi by a high concentration of detergents such as bile salts, (Monet, et al., 1975).

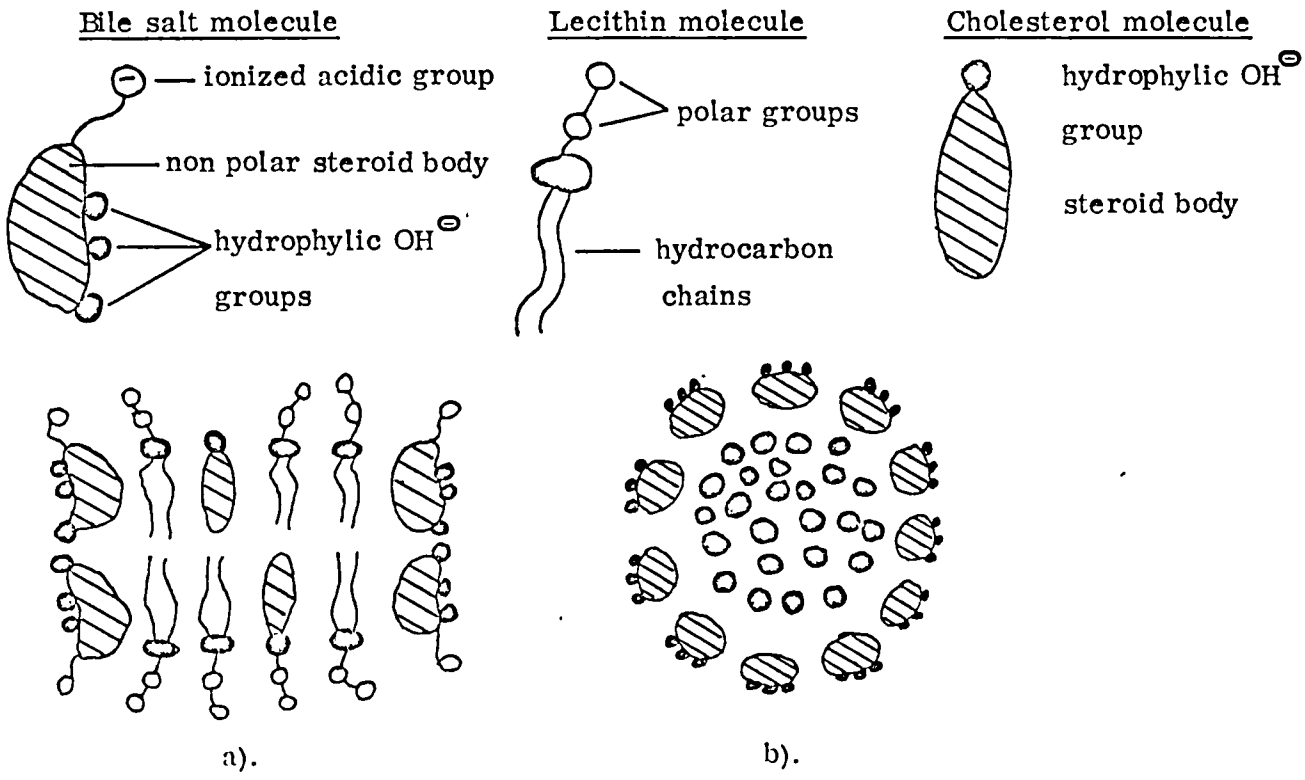
c) Bile pigments

The bile pigments are products of hepatic haemoglobin breakdown, bilirubin being the major bile pigment found in the bile of higher animals. Of the total bilirubin synthesised, about 70% is formed from haemoglobin, 15% from haem and 15% from other tetrapyrroles. Once formed in the liver, bilirubin can be transported in the blood bound to plasma albumin or conjugated in the liver and excreted in the bile. Two types of bilirubin conjugates have been characterised in the bile on the basis of their susceptibility to alkaline hydrolysis, (Billing et al, 1957). Between 85 and 90% of bilirubin conjugates were found to be alkali-labile while the remaining 10-15% were alkali-stable. Of the alkali-labile

conjugates, the bulk have been shown to be mono and diglucuronides, (Kuenzle, 1976). The remainder of the alkali-labile conjugates have recently been characterised and include xylosides and glycosides of bilirubin in dog bile and disaccharide containing conjugates in human bile, in all about 20 conjugates (Heirwegh, et al., 1975 and Kuenzle, 1975).

The alkali-stable conjugates were originally thought to be sulphates

Figure 1.6 - Models proposed for the structure of mixed micelles (Small 1970)
a) Bile salt-lecithin-cholesterol mixed micelle(longtudinal view)
b) Bile salt-lecithin-cholesterol mixed micelle(cross-section)



(Noir and Nanet, 1966), but other workers have found phosphates, (Tenhunen 1965), ethereal and N-glucuronides, (Gregory and Watson, 1962), derivatives involving glycine, (Jirsa and Vecerek, 1958a), taurine, (Jirsa and Vecerek, 1958 b) and methyl residues (Isselbacher and McCarthy, 1959). Recent opinions on their identity suggest that these early claims for characterisation of the alkali-stable conjugates do not stand up to critical evaluation as a result of their low purity, (Kuenzle, 1976).

Defects in bilirubin metabolism or its hepatic uptake, transport or excretion

resulting in plasma bilirubin exceeding 1 mg/100 ml (20 μmol/l) gives rise to the clinical condition of jaundice.

d) Inorganic ions in the bile

Bile has been shown to be isotonic with respect to plasma, but tonicity is subject to minor variations, (Prestig et al., 1962). An apparent difference between total solute concentration and osmotic activity can be explained by the fact that bile salts exist principally in the form of large polyionic aggregates or micelles, (Carey and Small, 1970). Bile concentration of Na, K, Ca and Mg are usually comparable with plasma concentration although water reabsorption by the gall bladder may result in slightly higher concentrations of these cations than occurs in plasma. The major inorganic anions of the bile, bicarbonate and chloride occur at higher concentrations in hepatic bile than in gall bladder bile due to either reabsorption by the gall bladder or ductal and/or ductular reabsorption from the biliary tree.

e) Proteins and enzymes in the bile.

Protein occurs in the bile in small amounts; Hauton et al., (1965) having identified albumins, γ-globulins and glycoproteins in the bile. The manner in which proteins enter the bile is not known but may be a result of the leakage of protein containing interstitial fluid across the bile canalicular membrane as many of these biliary proteins such as γ-globulins are not normally synthesised in the liver. The bile contains several enzymes including alkaline phosphatase and β-glucuronidase at concentrations higher than occur in plasma and acid ribonuclease and lactate dehydrogrenase at concentrations lower than occurs in plasma.

The origin of lipids, proteins and enzymes present in the bile in low concentrations is not known, but Carey and Small, (1970) proposed that the membrane solubilising properties of bile salts may account for their presence in the bile by the dissolution of the bile canalicular membrane during bile secretion. A comparison of the composition of the bile and that of canalicular membranes showed that polypeptide, lipid and protein composition differed radically however certain components notably glycoproteins enzymes were identical and may have been released into the bile as a result of detergent action, (Evans et al., 1976).

The Mechanisms of Bile Secretion

The precise manner in which bile is formed is not known. In evaluating the liver's excretory functions, physiologists have, in the past, compared them with those of the kidney, which at that time were better understood. Our knowledge of the renal excretion process at the present time still remains superior to the understanding of the mechanisms of bile formation. Physiologically the liver is primarily a metabolic organ with secondary excretory activity whereas in the kidney the roles are reversed. Insight into the excretory function of the liver is complicated by the probability that the metabolic and excretory roles are more intimately related in the liver than occurs in the kidney. Despite the differences in their primary functions, the liver and kidney show some remarkable similarities in their excretory characteristics such as their response to choleretics, diuretics and the clearance of dyes such as phenol red and fluoroscein (Sperber, 1959) suggesting that for certain compounds, similar processes are involved in their excretion in both bile and urine. Techniques employed successfully in elucidating renal function such as stop flow analysis and micropuncture of kidney tubules for the collection of primary urine (Lassiter, 1975) have not been applied with much success in the liver. It has been said by Brauer (1965) that the major breakthrough in renal physiology occured through the ability to sample primary urine and until similar techniques become available for collecting primary bile there can be no major advances in the understanding of bile secretion, a view which is still held by workers in this field of research.

Early attempts to define the mechanisms by which compounds passed from blood to the bile were reviewed by Brauer (1959). He classified the components of the bile into three groups on the basis of their relative concentrations as found in plasma and bile. Compounds of Class A included Na^+, K^+, Cl^- and glucose where their plasma/bile ratios were of the order of 1:1. Class B compounds were characterised by bile concentrations greatly exceeding their plasma concentrations by a factor of 10 - 1000 times and included bile salts, bile pigments and dyes such as bromosulphophthalein, (BSP). Compounds of Class C such as insulin, sucrose, phosphate, muco and plasma proteins showed plasma concentrations greatly exceeding those to be found in the bile.

The similarity in the plasma/bile ratio of Class A compounds suggested that a diffusion or filtration mechanism from plasma to bile would explain their transfer. Evidence obtained primarily through work in the isolated perfused liver suggested that active transport rather than diffusion or filtration was involved in the transfer of both Class A and B compounds from plasma to bile and thus forming bile.

The mechanism of bile secretion proposed by Brauer was in part challenged by Sperber (1959). While Sperber would agree with Brauer that no mechanism comparable with glomerular filtration exists in the liver, he does not rule out the possibility that osmotic filtration contributes to bile formation. He suggests that the primary event would be the active transport of bile salts and other Class B components of the bile into the bile canaliculi, the osmotic effect of which would result in a flow of water, dissolved molecules and ions into the canaliculi. A direct quantitative relationship between bile flow and bile salt concentration has been observed in the dog (Prestig, et al., 1962), the rat, (Boyer and Klatskin, 1970, Klaassen, 1971) and man, (Schersten , et al., 1971). Although the so called 'bile salt dependent' fraction is generally accepted as being involved in canalicular bile flow, there is evidence to suggest other processes may contribute to the overall flow. It can be shown that the bile flow remains high in bile duct cannulated rats where the bile salt pool is rapidly depleted (Light et al., 1959) and in the isolated perfused liver where no enterohepatic circulation of bile salts occurs (Boyer and Klatskin, 1970), which has suggested the presence of a 'bile salt independent' fraction. Studies in rabbits have shown a large hepatocytic bile salt independent fraction of 60μl/min/Kg body weight that is 60% of the spontaneous flow (Erlinger et al., 1969). Similar bile salt independent flows have been reported in dogs, (Wheeler et al.,1968), rats (Boyer and Klatskin, 1970, Berthelot et al., 1970) and in man (Boyer and Bloomer, 1974). The exact nature of this fraction is unclear, but Erlinger et al., (1969, 1970) have suggested that it involves the transport of sodium across canalicular membranes linked with Na^+/K^+ ATPase, an enzyme controlling the movements of sodium and potassium and shown to be present in canalicular membranes (Song et al., 1969). The dependence of bile flow on cell ATP and Na^+/K^+ ATPase levels has been indicated by Slater and Delaney (1970). Impairment of the Na^+/K^+ ATPase has been seen to result in higher

levels of intracellular sodium (Elshove and Van Rossum, 1963). Inhibitors of the Na^+/K^+ ATPase such as ouabain and diuretics (Graf et al., 1972, a,b) resulted in an increased bile flow in both isolated perfused rat livers and in intact rats, ouabain increasing bile secretion by up to 277% of the basal level in a dose dependent fashion. A recent study with ouabain has suggested that it acts by cotransporting sodium with ouabain into the cell rather than inhibition of the Na^+/K^+ ATPase (Graf and Peterlik, 1976). The suggestion that the major thermogenic effects of thyroid hormone is via the regulation of Na^+/K^+ ATPase (Izmail-Beigi et al; 1970) prompted Boyer (1976) to study the effects of thyroxine on bile flow. He observed that both the bile salt independent flow and Na^+/K^+ ATPase levels were increased in hyperthyroid animals and both were reduced in hypothyroid animals, the later situation restored to normal levels by the administration of L- thyroxine. The probability that the bile salt independent flow is related to sodium transport and Na^+/K^+ ATPase activity has recently given rise to suggestion that the bile salt dependent flow may simply be due to the activation by bile salts of the cation-adenine nucleotide mechanism involved in the bile salt dependent flow (Taylor, W., 1976, Soloway et al., 1973). In summary, it appears that bile secretion is initiated by active transport of bile salts and another solute, possibly sodium. The osmotic gradient created by the active transport of these solutes would then initiate the passive movement of water (Diamond and Bossert, 1968, Forker, 1968).

Recent study of the widely accepted 'straight line' relationship between bile salt concentration and bile flow now suggests that it cannot be adequately represented by a single regression line and that extrapolation to determine the bile salt independent flow is not valid, (Balabaud et al., 1977). These findings would indicate that it is not possible to assess the bile salt dependent and independent fractions as though they were totally separate.

A third fraction thought to contribute to bile secretion is the ductal and / or ductular bile salt independent flow. Ductal or ductular secretion was first demonstrated in response to the choleretic hormone secretin in several species including man, (Grossman et al., 1949) whichresulted in addition of an aqueous solution of inorganic electrolytes especially bicarbonate and chloride to basal bile flow without modifying bile salt excretion, (Prestig et al., 1962). It is thought

that this particular bile salt independent fraction is ductular or ductal rather than hepatocytic, (Wheeler et al., 1968). In the absence of secretin, the secretory function of bile ducts is unclear, but in certain clinical conditions which result in proliferation of the bile ducts such as Caroli's disease and cirrhosis, a marked hydrocholeresis is observed (Turnberg et al., 1968, 1970). The use of techniques such as stop flow analysis and in vivo/in vitro bile duct perfusion in the absence of secretin has shown that there is a water transport towards the duct lumen following the active transport of solutes, sodium, chloride and bicarbonate ions across the bile duct wall, (Chenderovitch 1968, 1971 and 1972). Water reapsorption from the biliary tree has been observed following intra-biliary injection, (Fujimoto and Peterson, 1973.)

The difficulties in evaluating the relative contributions of each of the three fractions to the overall flow again emphasises the importance of developing a technique for the sampling of primary bile, which at present, is not forthcoming.

Biliary excretion

The factors which effect biliary excretion can be divided into physico-chemical factors such as molecular weight, polarity and structural requirements, metabolic factors, species differences and sex differences.

1. Physicochemical factors

a) Molecular weight

Studies showing that fat soluble substances of high molecular weight were excreted in the bile in preference to the urine (Sobotka, 1937) gave rise to the possibility that molecular weight may be a factor involved in biliary excretion. Brauer (1959) indicated that substances excreted extensively in the bile were often organic carboxylic acids of molecular weight in excess of 300. Sperber (1963), comparing the urinary and biliary excretion of compounds, stated that compounds excreted extensively in the urine had molecular weights of about 200 - 400 whereas those excreted in the bile had higher molecular weights. The study of biliary excretion of endogenous compounds (Dittmer, 1961) has shown that compounds of high molecular weight (500 - 1000) such as bile salts, bilirubin and conjugates of some steroid hormones were well excreted in the bile whereas amino acids, sugars and urea of lower molecular weight were poorly excreted in the bile.

Further evidence for the relationship between molecular weight and extent

of biliary excretion came from the study of the excretion of foreign compounds whereby increasing their molecular weights by the introduction of additional groups into the molecule has resulted in increases in their biliary excretion. Examples of such increased biliary excretion has been shown following iodination of anthranilic acid (Williams et al., 1965), bromination of phenolsulphonphthaleins, (Kim and Hong, 1962), para substitution of phenylsulphate with a cyclohexyl group, (Hearse et al., 1969) and the introduction of a thiazole group into sulphanilamides (Hirom et al., 1972 a), (see Table 1.3).

Early work in this department studying the physicochemical factors influencing the biliary excretions of xenobiotics has defined molecular weight thresholds for biliaryexcretion which have been found to vary according to species, (Millburn et al., 1967, Abou-El-Makarem, 1967, Hirom et al., 1972 b).

The relationship between molecular weight and biliary excretion is not thought to be a direct consequence of molecular size except at high molecular weights where an upper molecular weight threshold may exist for biliary excretion. The existence of an upper molecular weight threshold would agree with the findings of Brauer (1959)that macromolecules such as proteins, phospholipids, and polysaccharides of the molecular weight exceeding 5000 were not excreted to any significant extent or absent from the bile. The trend towards increasing lipid solubility with increases in molecular weight (Hirom et al., 1974) coupled with the tendency for lipid soluble compounds to be preferentially excreted in the bile rather than in the urine may go some way to explaining the relationship between molecular weight and biliary excretion, (Millburn, 1976).

Although most compounds conform to molecular weight thresholds, there are a few exceptions such as certain azo dyes with molecular weights in the order of 600 - 700 (Richardson, 1939) which are known to be poorly excreted in the bile. Smith (1973) has suggested that these particular azo dyes form an aggregate of molecular weight approaching 8,000 and may exceed the maximum threshold for biliary excretion.

From the study of the relationship between molecular weight and biliary excretion, it becomes apparent that the bile and urine are complementary pathways for the excretion of xenobiotics (Hirom et al., 1976). Urinary excretion is greatest for compounds of low molecular weight and decreases with increasing

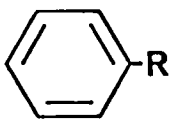
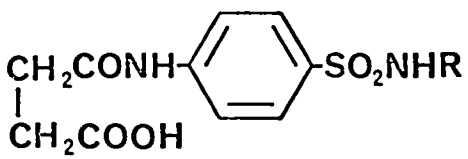
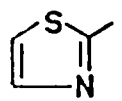
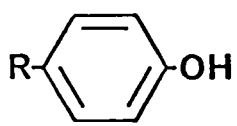
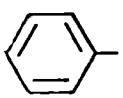
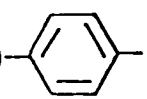
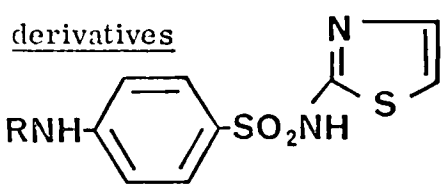
STRUCTURE	COMPOUND	R=	Mol Wt	% bile	Ref
Monosubstituted benzenes 	Benzene	H	78	1	Abou-El-Makarem (1967)
	Toluene	CH_3	92	2	
	Aniline	NH_2	93	6	
Sulphonamides 	Succinylsulphanilamide	H	272	5	Hirom et al., (1972 a)
	Succinylsulphacetamide	$COCH_3$	314	4	
	Succinylsulphathiazole		355	50	
Monosubstituted phenols 	Phenol	H	94	1	Abou-El-Makarem (1967) Millburn et al., (1967)
	4-hydroxylbiphenyl		170	37	
	4'4 dihydroxybiphenyl	HO- 	185	65	
Sulphathiazole derivatives 	Sulphathiazole	H	255	4	Hirom et al., (1972 a)
	Oxalylsulphathiazole	HOOC.CO-	327	21	
	Succinylsulphathiazole	$HOOC(CH_2)_2CO-$	355	54	
	Glutarylsulphathiazole	$HOOC(CH_2)_3CO-$	369	74	

Table 1.3 Relationship between molecular weight and extent of biliary excretion in several groups of compounds of similar structure.

molecular weight while biliary excretion becomes more extensive. This is best demonstrated by the studies of excretion of compounds in animals in which their biliary or urinary excretion has been prevented by ligation of the bile duct or renal pedicles respectively, (Table 1.4).

Table 1.4 Comparison of excretion of compounds in intact, bile duct and renal pedicle ligated rats.

Compound	Mol Wt	Intact		RPL	BDL	Ref
		B	U	B	U	
I) Benzoic acid	179	1	-	5	-	Abou-El-Makarem (1967)
II) Glutarylsulphathiazole	369	42	47	87	84	Hirom (1970)
III) Indocyanine green	752	82	-	-	0	Millburn (1976)

* Figures represent the % of the administered dose excreted in the bile and / or urine of intact, bile duct ligated (BDL) rats or rats having ligated renal pedicles (RPL).

It appears that compounds of molecular weight close to the molecular weight threshold (II), bile and urine are complimentary pathways for elimination, but as the molecular weight of compounds diverge from the threshold value (I and II) they are increasingly handled by one pathway only.

The biliary excretion of monoquaternary cations also appears to be determined by molecular weight, but the minimum threshold molecular weight for cations (200 ± 50) is lower than the corresponding value for anions and shows no significant species variation unlike the variations observed with anions, (Hughes, et al., 1973). It is thought that the transport of quarternary ammonium compounds from blood to bile occurs by a process which is different from that for organic anions (Schanker and Solomon, 1963) which may explain the differences between molecular weight thresholds for cations and anions.

b) Polarity

Evidence suggests the need for a strong polar water soluble group in molecules for their extensive excretion in the bile, (Millburn et al., 1967). Such polarity requirements can be supplied by an ionizable group in the molecule such as a carboxylic or sulphonic acid group, a sulphate or quarternary ammonium group allowing the molecule to exist as water soluble cations or anions at physiological

p H or as a water soluble sugar residue as occurs in glycosides as shown in Table 1.5. For many compounds, the polar group is incorporated into the molecule as a result of metabolic conjugations with glucuronic acid, or amino acids or glutathione which then allows them to be excreted in the bile as conjugates. Prevention of their conjugation either by chemically altering the molecule (Jirsa et al., 1968) or by inhibition of enzymes involved in conjugation (Levine et al., 197b) can reduce the extent to which such compounds are excreted in the bile. Compounds containing polar groups as an integral part of their structure are often excreted in the bile unchanged, examples being several of the cardiac glycosides (Smith, 1973). Compounds not possessing polar groups, but fulfilling the molecular weight requirements for biliary excretion such as dieldrin (mol. wt. 381) are not excreted in the bile (Williams et al., 1965). More direct evidence for the necessity of a polar group in biliary secretion comes from the systematic studies of biliary excretion of sulphathiazole derivatives which suggests the need for a polar carboxyl group in the molecule for the extensive biliary excretion of these compounds, (Hirom et al., 1972 a).

c) Structural requirements

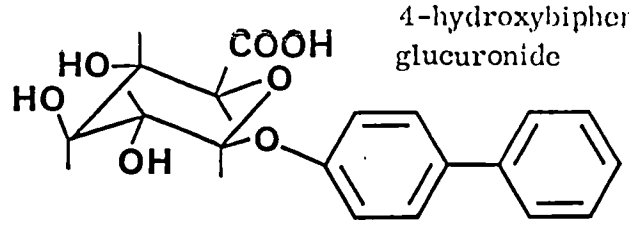
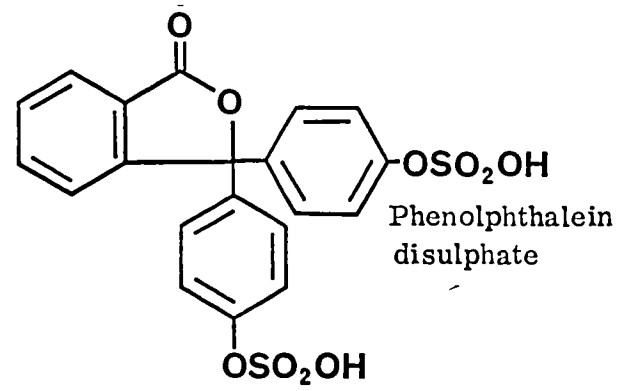
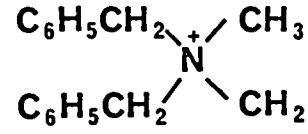
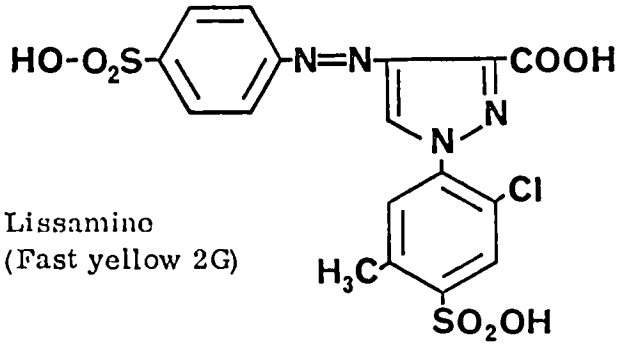
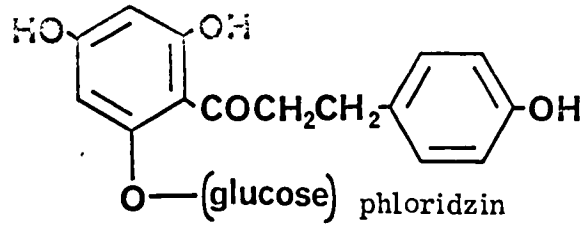
It can be shown thatby bringing about certain structural changes within a compound so as to alter the relative positions of particular groups, while retaining a similar molecular weight and polarity, the extent to which the compound is excreted in the bile can be significantly altered.

A change in the position of a single SO_3^- group in several sulphonated dyes was seen to result in a 4 - 8 fold change in the extent of their biliary excretion, (Iga et al., 1970, 1971). Similarly, structural changes affecting the biliary excretion of other compounds have been reported, (Ryan and Wright, 1961, Williams et al., 1965, Hirom et al., 1972a and Millburn et al., 1967).

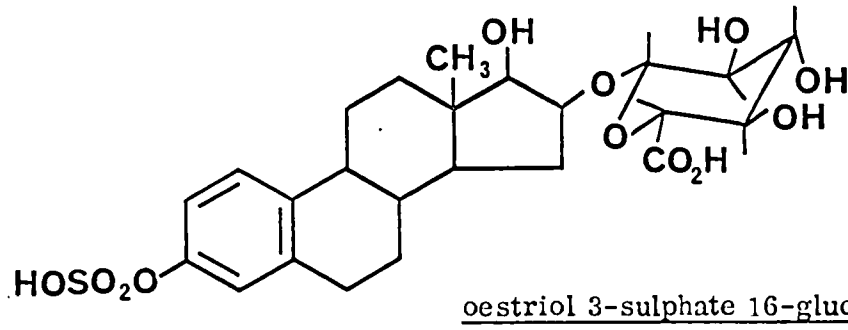
2. Metabolic factors influencing biliary excretion

It has been stated earlier that a compound may attain the necessary polarity requirements for an extensive biliary excretion as a result of its conjugation with a conjugating agent possessing a strongpolar group such as the carboxyl grouping of glucuronic acid. Besides providing the polarity requirements, conjugation will also give rise to an increase in molecular weight, which, depending on the molecular weight of the compound and the conjugating agent may significantly increase the extent to which the compound is excreted in the bile. The commonest conjugates excreted in the bile are glucuronides and glutathione conjugates

Table 1.5 Polar groups important for the extensive secretion of compounds in the bile

Group	Ion structure	Example
Carboxyl	$-C(=O)-O^-$	 4-hydroxybiphenyl glucuronide
Sulphate	$-OSO_2-O^-$	 Phenolphthalein disulphate
Quarternary ammonium	$-N^+(R)_3$	 Tribenzylmethyl-ammonium
Sulphonate	$-SO_2O^-$	 Lissamine (Fast yellow 2G)
Glycoside	various sugars	 phloridzin

which, in combination with a compound, will increase its molecular weight by 176 and 306 respectively where a single glucuronic acid or glutathione group is involved in the conjugate. If more than one conjugating agent grouping is involved in the conjugate, such as oestriol 3-sulphate 16-glucuronide, a correspondingly greater increase in molecular weight is achieved.



oestriol 3-sulphate 16-glucuronide

Examples of conjugation resulting in an increase in biliary excretion are shown in Table 1.6, where conjugation has been depressed by the inhibitor SKF, 525A (Levine et al., 1970b). Compounds normally excreted in the bile as conjugates (B)

Table 1.6 Effect of SKF 525A on the biliary excretion of compounds

Compound		Mol. Wt.	Normal % bile	SKF525A % bile
Succinylsulphathiazole	A	355	20	20
Phenolphthalein glucuronide	A	495	40	40
Stilboestrol	B	268	40	22
Phenolphthalein	B	318	82	70

* Values represent the % of the administered dose excreted in the bile of normal and SKF525A treated rats.

show a reduced biliary excretion following SKF525A treatment whereas compounds normally excreted in the bile unchanged (A) show no change in biliary excretion following similar treatment.

3. Species differences in biliary excretion

It has been stated earlier that for organic anions there was a marked species

variation in the minimum molecular weight threshold for biliary excretion which was not observed with cations. Evidence suggests that compounds of molecular weight up to 300 are poorly excreted in the bile of most species and that compounds of molecular weight exceeding about 500 are extensively excreted in the bile of most species. A species variation in biliary excretion only becomes significant for compounds of molecular weight of approximately 300 - 500 which coincides with the range of estimated molecular weight thresholds for biliary excretion for most of the species studied. It is known that many compounds exhibit considerable species variation in the manner and extent to which they are metabolised, which, for reasons stated earlier, may ultimately affect the extent to which they are excreted in the bile, (Hirom et al., 1977). As a species variation has been observed in the extent to which certain compounds are excreted in the bile unchanged, it appears that species variation in metabolism is not the only contributing factor to the observed species differences in biliary excretion, see Table 1.7. The reasons for species variation in biliary excretion of compounds within the molecular weight range of 300 - 500 is unclear but does not appear to be due to physiological or anatomical differences between species, (About-El-Makarem, 1967). The most likely explanation for the species variation is thought to be the biliary excretion process itself such as the rate and nature of the transfer of compounds between the blood and bile, (Millburn, 1976).

Table 1.7 Species variation in the biliary excretion of compounds

Compound		Mol Wt (in bile)	% excreted in bile in: - rat	rabbit	guinea pig	cat	man
Benzoic acid	I	179	1	1	2	1	-
Morphine	I	461	49	8	48	9	2
Phenolphthalein	I	495	85	25	22	13	26
Succinylsulphathiazole	II	355	36	1	6	7	-
Lissamine fast yellow	II	502	90	84	75	-	-
Indocyanine green	II	752	82	94	97	-	66

* I - excreted in bile as conjugates
II - excreted in bile unchanged

(Chernov and Woods (1965), Abou-El-Makarem (1967), Hirom et al., (1972b), Hirom et al., (1977), Pekanmaki and Salmi (1961).

4) Sex differences in biliary excretion

The subject of sex differences with respect to biliary excretion has received little attention and has only been reported in the rat with a few compounds. Tartrazine has been shown to be excreted to the extent of 13% in male rats as compared with 29% for female rats, while identical studies in guinea pigs and rabbits revealed no significant difference between the sexes. Similarly for the dye lissamine fast yellow, this compound was excreted to the extent of 80% in male rats and 90% in femalerats and again identical studies in guinea pigs and rabbits showed no significant differences between sexes, (Gregson et al., 1972). Following treatment with oestradiol, the biliary excretion of tartrazine in the male rat has been shown to be increased to the extent observed in female rats while the treatment of femalerats with testosterone decreases the biliary excretion of tartrazine to that found in normal males, (Bertagni et al., 1972). The female dominance in extent of biliary excretion in the rat does not occur for chlorothiazide where male rats excreted more of the drug in the bile than female rats, (Smith, 1973). Sex differences in the biliary excretion of certain compounds can be attributed to differences in metabolism between the sexes, but for compounds excreted in the bile unchanged, sex differences are not adequately explained solely by differences in metabolism and may be due to variation in the biliary excretion process itself.

Hepatic Clearance

The term 'hepatic clearance' refers to the overall transport of compounds from the blood to bile via the liver, combining the processes of hepatic uptake and biliary excretion. Most organic compounds with certain exceptions are thought to take a trans-cellular route between the blood and bile (see Figures 1.4 and 1.7). The overall rate of transport between the blood and bile will depend on the individual rates associated with i) hepatic uptake, ii) transcellular transport and iii) exeretion from the hepatocyte into the bile. More specific constraints that may affect the rate of hepatic clearance are the possibility of protein binding and / or metabolism of the compound within the hepatocyte.

In order to ascertain the effect of these limiting factors on hepatic clearance rate, so called 'inert' substances have been studied. These inert substances such as mannitol, erythritol and creatinine have been shown to exhibit high

clearance rates from the liver associated with a direct passage through the liver and do not appear to be affected by protein binding or reabsorption from the biliary tree, nor are they metabolised, (Wheeler et al., 1968, Forker 1967). Evidence supporting their suggested 'direct' passage through the liver comes from the similarity in their clearance rates, despite the structural differences between the various 'inert' substances studied. The rate at which inert substances are cleared is particularly high when compared with other compounds and is thought to be a result of a special carrier mechanism existing for these substances or their passage along a theoretically faster and more direct extra-cellular route, (Forker, 1967).

Mechanisms of hepatic clearance

In general, the processes by which xenobiotics are excreted in the bile are obscure. However the mechanisms which have been proposed for the secretion of bile components discussed earlier are most probably also involved with the excretion of xenobiotics in the bile, (Millburn, 1976).

Compounds of Class B (Brauer, 1969) are most applicable to xenobiotics excreted in the bile which characteristically are polar, of molecular weight in excess of 300. A theory has been proposed to explain the findings that compounds of molecular weight below 300, are poorly excreted in the bile and on administration via intra biliary or retrograde infusion into the bile duct, are absorbed by the liver and can appear in the blood and urine. Compounds of molecular weight in excess of 300 which are extensively excreted in the bile, are not generally found to be absorbed by the liver following retrograde infusion. This has been suggested to be a result of a selective reabsorption process from primary bile for low molecular weight compounds by the biliary tree, (Clarke et al., 1971). A second theory suggests that many compounds excreted in the bile are amphipathic and as such may combine with the micellar complex of bile salts, cholesterol and phospholipids resulting in a concentration gradient between the hepatocytes and the bile along which compounds could be transported into the bile, (Hirom, 1970).

Recent views, which take into account the saturation and other kinetic characteristics of the hepatic clearance of organic anions, suggest the involvement of a carrier process which is discussed later in the context of hepatic uptake, (Paumgartner, 1976). The existence of a separate mechanism for the clearance

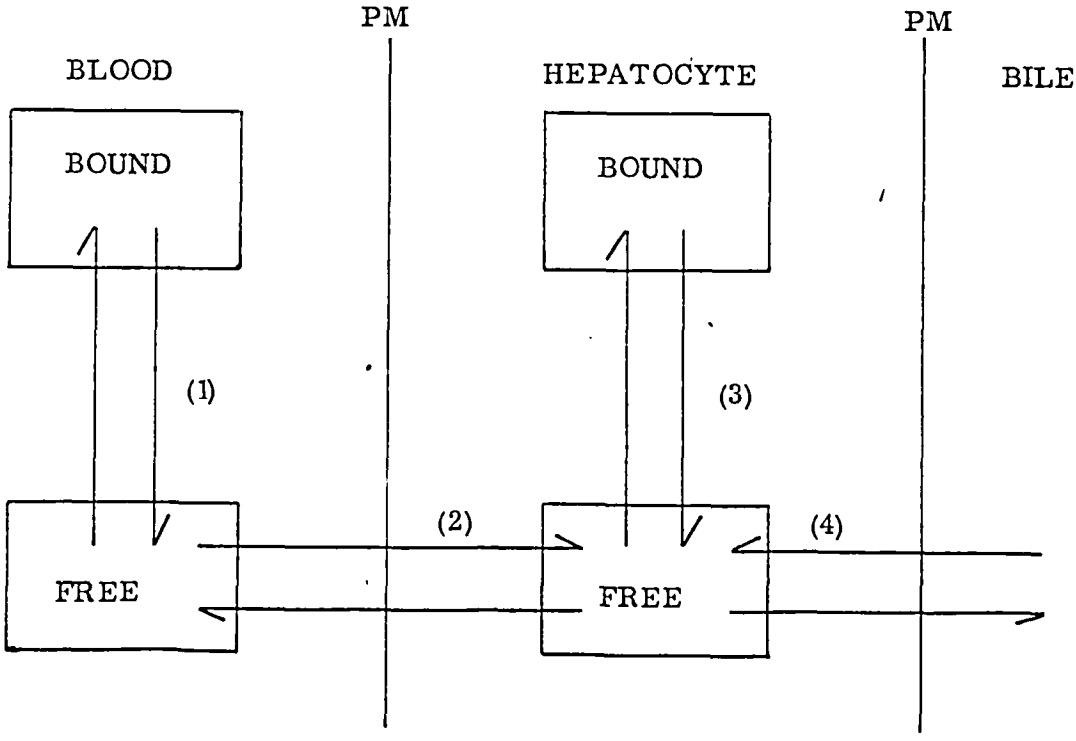
of cations has been proposed following the observation that although cations themselves compete for hepatic clearance, their clearance is not influenced by anions, (Schanker and Solomon, 1963).

Hepatic uptake

Hepatic uptake can be defined as the process by which compounds are transported from the blood into hepatocytes across a plasma membrane(s), separating the two compartments. This superficially simple process is complicated by the combined influences of plasma protein binding on the one hand and on the other intra-cellular processes such as cytoplasmic protein binding and biliary excretion, any of which may be rate-limiting to hepatic uptake.

Sperber (1959) saw hepatic uptake as part of a dynamic process under the influence of at least four potentially rate limiting steps across a cell separating the blood from the bile, (see Fig.1.7).

Fig.1.7 Schematic representation of hepatic uptake



Hepatic uptake is characterised by high specificity and the ability of the process to attain saturation. The mechanism by which compounds cross the plasma membrane(s) to enter the hepatocyte is not known, but is thought to be either a non-ionic diffusion involving cytoplasmic binding proteins (Arias et al., 1976) or a carrier mediated process (Paumgartner and Reichen, 1976).

a) Non-ionic diffusion and the involvement of cytoplasmic proteins

The possibility that non-ionic diffusion was the mechanism involved in hepatic uptake resulted from the identification of hepatic cytoplasmic binding proteins from the 100,000g supernatant of rat liver homogenates following gel filtration procedures, (Levi et al., 1969). Workers supporting this mechanism envisage the plasma and hepatocyte as two compartments separated by a plasma membrane with free unbound compound in equilibrium between the two compartments. Compounds cross the membrane by non-ionic diffusion so that the affinities of the compound for plasma and cytoplasmic proteins could be similar in this model, (Levi et al., 1969, Arias et al., 1976). The specificity requirements of hepatic uptake was satisfied in this model by the demonstration that cytoplasmic binding proteins themselves are specific in their binding properties (Arias et al., 1976). Saturation characteristics of hepatic uptake have been satisfied in this model by the limitations in the number of binding sites available on the cytoplasmic proteins. Initial binding studies with bilirubin and bromosulphophthalein (BSP) showed that these compounds were bound preferentially to three binding proteins occuring in the cytoplasm of hepatocytes termed X, Y and Z, of which Y and Z bound the greater proportion of the total bound bilirubin and BSP, (Levi et al., 1969). Studies with a wider range of compounds showed that Y protein was more specific in its binding properties than Z, and on this basis, Y protein was suggested to be more involved in selective hepatic uptake than was Z, (Levi et al., 1969). Y protein, known as 'ligandin', (Ketterer et al., 1971) and Z protein were not found to be exclusive to the liver and were identified in the small intestine (Levi et al., 1969) and the kidney (Fleischner et al., 1972) and known to occur in a wide range of species (Levine et al., 1971). Substantiation for the involvement of cytoplasmic binding proteins in hepatic uptake was demonstrated by the observation that compounds known to interfere with hepatic uptake such as flavaspidic acid-N-methyl glucaminate, bunamiodyl and iodipamide all compete for BSP binding

to Y and Z protein, (Levi et al., 1969). Both ligandin and Z protein have been shown to be inducible following pretreatment with dieldrin, DDT, 3-methylcholanthrene, benzpyrene, allylisopropylacetamide (Reyes et al., 1971) and phenobarbital (Fleischner et al., 1972), the later resulting in a 280% increase in hepatic concentration of ligandin. The increases in hepatic ligandin levels were all associated with an increased hepatic uptake of BSP and bilirubin. Ethinyl oestradiol known to induce cholestasis (Karkavy and Javitt, 1969) was shown to reduce levels of hepatic ligandin and Z protein with a parallel reduction in the hepatic uptake of BSP (Reyes et al., 1971).

Ontogenic studies in newborn guinea pigs, rats, monkeys and man showed impaired hepatic uptake of BSP, bilirubin and other organic anions which was associated with a virtual absence of ligandin in the livers of the newborn of these species which increased to adult levels within the first few weeks of birth, (Levi et al., 1970 and Levine et al., 1971). The identity of ligandin with two other proteins, corticosteroid Binder I (Singer et al., 1970) and carcinogen-binding protein (β-ABP), (Ketterer et al., 1967) was shown, indicating that ligandin was also capable of binding several steroid hormones and certain carcinogens and their metabolites, (Fleischner et al., 1971). Identification of a fatty acid-binding protein in the small intestine and other tissues utilizing fatty acids,including the liver, myocardium, adipose and kidney, showing characteristics of cytoplasmic binding proteins, has given rise to the suggestion that this protein may be identical to Z protein, (Ockner and Manning, 1974). Z protein had previously been shown to occur at higher levels than ligandin in the small intestine where it was thought it may have a role in intestinal absorption of compounds undergoing EHC, (Levi et al., 1969).

Ligandin has recently been shown to be identical with the enzyme glutathione S-transferase B, an enzyme involved in glutathione conjugation. Ligandin thus possesses both binding and enzymic properties, however the ligands it binds are not necessarily substrates for its enzymic functions and have been shown to inhibit the enzymic capacity of ligandin, (Habig et al., 1974).

The binding capacity of ligandin was originally thought to be restricted to organic anions, but is is now known to be capable of binding most ligands possessing electrophilic sites.

It is at this site that substrates for its enzymic action are attacked, (Habig et al., 1974). To summarise, the cytoplasmic binding proteins have been shown to exhibit many of the characteristics associated with hepatic uptake which strongly suggests their involvement in this process.

b) Carrier mediated processes

The kinetics of hepatic uptake have been studied extensively by Paumgartner and Reichen (1976) who suggest that hepatic uptake processes are characteristic of carrier mediated transport. The hepatic uptake of BSP (Goresky, 1975), taurocholate (Glasinovic et al., 1974, Reichen and Paumgartner, 1975), bilirubin (Robinson et al., 1971), indocyanine green (ICG) (Paumgartner 1975) and bile acids (Paumgartner and Reichen, 1976), show kinetics that are consistent with carrier mediated transport. For bile acids, the maximum rate of uptake appears to be effected by structural differences in the molecule, the number of hydroxyl groups seeming to alter the rate such that tri-hydroxy bile acids are taken up by the liver at twice the rate as for dihyroxybile acids, (Paumgartner and Reichen, 1976).

A comparison of the maximum uptake velocities with steady state excretory transport maximum suggests that the hepatic uptake is not the rate limiting step in the overall transport of compounds from blood to bile. The hepatic uptake capacity of taurocholate has been shown to exceed the excretory capacity by 4 times, (Paumgartner et al., 1974), by 8 times for bilirubin, (Robinson et al., 1971) and up to 20 times for ICG, (Paumgartner, 1975). Evidence for the inhibition between individual bile acids for hepatic uptake would indicate that there may be a common carrier system for bile acids (Paumgartner and Reichen, 1976). Absence of inhibition between taurocholate and ICG (Paumgartner and Reichen, 1975) and between bilirubin and taurocholate for hepatic uptake (Paumgartner and Reichen, 1976) suggests the possibility of different carrier systems for ICG and bilirubin to that for bile acid transport. Although the nature of the carrier mediated mechanisms are unknown, their involvement in hepatic uptake processes is supported by several workers, (Goresky 1964, Scharschmidt et al., 1974 and Paumgartner and Reichen, 1976). Despite argument as to whether non-ionic diffusion or carrier mediated transport is the primary event in hepatic uptake, the involvement of hepatic binding proteins in the process appears to be

accepted by most workers. The major difficulty in accepting the view that the primary event could be non-ionic diffusion is that for bilirubin at least, it's binding affinity to ligandin is less than that for plasma albumin, (Arias, 1972, Arias et al., 1975, Bloomer et al, 1973). Under such conditions an equilibrium between blood and hepatocyte directed towards the hepatic uptake of bilirubin would be difficult to envisage in the absence of an 'active' transport system such as the carrier mediated mechanisms as proposed by Paumgartner and Reichen, (1976).

The Intestinal Phase of Enterohepatic Circulation

Having been excreted into the duodenum via the bile, a compound or its metabolites may be subjected by up to three distinct processes on its passage down the gastrointestinal tract:

(i) metabolism within the gut lumen (gut microfloral metabolism)

(ii) intestinal absorption

(iii) metabolism within the gut wall.

As bile enters the gastrointestinal tract at the level of the duodenum we are mainly concerned with events occuring in the small and large intestines rather than the stomach.

Anatomy and histology of the gut.

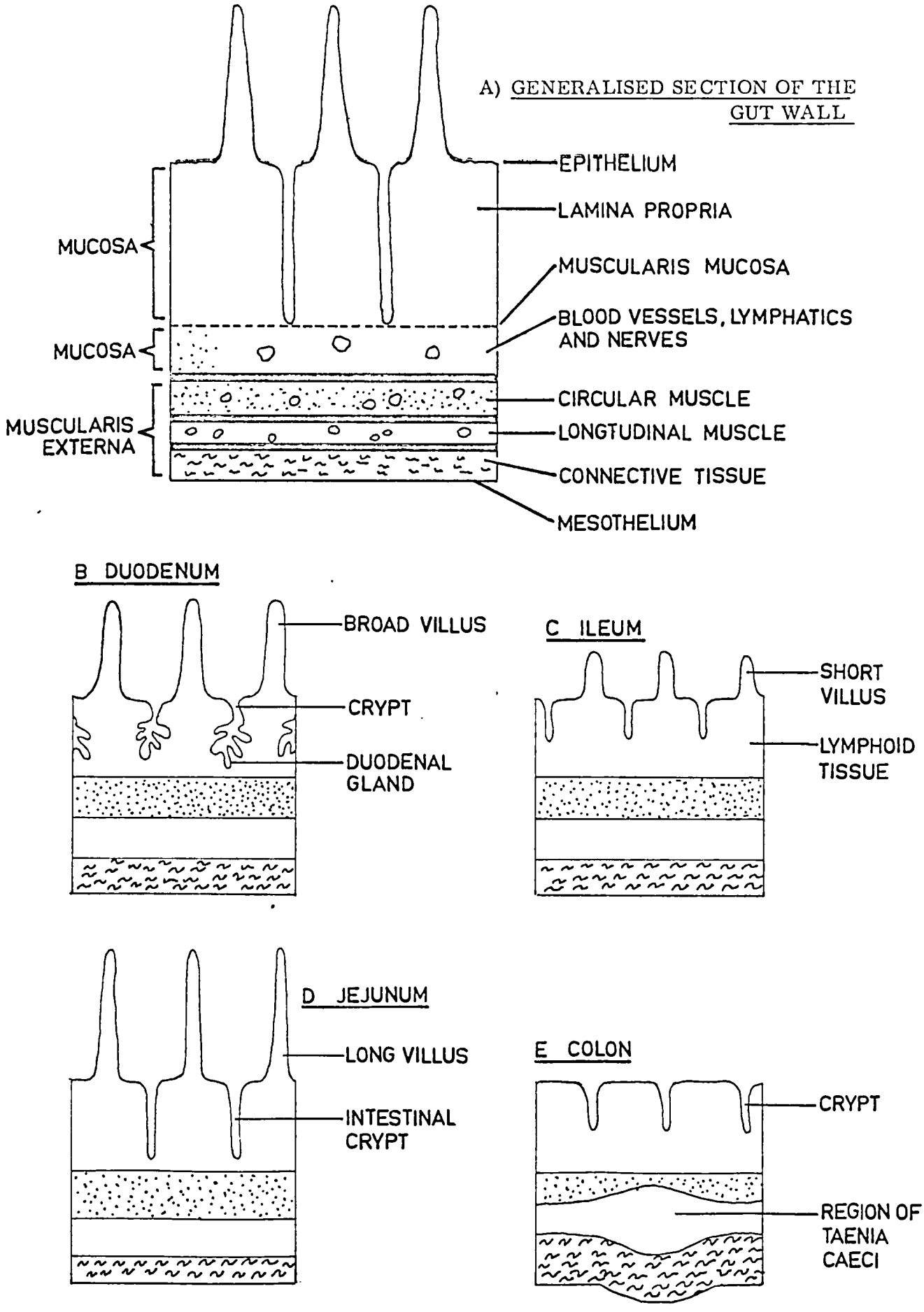
The section of the gastrointestinal tract below the stomach can be divided anatomically into the duodenum and the small and large intestine. The duodenum starts immediately below the pyloric sphincter and continues for a distance of approximately 22 cm in man, (Blackenhorn 1955) and 10 cm in the rat, (Lambert, 1965). The duodenum can be immediately recognised by its thick walls resulting from circular mucosal folding. The small intestine continues from the distal end of the duodenum for a distance of about 285 cm in man (Blackenhorn,1955) and 100 - 120 cm in the rat, (Fabry and Kujalova, 1960). The small intestine is divided into two sections, a proximal portion known as the jejunum which is broader and thicker walled than the distal section of ileum.

The large intestine is divided into the caecum, colon and rectum, starting below the ileocaecal valve and continuing for a distance of approximately 110 cms in man, (Blackenhorn,1955) and 15 - 20 cms in the rat, (Schanker, 1959). It is characterised by strips of longitudinal muscle or taenia caeci which extend from the caecum down to the rectum where they spread to form a continuous sheet of muscle.

Histological structure

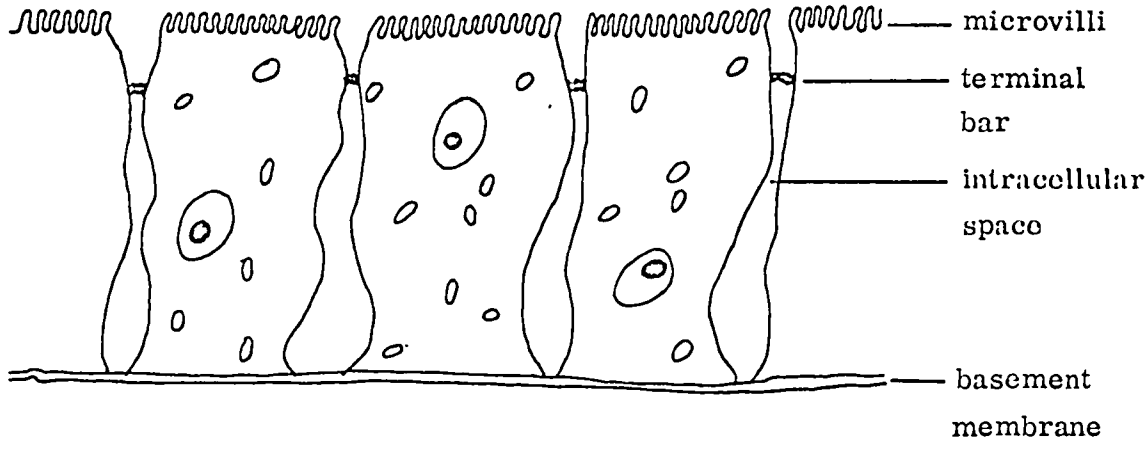
The histology of the gastrointestinal tract varies depending on the particular section examined, but can be generalised as shown in Fig. 1.8 (a). Major histological differences in the various sections of the tract occur at the mucosal level which is directly related to the absorptive capacity of the particular section (see Fig 1.8 b - e). The absorptive area of the gut is restricted to the

Fig 1.8 Anatomical and Histological Structure of the Gut



mucosa which is composed of finger like projections or villi which contain arterial, venous and lymphatic vessels for the transport of absorbed substances. The epithelium of the villus is composed of columnar absorptive cells, the luminal surfaces of which are composed of a brush border of microvilli which serve to increase the surface area available for absorption (20 - 40 m^2 in small intestine) as shown in Fig. 1. 9.

Fig. 1. 9 Section of epithelium of a villus



The large intestine is generally deficient in mucosal brush border, but certain absorptive areas are equiped with a brush border of microvilli.

1) Metabolism within the gut lumen (gut microfloral metabolism).

a) Nature and distribution of microflora

Approximately 60 different species of microorganisms have been identified from the gastrointestinal tract of man, (Donaldson, 1964); the dominant species being the bacteroides and bifidobacterium, (Syded, 1972), (see Table 1. 8). In man, the duodenum, jejunum and upper ileum are sparcely populated with microorganisms of which there are only a few different species, mainly gram-positive bacteria, coliforms and bacteroides, the rabbit showing a similar pattern in this region of the gut, (Drasar, et al., 1970).

Table 1.8 The common species of microorganisms found in the gut (Scheline, 1973).

MICROORGANISMS		TYPE
a) Bacteroides		Gm -ve rods, anaerobic
b) Lactobaccili	1. Anaerobic 2. Aerobic	Gm +ve rods
c) Fusobacterium		Gm -ve anaerobic rods
d) Enterobacteria	1. E. coli 2. Aerobacter 3. Klebsiella 4. Proteus 5. Providence group	Gm -ve aerobic or facultatively anaerobic rods.
e) Clostridia		Gm +ve anaerobic rods
f) Streptococci	1. Enterococci 2. Anaerobic	Gm +ve aerobic or facultatively anaerobic cocci. Gm +ve cocci.
g) Pseudomonads	1. Pseudomonas 2. Alcaligenes faecalis	Gm -ve aerobic motile rods
h) Staphylococci		Gm +ve, aerobic or facultatively anaerobic cocci.
i) Veillonella		Gm -ve, anaerobic, cocci.
j) Yeasts		

The distal ileum can be thought of as a transition zone from the point of view of numbers of microorganisms present, above which there is a sparce population and below which large numbers of microorganisms are to be found in most species. Below the ileocaecal valve there is a significant increase in the number and variety of microorganisms with anaerobic predominating over aerobic and facultative forms and consisting of mainly bacteroides and bifidobacteria. In addition to the longitudinal variation in microfloral population, a variation in the cross-sectional distribution has been shown where many bacteria are found to be associated with the intestinal mucosa, (Savage, 1970).

Although there is a general similarity in the microflora of different species of mammals, variations do occur as do differences in the distribution of microbial populations between species. An example of species variation in distribution of microorganisms is the high concentration of microflora in the upper gastrointestinal tract of rats and mice in comparison to the sparse population found in this region in man and rabbit.

Changes in the size of type of microorganisms in the gut microfloral population may occur with changes in diet (Donaldson, 1964), changes in environment and physiology (Dubos et al., 1967), starvation and age (Smith, 1965), disease (Tabaqchali, 1970) and on addition of foreign compounds (Finegold, 1970). The ability of antibiotics and related compounds to modify the gut flora, can provide a useful experimental tool for the study of the drug metabolising effects of the gut microflora.

b) Microfloral metabolism

Microorganisms of the gut flora are able to metabolise a wide range of foreign compounds by several different mechanisms as shown in Table 1.9. From the point of view of our work on EHC and the type of compounds we have studied, only the hydrdyc activity of microorganisms is relevent and so the hydrolysis of glucuronides is described.

c) Gut microfloral hydrolysis of glucuronic acid conjugates

The hydrolysis of glucuronides in the gastrointestinal tract is a result of the hydrolytic activity of the enzyme β-glucuronidase which is capable of splitting the glucuronic acid group from the conjugated compound. β-glucuronidase was first shown to occur in the bacteria of E. coli, (Buehler et al.,

Table 1.9 Classification of metabolic reactions involving gut microflora

(Scheline, 1973)

A. Hydrolysis of	1. Glycosides	a. glucuronides
		b. other glycosides
	2. Sulphate esters	
	3. Amides	
	4. Esters	
	5. Sulfamates	
	6. Nitrates	
B. Dehydroxylation		
C. Decarboxylation		
D. Dealkylation		
E. Dehalogenation		
F. Deamination		
G. Heterocyclic ring fusion		
H. Reduction of	1. double bonds	
	2. nitro groups	
	3. azo groups	

1951) however, its scarcity amongst the total microbial population of the gastrointestinal tract indicated that it could not account for the total β-glucuronidase activity known to exist in the gut. Later evidence suggested that β-glucuronidase activity of the anaerobic bacteria bacteroides, bifidobacteria and lactobaccili could be held largely responsible for the bulk of β-glucuronidase activity in the gut. Studies in specific pathogen free (SPF) rats suggested that although anaerobic bacteria were responsible for the major portion of the total β-glucuronidase activity in the gut, a portion must result from sources other than those of microbial origin, possibly from the bile (Smith, 1973) or from the gut wall itself, (Eriksson and Gustaffson, 1970a).

The β-glucuronidase activity of gut microflora will depend primarily on the number of bacteria present in the population containing the enzyme and this may vary with species and with certain other factors as stated earlier such as diet, age, etc. Hanninen et al., (1968a) showed that the β-glucuronidase activity in the rat intestine increased linearly by 41% from the proximal to the distal end which may reflect the longitudinal distribution of bacteria containing the enzyme.

The significance of the microbial hydrolysis of glucuronides is considerable when it is appreciated that the majority of drugs known to undergo EHC are excreted in the bile as glucuronides. β-glucuronidase is not unique to the gastrointestinal tract and has been shown to occur in the bile (Smith, 1973), lung, spleen, thymus and liver (Aitio, 1973). The functional significance of this enzyme in these tissues is unknown, but it has been suggested that the presence of β-glucuronidase in target organs could hydrolyse hormone conjugates to release their pharmacologically active forms, (Fishman, 1961).

d) Gut microfloral hydrolysis of sulphate esters

Although relatively few compounds are excreted in the bile as sulphate esters when compared with those excreted as glucuronide or glutathione conjugates, sulphoconjugates are the primary biliary metabolites of many phenolic and steroid compounds, several of which are known to undergo EHC. Compared with the wealth of evidence supporting the microbial hydrolysis of glucuronides, there are relatively few reports of microbial hydrolysis of sulphate esters. An analogue of biscodyl known to be excreted in the bile as

a sulphate has been shown to be hydrolysed in the gut only to the extent of 4% of the dose in rats (Forth et al., 1972) which along with similar reports for other compounds would suggest that the sulphatase activity of the gut microflora is low. Sulphatase activity has been attributed to certain species of enterobacteria including Pr. Vulgaris, Proteus, Reggeri and A. Aerogenes, (Scheline, 1973). Several sulphoconjugates originally thought to be hydrolysed by microbial sulphatases such as sodium estrone sulphate, have later been shown to be hydrolysed within body tissues rather than by the gut microflora, (Dolly et al., 1971), which suggests that the hydrolysis of sulphate esters by gut microorganisms may be of limited importance.

2. Absorption from the gut

The absorption process by which compounds pass from the gut lumen across the intestinal mucosa into blood or lymphatic vessels can conveniently be divided into three phases:-

a) Passage of compound from the gut lumen across the mucosal membrane of the mucosal epithelium - the primary barrier to compounds being absorbed and hence the most studied phase of absorption.

b) Movement of compounds across the cytoplasm of the mucosal epithelium and basal membranes towards the blood and lymphatic vessels - the processes involved being unknown.

c) Uptake of absorbed compounds into blood and lymphatic vessels.

The passage of compounds from the lumen across the mucosal membrane

It is thought that at least three alternative routes exist for the passage of compounds from the gut lumen across the mucosal membrane:-

A) A carrier route associated with 'active' transport systems.

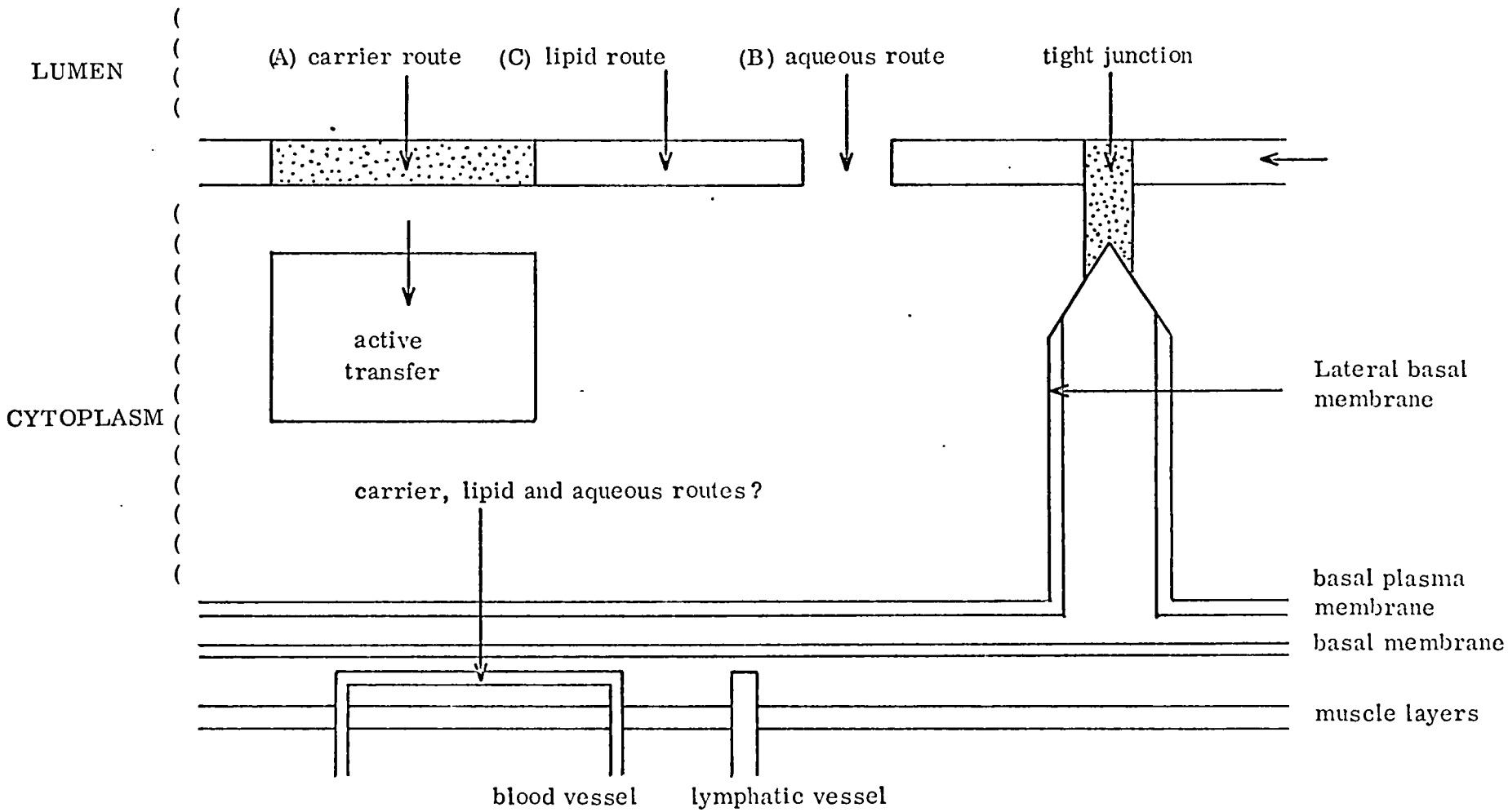
B) An aqueous route - restricted to polar compounds of small molecular size.

C) A lipid route restricted to lipid soluble compounds, as shown in Fig. 1.10.

A The carrier route

This particular route has received the greatest attention as it is responsible for the transport of amino acids and sugars. Carriers, specific for individual monosaccharides and amino acids have been postulated in order to explain the variation in absorption rates shown by the individual sugars and amino acids (Matthews, 1972, Milne, 1972 and Kohn et al., 1965). The absorption of amino acids and sugars have been shown to exhibit saturation characteristics which would indicate that they are transported by 'active' energy requiring mechanisms

Fig. 1. 10 - Uptake processes occuring across the intestinal mucosa



or facilitated diffusion, (Parsons and Boyd, 1972). Fujita et al., 1972) identified Na+/K+ ATPases in intestinal mucosal membranes which may be associated with the absorption of water, solutes, amino acids and sugars, (Semenza, 1972). Another possible source of energy is that which may be derived from an ion moving down a concentration gradient as there is evidence to suggest a linkage of this nature between sodium, amino acids and sugars, (Sladen and Dawson, 1969; Schultz and Curran, 1970).

The nature of the specific transport systems for amino acids and sugars are thought to be carrier proteins (translocases or permeases) and although certain proteins of this type have been isolated from intestinal tissue, they have yet to be positively identified as the specific carriers, (Semenza, 1972).

Unlike the situation existing for amino acids and sugars, very few foreign compounds are thought to be associated with the carrier route, but there are reports to suggest that cardiac glycosides and quarternary ammonium compounds are absorbed via an 'active' carrier route, (Lauterbach, 1972, Damm et al., 1975).

B. The aqueous route.

The so called 'aqueous route' has been suggested by Diamond (1971) to be the course taken by solutes and water via pores or localised polar regions in the intestinal mucosal epithelial membrane (Fig. 1.8). The simplest concept of this route was envisaged by Smyth (1966) as water filled channels along which hydrophylic substances pass down a concentration gradient. Apart from polarity requirements, compounds taking the aqueous route appear also to be limited to those of small molecular size as a result of the small pore size. It is thought that compounds having more than three carbon atoms cannot enter the mucosal membrane by this route (Smyth, 1966). With such entry limitations it seems unlikely that amino acids, sugars and most foreign compounds would be acceptable for transport by this route.

C. The lipid route

The absorption formost foreign compounds and non-electrolytes by the intestinal mucosa is via the lipid route by a process which has been termed non-ionic diffusion across the lipid phase of the intestinal mucosal membrane, (Schanker, 1962). A compound transported by this route must be lipid soluble but in addition must have some degree of water solubility, there being some

optimal relationship between these two requirements which enables the compound to pass through both the lipid and aqueous phases it must encounter on passage th rough the intestinal mucosa to the blood stream or lymphatics, (Penniston et al., 1969). This implies that increases in the lipid solubility of a compound beyond the optimal relationship would no longer be accompanied by increased absorption rate whieh may explain why it is that a drug such as dicumerol despite its high lipid solubility, is poorly absorbed from the intestine, Brodie 1964), Conversely it can be demonstrated that increasing the polarity of a compound beyond the optimum will reduce the rate it can be absorbed as is illustrated by morphine, codeine and thebaine, where morphine having 3 OH groups is absorbed from the intestine more slowly than codeine (2 OH groups) whereas thebaine,having no OH groups, is the most rapidly absorbed of the three (Nogami, 1968). The partition coefficient between a drug and a solvent system has been widely used in assessing the lipid solubility of the drug in relation to their intestinal absorption rates. The poor correlations between the partition coefficientof a drug and its intestinal absorption rate may be due to the diffieulty in finding a model solvent system which is representative of the lipid phase of intestinal membranes (Kurtz, 1971) or that the partition coefficient refers to an equilibrium state whereas drug absorption from the intestine is a dynamie process (Dolvisio et al., 1970)

As many drugs are weak acids or weak bases their absorption will depend upon the degree of ionisation they exhibit in the various seetions of the gut. Generally speaking only non-ionised molecules are able to pass through the lipid phase of the intestinal mucosal membrane, hence the term non-ionic diffusion, whereas the ionised form is not able to do this due to its small lipid solubility. If the pH of the intestinal lumen differs from that of the plasma, which only occurs to a significant extent in the stomaeh and duodenum, then in circumstances where compounds have pKa's close to that of plasma, the luminal pII may alter their degree of ionisation and eonsequently affect the extent of their intestinal absorption.

Passage of compounds aeross the cytoplasm and basal membranes of the intestinal mucosal epithelia.

As stated earlier the proeesses involved in this phase of absorption are unknown, but may be simple diffusion for most compounds along a concentration

gradient towards blood and lymphatic vessels.

Uptake of absorbed compounds into blood and lymphatic vessels

Having passed through the intestinal mucosal epithelium, an absorbed substance enters either the blood or lymph and in the former case, a concentration gradient for the substances will be set up between the serosal and mucosal sides of the mucosal epithelia. The steepness of the gradient is dependent on blood flow rate, but the slower the drug permeates the mucosal epithelium the less effect the blood flow will exert on the overall rate of absorption.

Transport of absorbed substances in the lymphatic system appears to be limited to chylomicrons, cholesterol, fat soluble vitamins and certain proteins, (Senior, 1964). The low flow rate of lymph, which is 500 - 700 times slower than blood flow, may partially explain why the lymphatic system is in general relatively unimportant for the transport of absorbed drugs, (De Marco and Levine, 1969 and Sieber et al., 1974).

3. Metabolism in the gut wall

The gut wall is capable of a wide range of metabolic reactions of both Phase I and Phase II types. The phase I reactions under the influence of mixed function oxidase enzymes known to occur in the gut wall have been classified by Aitio (1973) and include N-demethylation, O-demethylation, deethylation, aromatic hydroxylation, side chain cleavage, side chain oxidation, desulphuration, sulphoxidation and azo reduction. Phase II reactions occuring in the gut wall include conjugations with glucuronic acid and sulphate (Hietanen and Hanninen, 1974, Bennet et al., 1975 and Powell et al., 1974).

The implications of gut wall metabolism pharmacologically and toxicologically, become apparent when it is appreciated that most drugs loose some or all of their pharmacological activity as a result of metabolism especially conjugation reactions. As many drugs are administered orally, they may undergo metabolism in the gut during their absorption and thus may loose a proportional amount of their pharmacological activity and toxicity before reaching their pharmacological site of action within the body. The extent to which a drug is metabolised by the gut wall will depend on:

i) the affinity of the drug for the drug metabolising enzymes present in the gut wall.

ii) rate of metabolism occuring

iii) the absorption characteristics of the drug - the slower the rate of absorption, the more likely it is to be metabolised by the gut wall.

a) Conjugation with glucuronic acid

Glucuronidation was first identified in duodenal slices by Hartiala (1954) in conjunction with studies of the nature of duodenal and pyloric ulceration resulting from the oral administration of the drug cinchophen. It was shown that cinchophen induced glucuronic acid depletion in both hepatic and intestinal tissues resulting from the conjugation of glucuronic acid with cinchophen. Glucuronidation of cinchophen in the duodenal wall is thought to occur at the expense of the incorporation of glucuronic acid into mucopolysaccharides which are major constituents of the protective mucous lining of the gut. Depletion of the duodenal mucous lining is known to induce ulceration thus the glucuronic acid depleting action of cinchophen may precipitate this condition, (Hartiala, 1961).

The ability of the gut wall to conjugate drugs has been shown to vary along the gastrointestinal tract, (Hartiala et al., 1964). A later study has indicated that UDP-glucuronyltransferase activity, as measured by the rate of conjugation of p-nitrophenol and o-aminophenol, was at a maximum in the duodenum and progressively decreased on moving towards the colon which exhibited only 30% of the duodenal rate, (Hanninen et al., 1968a). In the same study, the β-glucuronidase activity of the gut wall was found to be minimal in the duodenum gradually increasing towards the colon which on first appearances could account for the decrease in UDP-glucuronyl transferase activity. Inhibition of β-glucuronidase by saccharo-1,4-lactone did not result in the expected stimulation of UDP-glucuronyl transferase activity and it seemed unlikely that with a pH optimum of 4, β-glucuronidase would be particularly active at cellular pH. Two possible explanations for the distribution of UDP-glucuronyl transferase activity in the gastrointestinal tract have been proposed, firstly that as this enzyme requires a continuous energy supply, the decrease in activity towards the colon may be the result of a steep energy gradient along the gut, (Hanninen et al., 1964, 1968a). Secondly that as the majority of compounds are absorbed in the upper intestine and as UDP-glucuronyl transferase is known to be an inducible enzyme

Hanninen (1966), absorption of compounds may induce the enzyme resulting in high UDP-glucuronyl transferase activity in this region of the gut, (Hanninen and Aitio, 1968b).

Several comparisons have been made between the glucuronidation capacity of the liver and that of the gut wall and other tissues known to contain UDP-glucuronyl transferase,(see Table 1.0).

Table 1.10 Comparison of UDP-glucuronyl transferase activity in various tissues

Tissue	Amount of glucuronide formed as % of liver production			
	a	b	c	d
Liver	100	100	100	100
Kidney	20	-	32	31
Duodenum	35	142	57	89

*a - Miettinon and Leskinen (1963) p- nitrophenol -♂ Wistar rats - microsomes
b - Chhabra and Fouts (1974) " " "
c - Aitio (1973) 4-methyl umbelliferone -♂ CD rats - microsomes
d - Hanninen et al., (1968a) o-aminophenol -♂ Wistar rats - tissue slices

The variation in the various estimates of enzyme activity shown in Table 1.10 may result from the use of different substrates in certain cases and a slight variation in experimental techniques. Comparisons of this nature are useful in attempting to evaluate the role of the gastrointestinal tract in the overall metabolism of a compound in the body and on this basis alone, it would appear that the gastrointestinal tract could provide a considerable proportion of the total metabolic conjugation with glucuronic acid especially for orally administered compounds.

A species variation in UDP-glucuronyl transferase activity of the gut wall has been demonstrated, highest activity occuring in the rabbit and rat, while the mouse and guinea pig show activities of about 80% lower, (Hietanen and Vainio,

1973). Glucuronic acid conjugation in the gut wall has been shown to be absent in the cat (Hietanen and Vainio, 1973) which seems to be in agreement with the apparent inability of the cat to conjugate with glucuronic acid in other tissues such as the liver, (Hirom et al., 1977). Changes in the composition of the diet in rats has been shown to alter UDP-glucuronyl transferase activity up to 30% and is thought to be a result of altered gastrointestinal function, (Hietanen and Hanninen, 1974).

b) Conjugation with sulphate

The intestinal capacity for sulphoconjugation has generally been accepted to be small in comparison with glucuronidation for most compounds, but certain exceptions exist. Conjugation with sulphate in the gut has been reported as being the major route of metabolism for certain phenols, (Powell et al., 1974), tyramine (Wong, 1976) and several steroid hormones, (Taylor, 1971a). Competition for sulphoconjugation in the gut between isoprenaline and salicylamide has been reported, (Bennet et al., 1975), their conjugation with sulphate in the gut thought to be important in the overall metabolism of these particular drugs, (Conolly, 1970; Levy and Matsuzawa, 1967).

The EHC of endogenous compounds

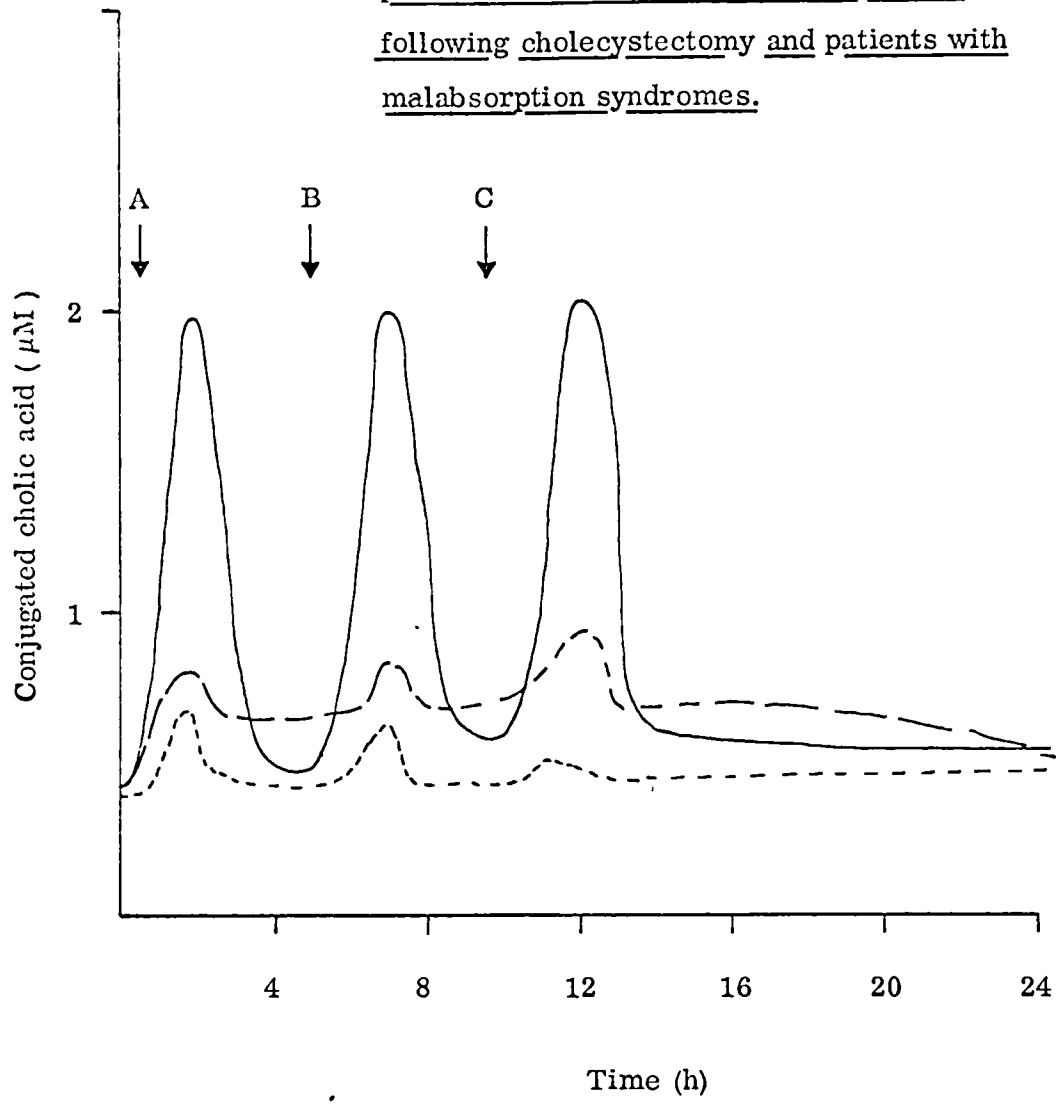
Endogenous compounds known to undergo EHC include several components of the bile such as bile acids and their salts, (Hofmann, 1976), cholesterol (Siperstein et al., 1952 a, b), phospholipids (Boucrot, 1972) and bilirubin metabolites (Watson 1963), in addition to several vitamins and certain steroid hormones. The physiological significance of EHC of bile constituents is that it aids the absorption of fats from the intestinal lumen, serves as a control for the synthesis of cholesterol and bile acids by negative feed back and lastly, conserves the body pool of the essential constituents of the bile. The physiological significance of the EHC exhibited by certain vitamins and steroid hormones is unknown as they have no known site of action in the intestine and thus it has been assumed that their EHC is merely a conservatory mechanism.

A) Constituents of the bile

i) Bile acids and their salts

Of the bile components involved in EHC, the bile acids and their salts have been of the most interest to workers in the field of EHC. In man, it has been estimated that the body pool of bile salts amounts to 3 - 5g which recirculates 6 - 10 times daily (Borgstrom et al., 1957). The exact number of recirculations of bile salts is dependent on the feeding habits of the individual as during fasting, the majority of the bile salt pool is retained in the gall bladder only being released in bulk as a result of the ingestion of food and release of digestive enzymes into the gut lumen, (see Fig. 1.1 1). In the absence of gall bladder stimulation it has been shown that there is still a significant EHC of bile salts and although the pool is reduced in size, (Northfield and Hofmann, 1975), bile salt synthesis remains unchanged (Hepner, 1975). It has been estimated that at any one time approximately 85% of the bile salt pool is present in the intestinal lumen, 10 - 12% in the gut wall and only 2% in the liver, except during fasting when the majority is retained in the gall bladder (Norman and Sjovall, 1958). In man the daily loss of bile salts is estimated to be 20 - 25% of the body pool (Bergstrom, 1962) mainly to the faeces; only 2% is lost in the urine (Grundy et al., 1965). The portion lost is replaced by hepatic synthesis of new bile salts which is controlled by the negative feed back of bile acids and their salts inhibiting the synthesis of cholesterol, the precursor of bile acids, (Bergstrom

Fig. I.10 The Effect of EHC on the plasma cholic acid profile in normal volunteers and patients following cholecystectomy and patients with malabsorption syndromes.



Test meals were injested at points A, B and C above in normal volunteers (———), patients following cholecystectomy (— —) and patients with malabsorption syndromes. (------)

(La Russo et al., 1974)

and Danielsson 1958, Shefer et al., 1970). Daily synthesis of bile acids has been shown to be 600 - 900 mg/day of which 2/3rds is cholic acid and 1/3rd chenodeoxycholic acid and since the pool size for both acids is similar, the lower turnover rate for chenodeoxycholic acid suggests it is more efficiently absorbed from the gut than cholic acid.

The major bile acids, cholic and chenodeoxycholic acids, are excreted in the bile exclusively as glycine and taurine conjugates in the ratio of 2-3:1 in man (Abaurre et al., 1969). At intestinal pH of 5-7, a considerable fraction of the glycine conjugates (pKa 4.3 - 5.3) are in the unionized form, whereas the taurine conjugates (pKa 1.8 - 1.9) are almost entirely in an ionized form (Dowling and Small, 1968), and as the rate of absorption from the gut of ionized compounds is slow in comparison with unionized compounds, taurine conjugates rely on active transport in the ileum for their absorption while glycine conjugates are readily absorbed in the jejunum and colon, (Hislop et al., 1967).

The newly formed secondary bile acids, lithocholic and deoxycholic acids, entering the pool daily represent 50% and 3% of the chenodeoxycholic and cholic acid synthesis respectively. Assuming complete dehydroxylation of the primary bile acids entering the gut by the microflora, it can be estimated that about 20 - 30% of the secondary bile acids formed are absorbed (Hofmann, 1976). Studies in which lithocholic acid was administered orally to patients showed that after 12 hours, 60% of the lithocholyl glycine remained in EHC while only 20% of the sulpholithocholyl glycine remained in EHC. The body pool of lithocholate was found to be small (100 mg) as a consequence of its rapid faecal excretion thought to be due to hepatic sulphation of lithocholate which is not passively absorbed by the jejunum (Cowen et al., 1975). Tertiary bile acids formed by the hepatic metabolism of secondary bile acids may undergo EHC, but their contribution to the overall EHC of bile acids (Bergstrom and Danielsson, 1968, Hofmann, 1977), is small.

Following intestinal absorption, bile salts pass back to the liver in portal blood mainly bound to albumin and not via the lymphatic system, probably due to its slow flow rate as compared with portal circulation (Reinke and Wilson, 1967).

Up to 36% of the bile salts in the portal blood occur as the free acid in the rat (Cronholm and Sjövall, 1967), but it is likely that lower levels of free acids may be found in the portal blood of man as a result of the lower levels of microflora able to deconjugate bile salts as compared to the rat (Dowling, 1972). Serum bile concentration in systemic blood is low implying an efficient hepatic uptake process for bile salts and acids, any bile acids present in systemic blood occuring in the conjugated form.

In disease states such as blind loop syndrome, an intestinal disease, serum bile salt concentrations are elevated,occuring mainly as free acids. However the reason for their presence in this form is unknown (Lewis, 1969). In cholestasis and obstructive jaundice an increased serum bile salt level is observed as the free acids due to an impaired hepatic uptake, (Neale et al., 1971).

The mechanism regulating the EHC of bile salts is thought to be due in part to the negative feedback of bile acids and salts on their hepatic synthesis, possibly the concentration of bile acids and salts regulating the activity of the enzyme cholesterol 7α-hydroxylase. Experiments in bile duct cannulated monkeys indicate that bile acid synthesis from cholesterol is normally repressed and interruption of EHC may increase bile acid synthesis by up to 10 times (Dowling et al., 1970). The bile acid pool size is a result of bile acid secretion rate and recycling frequency and thus it is probable that bile acid secretion rather than the pool size is regulated homeostatically (Hofmann, 1977).

ii) Cholesterol

Indications that cholesterol may undergo EHC stemmed from evidence which showed that following the administration of labelled cholesterol in bile duct cannulated rats, 50% of the dose was excreted in the bile in 60 - 70h whereas in intact rats, the appearance of a similar amount was delayed (120 - 170 h), thought to be due to intestinal reabsorption of the biliary metabolites (Siperstein and Chaikoff, 1952a). The possibility of cholesterol EHC was later confirmed following the intraduodenal administration of cholesterol biliary metabolites to a bile duct cannulated rat of which about 70% was recovered in the bile (Siperstein et al., 1952b). It was further shown that cholesterol metabolites absorbed in the intestine entered portal blood rather than being

taken up by the lymph as is the case with cholesterol itself. The suggestion that the cholesterol concentration in blood returning to the liver after EHC is responsible for the regulation of hepatic cholesterol synthesis, in a similar manner to the homeostatic regulation of bile acid synthesis, has been proposed. Grundy et al., (1969) showed that the rate of hepatic cholesterol synthesis was dependent upon the amount of cholesterol absorbed by the intestine.

iii) Phospholipids

It has been shown that ingested phospholipids can be hydrolysed in the gut by the pancreatic enzyme phospholipase A_2 to release fatty acids (Bellville and Clement, 1966) and absorbed by the intestine to enter the lymph as chylomicrons, (Nisson, 1968). In contrast, it has been observed that after intraduodenal administration of bile containing labelled ^{3}H-oleic acid, of which 70% was incorporated into phospholipids, to rats, little of the label was recovered in the lymph, whereas the portal blood contained high levels of the label, (Boucrot and Clement, 1969, 1971). Further they found that bile phospholipids were resistant to phospholipase A_2. These findings suggested that unhydrolysed biliary phospholipids may be absorbed by the gut returning to the liver in the portal blood. Later experiments confirmed an EHC of bile phospholipids by comparing the disposition of ^{14}C with ^{3}H in a mixture of ^{3}H bile phospholipids and ^{14}C oleic acid administered intraduodenally to bile duct cannulated rats. It was found that none of the ^{14}C label appeared in the bile whereas 10 - 25% of the ^{3}H label was excreted in the bile, suggesting that bile phospholipids enter EHC and are prevented from dilution in a larger pool of lipids, implying the existence of a specific hepatic pool of biliary phospholipids (Boucrot, 1972); such a pool has been proposed by Nilsson and Scherstein (1970).

iv) Bilirubin and its metabolites

Bilirubin is known to undergo bacterial reduction in the colon to form urobilinogens (Watson, 1963) which can be partially reabsorbed by this region of the gut and re-excreted in the bile and to a lesser extent in the urine (Fenton, 1945). Confirmation of early studies that bili rubin metabolites undergo EHC was established using a labelled urobilinogen ^{14}C-meso-bilirubinogen. Intravenous administration of this metabolite resulted in the

appearance of 85 - 90% of the dose in the bile and less than 10% in the urine within 3 hours. Administration intraduodenally showed that 50 - 60% of the dose was recovered in the bile as intact mesobilirubinogen (Lester and Schmid, 1964).

B) Vitamins

Vitamins known to undergo EHC are A, B12 and folic acid.

i) Retinol (Vitamin A)

On parental administration of retinol to rats, 15 - 20% of the dose appeared in the bile in 24 hours as water soluble metabolites thought to be hydroxylated derivatives of retinoic acid, while 30% of the dose was retained in the liver as retinol ester and a small amount as free retinol (Zachman and Olson, 1964). In the same study, comparison of retinol in the whole animal with that in isolated perfused liver showed that 1 hour after the start of perfusion, 10% was excreted in the bile as water soluble metabolites, the liver retaining 20 - 25% as retinol ester.

Demonstration of retinol EHC was achieved following the injection of bile containing retinol metabolites into duodenal loops of bile duct cannulated rats, 30% of the administered dose being recovered in the bile (Zachman and Olson, 1964).

ii) B_{12}

Early studies of the disposition of vitamin B_{12} in man suggested that very little was excreted, less than 1 μg / day in the urine (Heinrich, 1954). The use of labelled ^{56}Co - B_{12} showed that the urinary route was of minor importance for this vitamin in man, whereas faecal excretion approached 12 μg / day (Grasbeck et al., 1958). Studying the biliary excretion of B_{12} suggested a figure of 43 μg / day, thus with faecal excretion of 12 μg / day, these results imply that over 2/3rds of the B_{12} excreted in the bile was reabsorbed by the gut (Grasbeck et al., 1958), the reabsorption possibly aided by B_{12} binding factor which has been identified in bile, (Okuda et al., 1958).

iii) Folic acid

The evidence suggesting that folic acid can undergo EHC must be said to be tentative since no direct demonstration of EHC has been observed.

The evidence which exists is based on studies whereby bile was collected from patients with 'T-tube'cannulation of the bile duct which was found to contain folic acid at concentrations exceeding those occuring in the serum. 1 hour after injection of folic acid, a secondary plasma peak was observed which has been said to be indicative of the EHC of folic acid (Baker et al., 1965). In another study, mice were killed and prepared for autoradiography following intraperitoneal doses of folic acid, autoradiographs revealing the presence of folic acid in the gut lumen which appeared at a maximum concentration 1 hour after administration of folic acid (Herbert, 1965). The body pools of both folic acid and vitamin B_{12} are small and thus a mechanism such as EHC which may conserve the body pools could be of physiological significance.

C) Steroid hormones

The literature reveals a considerable number of reported instances in which steroid hormones undergo EHC in a variety of species. They can be divided into four groups on the basis of their structural and functional similarities as progesterones, oestrogens, corticosteroids, androgens and thyroxine. It will be shown that members of these groups show certain similarities in their EHC which appear to be common to most steroid hormones, but differences seem to occur between groups.

i) Progesterones

The naturally occuring progesterones known to undergo EHC are progesterone and pregnanolone, although metabolites of progesterones and synthetic derivatives have also been reported as undergoing EHC (Slaunwhite and Sandberg, 1961, Vermeulen et al., 1961, Peterson 1965 and Senciall, 1970). In man, about 30% of a parenteral dose of progesterone is found in the bile in patients with T-tube drainage while only 3% is recovered in the faeces which implies the reabsorption of a portion of the biliary metabolites in the gut (Slaunwhite and Sandberg, 1961). Examination of human biliary metabolites of progesterone revealed the presence of a major metabolite, pregnanediol, with smaller amounts of 5 α - pregnanediol, pregnanolone and progesterone (Chang et al., 1960) of which 70% could be hydrolysed and thus thought to be conjugates (Wiest et al., 1958). Pregnanolone is excreted in the bile as the ester glucuronide in rats, less than 4% occuring as unchanged

hormone, while in intact rats less than 8% appears in the urine and persists in the body for several days, (Soyka and Long, 1972). Intragastric infusion of pregnanolone biliary metabolites in bile duct cannulated rats resulted in the excretion in the bile of approximately 75% of the administered dose within 6 hours as two peaks (Long and Soyka, 1975). The initial minor peak of biliary excretion occured about 1 hour after infusion and is thought to represent a rapid absorption and reexcretion of unchanged pregnanolone while the second major peak occuring 2 - 6 hours after infusion may respresent the slower reexcretion of conjugated pregnanolone. Analysis of the gut lumen contents 6 hours after infusion showed that 25% of the infused does remained of which less than 35% was free pregnanolone, 28% as the 20β diol and the reaminder as more polar conjugates of pregnanolone.

The considerable EHC of pregnanolone in rats can also be shown for progesterone where 80% of a parenteral dose is known to appear in the bile and only 10 %recovered in the faeces implying a considerable reabsorption of the hormone (Shen et al. 1954) and this is confirmed by Grady et al., (1952). who estimated an intestinal reabsorption of biliary metabolites of up to 97%.

ii) Oestrogens

The oestrogens known to undergo EHC are oestrone, oestradiol and oestriol. In man, oestrone and oestradiol have been shown to be excreted in the bile to the extent of 30 - 50 % of the dose in patients with T-tube drainage mainly as glucuronides and sulphates of which over 90% is thought to be reabsorbed in the gut (Sandberg and Slaunwhite, 1957a, 1965). Studies of the disposition of oestrogens in other species suggests a marked species difference as to the extent of their EHC. About 80 - 90% of the administered dose of oestrone biliary metabolites is reabsorbed in the gut of the rat, guinea pig and man, while only 5% is reabsorbed in the rabbit (Sandberg et al., 1967). The low reabsorption of oestrone biliary metabolites in the rabbit is partially explained by the presence of single and double N-acetyl glucosamine conjugates of oestrone in rabbit bile which are not readily hydrolysed or absorbed by the gut (Layne et al., 1964). The recent identification of N-acetyl glucosamine conjugates of oestrone in human bile which appear to be ultimately reabsorbed by the gut in man does not explain the situation occuring in the rabbit (Taylor, 1971b).

Similarly oestriol has been shown to exhibit a species difference in the extent of EHC. In man and rabbit, 90% of the biliary metabolites are reabsorbed, 70% in the guinea pig, but only 36% in the rat, the poor reabsorption in the latter thought to arise from a resistance of the metabolites in rat bile to microfloral hydrolysis (Sandberg et al., 1967). Other species known to exhibit EHC of oestrogens are the cat (Pearson and Martin, 1966, Karim and Taylor, 1970) and the bull (Leung et al., 1975).

iii) Corticosteroids

The corticosteroid group of hormones known to undergo EHC include corticosterone, cortisone, hydrocortisone and aldosterone. In man, 10% of a parenteral dose of cortisosterone is excreted in the bile mainly as glucuronides (30 - 40%), unidentified polar metabolites and a small amount of free corticosterone, most of which is thought to be reabsorbed from the gut (Migeon et al., 1956, Hyde and Williams, 1957 and Peterson, 1965). EHC of corticosterone has also been identified in the rat (Eriksson and Gustafsson, 1970b), dog (Willoughby et al,1959) and cat (Taylor and Scratchard, 1962, 1963).

Cortisone is known to be excreted extensively in the bile of rats (70% of the dose) while only 20% appears in the urine. In intact rats faecal excretion of cortisone amounts to 56% and the urinary excretion increased to 40% suggesting that a portion of the biliary metabolites must be reabsorbed from the gut (Bocklage et al., 1955). There are very few reports of this hormone undergoing EHC in other species except in the mouse (Bradlow et al., 1954). In man, the urine appears to be the major route of excretion of coristone thus it is unlikely that any significant EHC of cortisone occurs in this species (Peterson, 1957).

Hydrocortisone or cortisol as it is sometimes known, is extensively excreted in the bile of rats and guinea pigs to the extent of 83% and 65% respectively while appearance in the faeces in intact animals was found to be 66% and 2 - 24% respectively. These results suggest reabsorption of hydrocortisone biliary metabolites in the gut of the rabbit is more efficient than in the rat which may in part be due to the presence of a considerable proportion of the easily absorbed unchanged hydrocortisone while rat bile consists primarily of the less readily absorbed conjugates (Wyngaarden et al., 1965). In contrast, other workers have found a virtually complete reabsorption of hydrocortisone

biliary metabolites from the gut of rats (Hyde and Williams, 1957).

Aldosterone is known to be extensively excreted in the bile, over 90% of a dose appearing in the bile of rats of which 25% appeared in the urine and 56% in the faeces after intragastric administration of aldosterone biliary metabolites suggesting that reabsorption from the gut of a portion occurs and the possibility of some EHC (McCaa and Sulya, 1966).

iv) Androgens

Androgens in general appear to be poorly excreted in the bile of all species and thus may undergo only a minimal EHC (Peterson, 1965, Sandberg and Slaunwhite, 1957b). Testosterone appears to undergo a considerable reabsorption from the gut of biliary metabolites; this has been shown in rabbits (Taylor and Scratchard, 1967, Taylor, 1971b) and in man , where an estimated 83% are absorbed (Sandberg and Slaunwhite, 1956), despite the low biliary excretion of this hormone in both species.

v) Thyroxine

Up to 25% of a parenteral dose of thyroxine can be shown to appear in the bile of rats. Comparison of urinary excretion and plasma levels of thyroxine in intact rats and bile duct cannulated rats suggests that an EHC of thyroxine is present in this species (Flock et al., 1962). Biliary metabolites have been shown to consist of thyroxine, tetraiodithyroacetic acid and iodine, but over 60% occured as glucuronide conjugates of thyroxine (Flock et al., 1962).

A summary of the EHC of steroid hormones

Steroid hormones are to some extent unique in that they are probably the only group of compounds in which EHC has been extensively studied over such a wide range of structurally similar compounds and in a large number of species.

Steroid hormones in general are extensively excreted in the bile of most species, exceptions being the androgens which are poorly excreted in all of the species studied. Biliary excretion of steroid hormones is generally low in man and primates when compared with other species. The variation in the extent of the EHC of steroid hormones appears to be a result of:-

a) The structure of individual steroid hormones which to some extent can determine the nature of the metabolites formed.

b) Species variation in the metabolism of steroids.

c) Species variation in gut microfloral metabolism, (Scheline, 1973). Hepatic metabolism of steroids generally includes initial phase I reactions usually hydroxylation followed by conjugation predominantly, glucuronic acid, however certain groups of steroid hormones , notably the oestrogens and neutral steroids, form conjugates with other substances, mainly sulphate, (see Table 1.11).

The nature of the biliary metabolites formed appears to be important in determining the rate and extent of their reabsorption from the gut since they possess varying degrees of lipid solubility. As most steroid hormones are excreted in the bile as a mixture of the unchanged hormone, free polar metabolites and their conjugates, variation in the rate of absorption of the individual metabolites from the gut have been shown. This can be seen as a biphasic re-excretion of certain steroids in the bile following their intra-duodenal or intragastric infusion of their biliary metabolites, (see Fig. 1.11).

Examples of species variation in the extent and nature of biliary metabolites formed have been described earlier and significantly effect the extent of EHC of steroid hormones. Once the biliary metabolites enter the gut lumen they are subject to the metabolic activity of the gut microflora which may render the biliary metabolites more or less readily absorbed from the gut. Conjugates of steroid hormones are generally less readily absorbed than the parent free hormone, (Inoue et al., 1969 a, b, Sandberg, et al. 1970), an exception being oestrone sulphate which has been shown to be absorbed more rapidly than free oestrone (Twombly, and Levitz 1960). As the biliary metabolites of most steroid hormones consist predominantly of conjugates, the capacity of the gut microflora for the hydrolysis of these conjugates will be important in determining the extent of reabsorption from the gut and hence the extent of EHC. A comparison of the microbial metabolism of pregnanolone and corticosterone by analysis of the gut luminal contents following the administration of labelled hormone in germ free rats showed that more free steroids were liberated from their conjugates in intact than in germ free rats (Eriksson and Gustafsson, 1970 a,b). Having been absorbed by the gut, steroid hormones or their metabolites may be subject to conjugation in the intestinal wall. Oestrogens have been shown to undergo conjugation with glucuronic acid in the rat intestine (Lehtinen et al., 1958 a, b) and similarly in isolated loops of human intestine (Diczfalusy et al.,

Table 1.11 Nature of biliary metabolites of certain oestrogens (a) and neutral steroids (b)

a)

STEROID	S	G	SG
2-methoxyestrone	+	++	0
Estrone	+++	+	0
15 α hydroxyestrone	+	+	0
Estriol	+	++	++
15 α hydroxyestradiol	+	0	0
Estradiol	0	+	0
2 - hydroxyestrone	0	0	0
16 α - hydroxyestrone	0	0	+

* S = sulphate, G = glucuronide, SG = sulphoglucuronide (Jirku, 1969) in man.
and Levitz

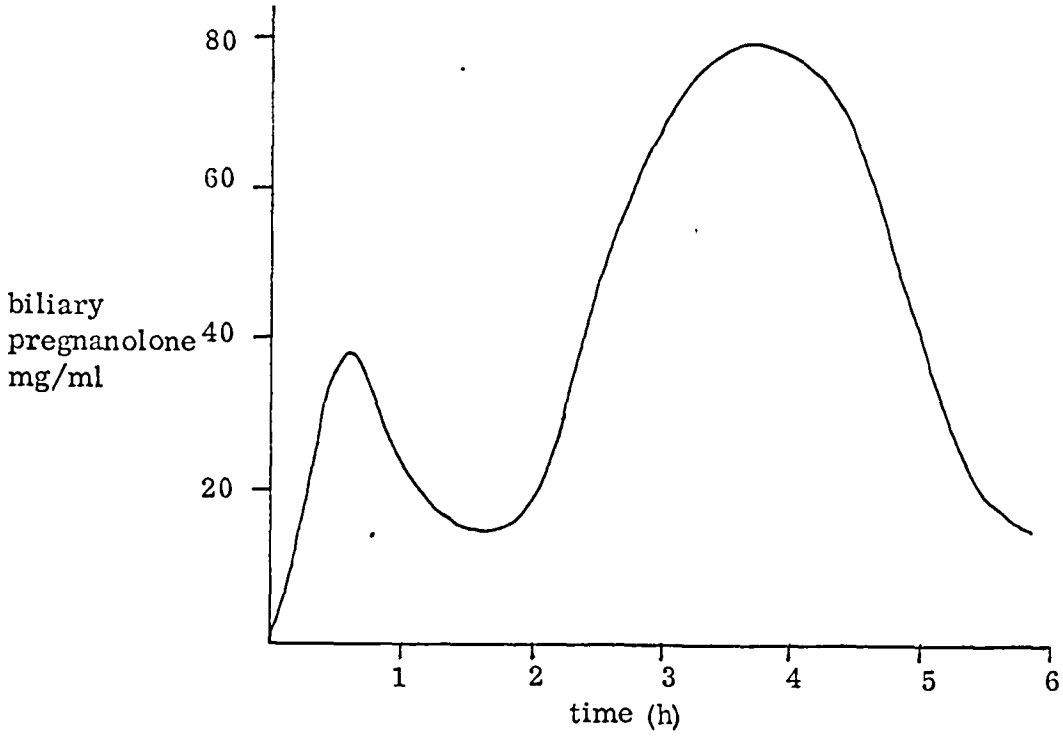
b)

STEROID TYPE	STRUCTURE	MS	DS	G
Androgen	$C_{12}O_2$	+	+	+
	$C_{19}O_3$	+	+	-
Progestagen	$C_{21}O_2$	+	+	+
	$C_{21}O_3$	+	+	+
	$C_{21}O_4$	+	-	-

*MS = monosulphate, DS = disulphate, G = glucuronide (Taylor, 1971a) in man.

1961, 1962).

The hepatic uptake of steroid hormones and their metabolites from portal blood, unlike the process for bile salts and acids, appears to be relatively inefficient for many steroids as much of the metabolites reabsorbed from the intestine appear in the urine, rather than being reexcreted in the bile. Estimation of the degree of hepatic extraction by comparison of the urinary excretion in bile duct cannulated and intact animals shows that oestriol urinary excretion increased from 11.4% in bile duct cannulated rabbits to 69% of the dose in intact rabbits (Sandberg, 1967) and similarly in man from 15 - 85% (Sandberg, 1957c) and for testosterone in the rabbit from 25 - 90% (Taylor, 1971b).



Re-excretion of bile in rats receiving i/g pregnanalone biliary metabolites. (4% unchanged, rest pregnanolone glucuronide). (Long and Soyka, 1975).

The Enterohepatic Circulation of Drugs and Other Xenobiotics

A considerable number of drugs and other xenobiotics have been reported to undergo EHC, some of which are listed in table 1.12. For the purposes of this review, no distinction is made between drugs and xenobiotics in general, unless stated.

Methods of estimating drug EHC

There are two possible approaches to the estimation of drug EHC, an indirect method derived from drug distribution studies in bile, urine, blood and faeces by which EHC can be identified, but not accurately quantified, whereas the direct methods involve the study of the individual phases of EHC enabling a precise quantitative value for drug EHC to be obtained.

a) Excretion pattern of drugs in the bile, urine, faeces and blood.

In the majority of reports in which drugs have been thought to undergo EHC, evidence has been based upon the excretion pattern of drugs in the bile, urine, faeces and blood. The standard procedure in metabolic studies for determining the disposition of drugs in laboratory animals is initially to measure the extent of their excretion in the urine and faeces of intact animals and secondly to repeat this in bile duct cannulated animals, where biliary excretion can be measured in addition to urinary and faecal excretion. Serial blood sampling from both intact and bile duct cannulated animals may also be carried out and can be useful in the identification of drug EHC. Using data obtained from such procedures described, a potential drug EHC may be indicated by: -

i) Biliary excretion rate in bile duct cannulated animals exceeding the faecal excretion rate in intact animals.

ii) Urinary excretion rate in intact animals exceeding the corresponding rate in bile duct cannulated animals.

iii) Systemic blood levels in intact animals exceeding the corresponding levels in bile duct cannulated animals.

These observations can be interpretated as an increased persistance of a compound within the body resulting from the retention of a portion of the dose within an enterohepatic cycle, which is not available for excretion until lost from the cycle.

Table 1.12 Some examples of xenobiotics shown to undergo EHC in the rat a) following intraduodenal infusion or intragastric administration of their biliary metabolites.

Compound	Intact		BDC		I/D or I/G			Biliary metabolites				References
	% U	% F	% B	% U	%B	% U	% F	FU	FM	GC	C	
Ampicillin	-	-	5	9	50	10	1	100	-	-	-	Stewart and Harrison (1961)
Butylated hydroxy toluene	6	7	52	-	30	-	-	-	52	28	16	Landomery et al (1976a,b)
Chlormethidone acetate	8	48	65	-	34	-	66	20	54	26	-	Hanasono and Fischer (1974)
DDA	-	-	97	1	67	-	-	-	-	100	-	Gingell (1975)
Etorphine	18	40	58	8	73	17	9	4	-	96	-	Dobbs and Hall (1968)
Fenclorac	43	47	20	-	20	65	-	?	?	?	?	Delong et al (1977)
Halofenate	35	26	88	-	61	-	-	-	100	-	-	Hucker et al (1971)
Hexachlorophene	8	70	31	-	28	15	57	5	-	95	-	Gandolfi and Buhler (1974)
Indomethacin	5	78	56	-	44	-	-	8	-	92	-	Hucker et al (1976)
Isoproterenol	75	-	38	62	1	8	-	-	-	100	-	Hertting (1964)
Mestranol	2	17	69	-	59	-	41	26	56	18	-	Hanasono and Fischer (1974)
Methaqualone	-	-	77	-	52	37	21	-	5	95	-	Polk et al (1974)
Norethidrone	15	37	80	-	64	-	36	6	33	60	-	Hanasono and Fischer (1974)
Pregnanolone	8	-	20	-	33	-	25	4	-	90	-	Long and Soyka (1975)
Stilboestrol	5	73	94	2	71	-	-	3	25	72	-	Fischer et al (1966)

Intact and bile duct cannulated (BDC) rats received compound i/v, i/p or orally, while I/D or I/G indicates animals received the respective biliary metabolites of the compound intraduodenally or intragastrically. %U, F, B = % of the administered dose recovered in the urine, faeces and bile respectively. FU, FM, G and C = % of the total biliary metabolites present as the free unchanged compound, free metabolites, glucuronic acid conjugates or other conjugates respectively.

Figure 1.12 Some examples of compounds thought to undergo EHC b) on the basis of excretion data in intact and bile duct cannulated animals

Compound	Species	Intact		BDC		Biliary metabolites				Reference
		U	F	B	U	FU	FM	G	C	
Bromobenzene	Rat	80	-	56	23	-	-	-	100	Sipes et al (1974)
Chloramphenicol	Rat	27	-	73	20	-	5	95	-	Glazko et al (1952) and Uesugi et al (1974)
Dapsone	Rat	45	22	32	27	-	48	62	-	Andoh et al (1974)
Dextromethorphan	Rat	13	27	84	-	-	46	37	24	Kamm et al (1967)
Furosemide	Rat	90	-	30	60	-	100	-	-	Wallin et al (1977)
Glutethimide	Rat	70	10	68	29	-	-	100	-	Keberle et al (1962)
Imipramine	Rat	47	30	75	-	2	11	87	-	Bickel and Weder (1968)
Indomethacin	Guinea pig	58	37	63	-	3	-	97	-	Hucker et al (1966)
Indomethacin	Monkey	27	3	48	-	6	-	94	-	Hucker et al (1966)
Mefloquine	Rat	1	14	46	1	-	-	100	-	Mu et al (1975)
Norethylnodrel	Rabbit	50	16	33	21	1	-	81	18	Aria et al (1962)
Pentazocine	Rat	30	11	77	-	10	-	90	-	El-Matzi and Way (1971)
Prontosil	Rat	81	2	21	-	70	20	-	-	Gingell et al (1971)
Propranolol	Rat	42	1	37	17	-			-	Hayes and Cooper (1971)

U, F and B = % of administered dose excreted in the urine, faeces and bile in intact and bile duct cannulated animals (BDC)
FU, FM, GC, C = % of total biliary metabolites present as the free unchanged compound, free metabolites, glucuronic acid or other conjugates respectively. V = metabolites present, but not quantitated.

The data may be used to evaluate the extent of EHC and the loss from the cycle; the following functions calculated: -

a) % biliary metabolites absorbed from the gut.

b) % absorbed metabolites lost from the EHC to the systemic circulation and appearing in the urine.

c) % absorbed metabolites lost from the EHC to the faeces.

d) % absorbed metabolites reexcreted in the bile.

Equating certain values enables estimation of the functions a) to d):-

% excreted in the bile (BDC) - % excreted in the faeces (I) = % absorbed from gut... (1)

% excreted in urine (I) - % excreted in urine (BDC) = % lost from EHC to urine.... (2)

From (1) and (2), it then follows: -

% absorbed from gut (1) - % lost from EHC to urine (2) = % excreted in the bile..... (3)

(where BDC = bile duct cannulated animals, I = intact animals and % = % of administered dose).

These estimates are based on the % excreted for each route over the same interval following administration of the drug. The criticisms of these methods for the identification and evaluation of EHC are as follows: -

a) Values for faecal excretion may not be representative of the total unabsorbed drug remaining in the gut lumen, especially when the value is obtained less than 24 hours after drug administration as defaecation may be delayed in some animals.

b) The estimation of EHC may be a result of several cycles.

c) Differences in systemic blood levels and urinary excretion between intact and bile duct cannulated animals may not be observed for drugs which are efficiently extracted from the portal blood by the liver.

d) Drugs undergoing limited EHC may not show differences in excretion rates between intact and bile duct cannulated animals.

e) Differences in systemic blood levels and urinary excretion between intact and bile duct cannulated animals for drugs poorly extracted by the liver may represent reabsorption of biliary metabolites from the gut and direct passage into the systemic blood rather than recirculation.

f) A comparison of values beteen intact and bile duct cannulated animals is not strictly valid unless the intact group are sham operated.

From the many reports of drug EHC, it is possible to differentiate two groups of drugs on the basis of the primary route of loss from EHC.

i) Drugs lost from EHC primarily in the urine - examples include pentazocine (El-Mazati and Way, 1971), propylthiouracil (Sitar and Thornhill, 1972), chlorpheniramine(Kamm et al., 1969), glutethimide (Keberle et al., 1962) and propranolol(Hayes and Cooper, 1971). Loss of a drug from EHC to the urine is indicative of a relatively inefficient hepatic extraction, but may also indicate an efficient absorption of biliary metabolites from the gut.

ii) Drugs lost from EHC primarily in the faeces - examples include chlormethidone acetate, norethidrone (Hanasono and Fischer, 1974), dextromethorphan (Kamm, 1967), and 5, 8, 11, 14 eicosatetraynoic acid (Stenlake et al., 1971). Loss from EHC via the faeces is indicative of relatively inefficient absorption of biliary metabolites from the gut in contrast to an efficient hepatic extraction of absorbed metabolites from portal blood.

A species difference has been observed for certain drugs as to the primary route of loss from EHC. Indomethacin is lost primarily to the faeces in dog, while loss is primarily to the urine in the guinea pig and monkey (Hucker et al., 1966). Norethylnodrel is lost primarily to the urine in the rabbit while primarily to the faeces in the rat (Aria et al., 1962, Hanasono and Fischer, 1974).

b) Evaluation of EHC after the introduction of biliary metabolites of a drug directly into the gastrointestinal tract.

The reexcretion of a drug in the bile following the absorption of its biliary metabolites from the gut is the most direct demonstration that the drug is undergoing EHC. Direct enteric administration of biliary metabolites in bile duct cannulated animals enables the direct estimation of biliary re-excretion to be made.

All variations on this particular approach to studying EHC involve the collection of 'donor' bile from a 'donor' bile duct cannulated animal to which the drug under study has been administered. The donor bile containing biliary metabolites of the drug is then administered directly into the gastro-

intestinal tract to a second 'recipient' bile duct cannulated animal. Enteric administration of donor bile to the recipient may either be an oral dose or a direct infusion into the stomach (intragastric i/g) or the duodenum (intra-duodenal i/d). A slight variation on this approach involves the 'linking' of the donor to recipient by passing the bile duct cannula of the donor animal directly into the duodenum of the recipient, (Siperstein et al., 1952 b). Other workers have administered the donor bile into 'intestinal sacs' of the recipient which are tied off sections of the intestine (Zachman and Olson, 1964). Although the 'linked' animals technique may be said to represent the more physiological situation when compared with i/d or i/g infusion, the extent of biliary excretion from the donor animal cannot be measured as a result of the 'link'. As reexcretion of biliary metabolites in the bile is a function of both the biliary excretion of the donor and the recipient, a value for the biliary excretion from the donor in the linked technique must be assumed from control experiments in which bile duct cannulated animals have recieved an identical dose of the drug to that administered to the donor in the 'link'.

In general, these methods allow an accurate estimation of biliary reexcretion, absorption of biliary metabolites from the gut, loss from EHC and hence an accurate evaluation of a single enterohepatic cycle. These methods are particularly useful for evaluating drugs undergoing a limited EHC such as isoproterenol (Hertting, 1964), of which only 1% of an i/d dose of donor bile can be shown to be reexcreted in the bile.

The extent and duration of drug EHC

The extent of drug EHC can be defined as the % of the administered dose involved in an EHC which is dependent upon a) the extent of biliary excretion, b) degree of absorption of biliary metabolites from the gut, and c) the extent of hepatic extraction of the absorbed biliary metabolites from the portal blood. If a drug were to undergo 100% EHC it would be totally contained within the cycle, recycling at infinitum. The drug iophenoxic acid may approach this theoretical maximum for EHC having a plasma half-life of at least $2\frac{1}{2}$ years, (Mudge et al., 1971). The rate at which a drug is lost from the cycle will dictate the duration of EHC, the latter may be estimated if the loss from a single cycle and the duration of a single cycle is known and has been determined for certain drugs in Table 1.13.

Table 13 Estimated duration of EHC (assuming a constant loss per cycle and cycle time of 8 hours).

Drug	% of drug remaining in EHC after n cycles					Duration
	n=1	2	3	4	5	(hours)
Etorphine	73	53	39	28	21	104
DDA	67	45	30	20	14	88
Norethidrone	64	41	26	17	11	80
Mestranol	59	35	21	12	7	72
Methaqualone	52	27	14	7	4	56
Indomethacin	44	19	8	4	2	48
Hexachlorophene	28	8	2	1	-	32
Imipramine	8	1	-	-	-	16

* DDA = bis(p-chlorophenyl)acetic acid

As stated earlier, EHC can increase the persistance of a drug within the body which is indicated by an increased half-life ($T\frac{1}{2}$) of the drug in intact animals as compared with that in bile duct cannulated animals. The $T\frac{1}{2}$ may be plasma or biliary half lives or a function of the total excretion rate, (Table 1.14)

Interruption of EHC

Any factor which interrupts drug EHC so as to reduce or eliminate the recirculation may give rise to a reduced persistence of the drug within the body, changes in the excretion pattern of the drug and possibly a reduction in the pharmacological and toxicological effects of the drug associated with EHC. Interruption of EHC occurs as a result of either certain pathological conditions or by the influence of drugs.

Pathological conditions

Although there are no reports of such conditions affecting drug EHC, there

Table 14 Comparison of the half-lives of drugs in intact animals with those in bile duct cannulated animals.

Drug	Species	½ life I	½ life BDC		Reference
Methyl proscillaridin	Man	51h	22h	T	Staud et al., 1975 Reitbrock, 1975.
Digitoxin	Dog	14h	6h	T	Katzung and Meyers, 1965.
Digitoxin	Rat	22h	7h	B	Abshagen et al., 1972.
Digitoxin	Rat	13.5h	7h	P	Abshagen et al., 1972.
Digitoxin	Man	9.3d	2.8d	T	Bazzano and Bazzano, 1972
Digoxin	Man	2d	2/3d	T	Bazzano and Bazzano, 1972

* P = plasma ½ life, B = biliary ½ life, T = total ½ life, I = intact animals, BDC = bile duct cannulated animals.

is ample evidence supporting their interruption of bile salt EHC thus drugs involved in EHC may equally be affected. Conditions acting at the hepatic level such as certain forms of jaundice and cholestasis are obstructive diseases which have been shown to prevent the biliary excretion of bile salts and hence their EHC by a cessation of bile secretion and,in such an event bile salts are excreted in the urine as sulphates (Hofmann, 1976). Conditions occuring at gastrointestinal level are either of an obstructive nature such as 'blind loop syndrome' (Dowling, 1972) and jejunal diverticulosis (Bewes et al., 1966) or malabsorption syndromes resulting from diseases of the ileum such as Crohn's disease (Meinhoff and Kern, 1968), coeliac disease (Low -Beer et al., 1971) and tropical sprue (Turner et al., 1970) or ileal resection and

by-pass. All such gastrointestinal conditions reduce or eliminate the absorption of bile salts from the gut (Dowling, 1972).

Drug induced interruption of EHC

In general, drug induced interruption of EHC occurs at the gastrointestinal level, but it can be shown that certain drugs are capable of blocking the hepatic uptake of other drugs. These hepatic uptake blocking drugs include flavaspidic acid, bunamiodyl, iodipamide, which are known to block the hepatic uptake of bile salts and other drugs excreted in bile (Levi et al., 1969) and this could interfere with drugs undergoing EHC.

Interruption of drug EHC at gastrointestinal level by certain drugs can occur by any of i) drug induced changes in gut microfloral metabolism, ii) drug induced malabsorption syndromes, and iii) binding of drug biliary metabolites to binding agents.

The drug induced gut floral metabolism is related either to the inhibition of specific bacterial reactions or to a depletion of the gut microbial population resulting from antibiotic pre-treatment. As a major proportion of drug biliary metabolites exist as glucuronides, the most likely gut microfloral reaction occuring with biliary metabolites is the hydrolysis of glucuronides by bacterial β-glucuronidase. The implications of this reaction in increasing the lipid solubility and hence the reabsorption from the gut of drugs has been stated earlier and has been inferred to be important in the EHC of certain drugs.

The contribution of bacterial hydrolysis of glucuronides to the overall EHC of drugs has been studied both by the use of specific inhibitors of β-glucuronidase such as saccharo-1,4-lactone and by antibiotic pre-treatment in order to reduce the population of gut flora and hence enteric levels of bacterial β-glucuronidase or in specific pathogen-free (SPF) rats which contain very few enteric microorganisms. The use of β-glucuronidase inhibitors appears to be relatively unpopular as compared with the widely used antibiotic pre-treatment regimes and only a few reports describe their use in evaluating the effects of interrupting EHC in this manner, an example being the reduction in the EHC of progesterone and phenobarbital following pretreatment with saccharo-1,4-lactone,(Marselos et al., 1975). The effect of antibiotic pretreatment

on the EHC of drugs has been reported for several drugs including etorphine which is excreted in the bile as a glucuronide and has been shown to undergo EHC in the rat (Dobbs and Hall, 1968). Their experiments revealed that the recovery in the bile of bile duct cannulated rats dried orally with etorphine glucuronide having been pretreated with antibiotic ampicillin was reduced by 50% as compared with controls that had not received antibiotic. Further studies showed that ampicillin pretreatment in rats gave a 10^6-fold reduction in the bacterial population of the caecum while the β-glucuronidase activity was found only to be 2 - 56% of control values (Dobbs and Hall, 1968). Similar results have been observed for several steroid hormones when comparing their EHC in normal and SPF rats (Eriksson and Gustafsson, 1970a) It is known that certain drugs including the antibiotic neomycin (Jacobson et al., 1960, Rothfield and Osborne, 1963) can give rise to malabsorption syndromes that may affect the gastrointestinal absorption of biliary metabolites involved in EHC.

The most widely used method of interrupting EHC appears to be the administration of the anion exchange resin cholestyramine and similar compounds which have been shown to bind with cholesterol, bile salts, certain hormones and many other drugs. Binding of compounds occurs in the intestinal lumen, following oral doses of cholestyramine, to form a complex which is poorly absorbed from the gut.

Digitoxin, which is known to undergo EHC in man (Okita et al., 1955), when administered in combination with cholestyramine gave a reduction in plasma half-life for digitoxin from 11.5 to 6.6 days when compared with controls receiving only digitoxin (Caldwell and Greenberger, 1971a). Similarly cholesterol, which is known to undergo EHC (Siperstein et al., 1952b) was found to be gradually depleted in patients receiving cholestyramine on a daily basis, plasma cholesterol levels falling by 6 - 38% of control values over a period of 2 - 34 weeks (Bergen et al., 1959, Hashim and VanItallie, 1965). As the interruption of drug EHC can be readily detected by changes in half life and increased faecal excretion of a drug, the use of antibiotics and cholestyramine can provide useful experimental tools for the estimation of drug EHC without the need to collect bile. The ability to evaluate drug

EHC without the need for bile duct cannulation is ideal for studies in man and in the estimation of the pharmacological effects resulting from EHC, the determination of which may be sensitive to the surgical procedure required for bile duct cannulation.

Species differences in drug EHC

There is little evidence of species difference in the extent of EHC of drugs as most of the quantitive work has been carried out in rats alone. A species difference in the primary route of loss from EHC has been discussed earlier but there is insufficient data to suggest a species difference in the extent of EHC although such a species variation may be predicted from consideration of the species differences shown for biliary excretion and the gut microflora discussed earlier.

Pharmacological implications of drug EHC

For many drugs involved in EHC, the cycling may give rise to their increased persistence within the body which may prolong or potentiate their pharmacological activity. The ability of EHC to influence the pharmacological activity of drugs depends primarily on the pharmacological potency of the drug metabolites as compared with that of the parent drug. It is well known that metabolism in general and particular conjugation tends to reduce the pharmacological activity of drugs such that most drug metabolites excreted in the bile possess little or no pharmacological potency. An example of this metabolic reduction in drug potency is the muscle relaxant, zoxazolamine, which is metabolised in the liver to a glucuronide which possesses no muscle relaxant activity (Ziegler et al., 1972). Changes in the pharmacological activity of drug biliary metabolites, many of which have lost much of their potency, may result from their interaction with the gut flora. Where a drug has lost its pharmacological activity solely as a result of conjugation, the deconjugation capacity of the gut flora may hydrolyse the conjugate to release the parent drug and thus restore its pharmacological activity. There are many reports of gut microfloral hydrolysis releasing potentially active metabolites from their pharmacologically inert glucuronides such as stilboestrol Clark et al., (1969, Fischer et al., 1970, 1973), morphine, (Walsh and Levine, 1975) and pentaerythritol trinitrate (Crew et al., 1971).

Many drugs lose their pharmacological activity via Phase I reactions and although they may be excreted in the bile as glucuronides, their deconjugation by gut microflora is unlikely to produce any significant restoration in pharmacological activity. Although the gut microflora via deconjugation tend to increase the pharmacological activity of most biliary drug metabolites, the gut flora are equally capable of rendering metabolites less pharmacologically active. Chloramphenicol, an antibiotic, is excreted in the bile as a pharmacologically inactive glucuronide and is both hydrolysed in the gut to form the active free drug and reduced to aromatic amines which possess no antibiotic activity. Both metabolites are subsequently reabsorbed by the gut and undergo EHC (Glazko et al., 1952). The passage of drugs or their metabolites through the gut wall during their absorption introduces the possibility of reconjugation of the active metabolites which may have been liberated from their inactive conjugates in the gut lumen. Drugs known to undergo EHC and reconjugation with glucuronic acid in the gut wall include stilboestrol (Fischer and Millburn, 1970, 1973), oestrogens (Taylor, 1971a) and testosterone (Kreek et al., 1963). As the EHC is by definition a closed cycle between the liver, gastrointestinal tract and portal circulation, a drug must be lost from EHC to the systemic circulation if it is to reach its pharmacological site of action, which for most drugs, lies beyond the confines of EHC. In order to reach the systemic circulation, a drug or its metabolites returning from the gut in the portal blood must pass through the liver. In the liver the drug may pass through into the systemic circulation unchanged or may undergo further metabolism prior to its release or alternatively be reexcreted in the bile. Stilboestrol has been shown to occur in the portal blood as both the free drug and the glucuronide as a result of gut luminal and intestinal wall metabolism. Stilboestrol occurs in systemic blood solely as the conjugate suggesting that the free drug present in the portal blood is reconjugated on its passage through the liver (Fischer and Millburn, 1970).

There are very few reports of drugs undergoing EHC appearing in the systemic blood as the free active drug or active metabolites except for the few drugs excreted in the bile unchanged and exceptional drugs such as

the cardiac glycosides (Katzung and Meyers, 1965).

Early studies showed that digitoxin and similar cardiac glycosides could undergo EHC in several species including man, the biliary metabolites being modified in the gut lumen and absorbed as non-polar cardioactive metabolites which are accessible to the systemic circulation(Okita et al., 1955 and Katzung and Meyers, 1965). More recent studies have investigated the effect of digitoxin EHC on its pharmacological response using the digitoxin induced reduction of left ventricular ejection time (LVET) and electromechanical systole (QS_2) as a measure of pharmacological response in man. The activity of digitoxin in normal subjects was compared with patients in which digitoxin had been interrupted by administration of cholestyramine. Results indicated that cholestyramine interruption of digitoxin EHC produced a more rapid return to base line values of digitoxin induced changes LVET and QS_2 than in controls. These effects could be correlated with plasma levels of digitoxin metabolites in a dose/response fashion (Caldwell and Greenberger, 1971a). In another study, the phenobarbitone and progesterone induced depression of locomotor activity in rats was shown to be reduced following interruption of the EHC of these drugs by the administration of saccharo-1, 4-lactone (Marselo et al., 1975).

Toxicological implications of drug EHC

In common with the pharmacological implications of EHC, toxicological implications are relatively unknown and although a possible relationship between EHC and toxicity has been implied by several workers (Williams,et al., 1965, Smith and Millburn , 1975), there is very little direct evidence to suggest such a relationship.

EHC generally involves the cycling of drug metabolites rather than the free drug and as metabolites are less toxic than their parent drugs, the probability of toxic metabolites being involved in EHC would on first appearances seem unlikely. The liberation of toxic metabolites arising from gut microfloral metabolism of biliary metabolites introduces a source of toxic compounds which may then undergo EHC.

Chloramphenicol, as mentioned earlier, is reduced in the gut lumen to arylamines which may be absorbed from the gut (Glazko, 1952) and have been

reported to be thyrotoxic in rats (Thompson, 1954). Aromatic amines metabolised in the liver to o-hydroxylamines are excreted in the bile as glucuronides and are hydrolysed in the gut to liberate the free o-hydroxylamines which are known to be carcinogenic (Walpole, 1962, Williams et al., 1965). Certain intestinal flora have, under particular dietary conditions, been shown to convert some steroids to aromatic hydrocarbon carcinogens which may undergo EHC (Hill et al., 1971).

Other reports which may be relevent to EHC are the observed increases in toxicity following bile duct ligation for drugs normally excreted in the bile and are known to undergo EHC such as glutethimide, stilboestrol, taurocholic acid and digitoxin (Klaassen, 1973a, b). Marked changes in toxicity were observed for these drugs following bile duct ligation, but it is unlikely that interruption of EHC would result in such extreme changes in toxicity.

The most convincing demonstration of toxicity induced by EHC is the increased toxicity of digitoxin in intact rats as compared with that in which the EHC of digitoxin has been interrupted by cholestyramine treatment (Caldwell and Greenberger, 1971b). The possible clinical use of cholestyramine and similar compounds in the event of digitalis intoxication has recently been evaluated using the ion exchange resin colestipol. This resin has been shown to be more effective in binding both digitoxin and digoxin in vitro in the presence of duodenal juice than was cholestyramine. Trials with colestipol in digitoxin and digoxin intoxicated patients showed that the resin was effective in reducing the toxicity of both drugs and the reduction in toxicity was paralleled by a decrease in plasma half-lives from 9.3 to 2.8 days for digitoxin and from 2 to 2/3rds days for digoxin (Bazzano and Bazzano, 1972).

The intestinal irritation and ulceration induced by the biliary excretion of unchanged indomethacin is thought to be enhanced by the prolonged exposured of the intestinal tract to the drug as a result of its EHC (Duggan et al., 1975).

CHAPTER TWO

Contents.

List of figures and tables.

Materials

Labelled compounds

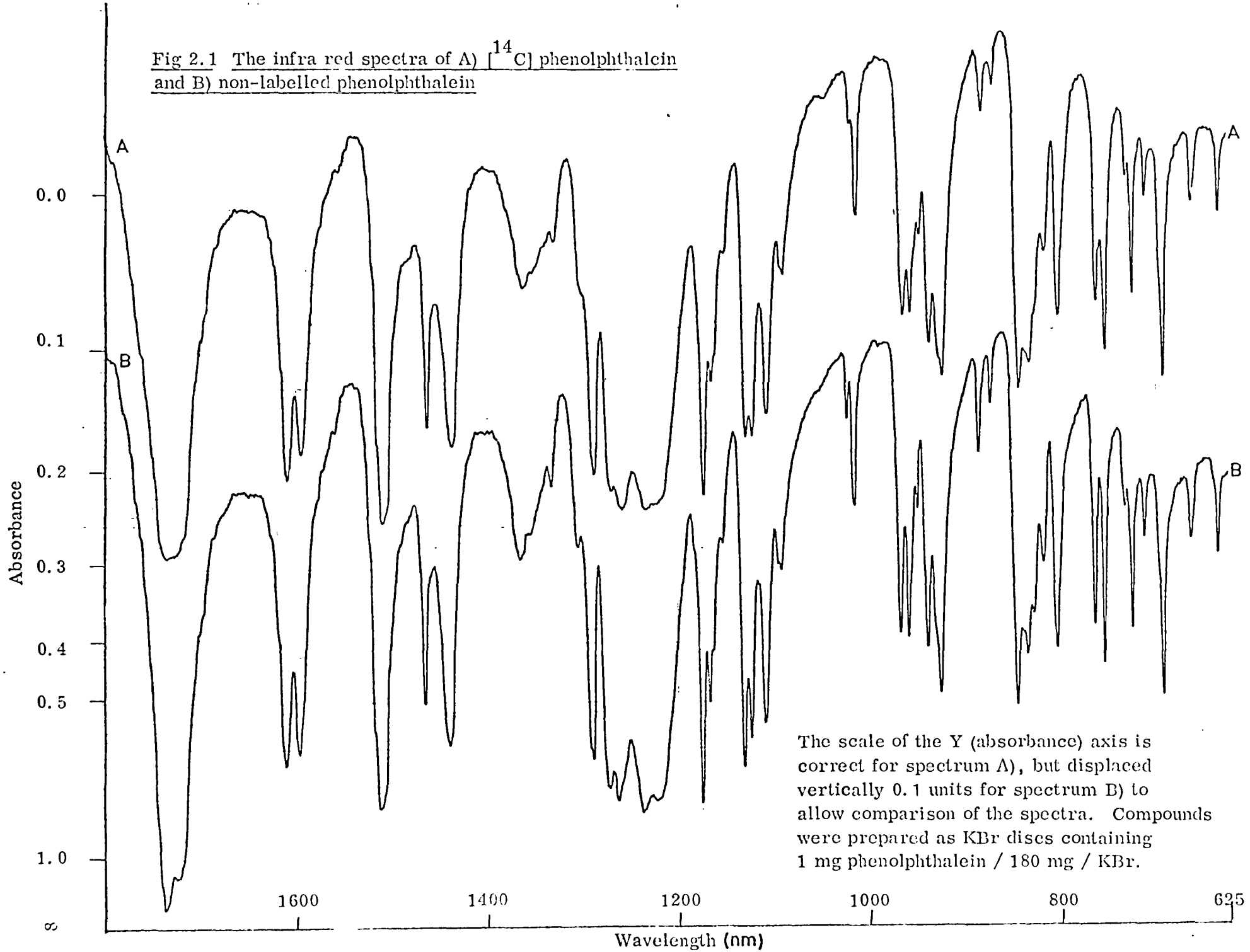
With the exception of [^{14}C]-Phenolphthalein and 13-hydroxy-[^{14}C]-Lysergic acid diethylamide(LSD), which we have synthesised, the other labelled compounds used in this study were obtained from other sources as listed in Table 2.1.

The synthesis of [^{14}C]-Phenolphthalein

This compound was synthesised by the condensation of phenol with [^{14}C]-Phthalic anhydride by a method similar to that described by Capel, (1974). 500 μCi of [carbonyl -^{14}C]-Phthalic anhydride was heated in a round bottomed flask at 120°C for 24 hours with 0.5 g of cold phthalic anhydride (British Drug Houses - BDH) and 2.5 g of phenol (Fisons) in the presence of anhydrous zinc chloride (BDH), stirring continuously. After heating, 50 ml of water was added and the resulting mixture boiled to remove all traces of phenol and then allowed to cool. The precipitate formed on cooling was filtered over sintered glass and the residue dissolved in 0.1M NaOH followed by further filtration to remove any insoluble by-products of the reaction. The filtrate was neutralised with dilute acetic acid and 0.1M HCl to form a white precipitate and left overnight. The precipitate was filtered off and dissolved in warm ethanol followed by refluxing for 1 hour with 5 g of decolourising charcoal. The charcoal was filtered off and the ethanolic mixture reduced in volume by 50% *in vacuo* to which was added 250 ml of water. This solution was heated to 80°C, then cooled and allowed to stand overnight, followed by a final filtration to remove the crystals of phenolphthalein which were dried and weighed.

The yield was 0.74 g, representing 52% of the theoretical yield and estimated to have a specific activity of 0.3 μCi/mg, equivalent to a radiochemical yield of 44.4%. The labelled compound was found to be chromatographically identical to authentic phenolphthalein (Sigma) and found to have a melting point of 252°C as compared with 251°C for the authentic compound and 251°C for a mixture of both the labelled and authentic compounds. The infrared spectra of [^{14}C]-Phenolphthalein and the authentic phenolphthalein were compared, (see Fig 2.1) following scanning of potassium bromide discs

Fig 2.1 The infra red spectra of A) [^{14}C] phenolphthalein and B) non-labelled phenolphthalein



The scale of the Y (absorbance) axis is correct for spectrum A), but displaced vertically 0.1 units for spectrum B) to allow comparison of the spectra. Compounds were prepared as KBr discs containing 1 mg phenolphthalein / 180 mg / KBr.

Table 2.1 Structure and radiochemical properties of labelled compounds

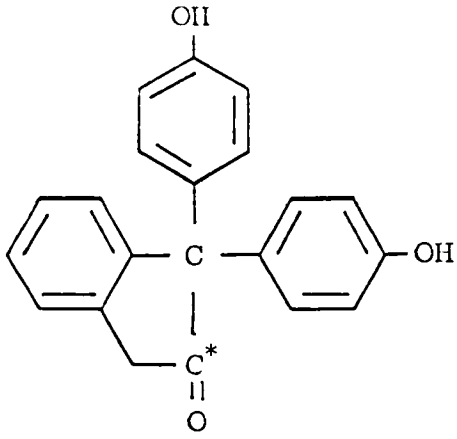
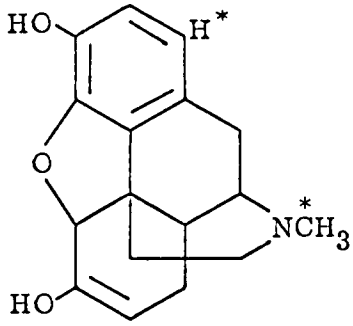
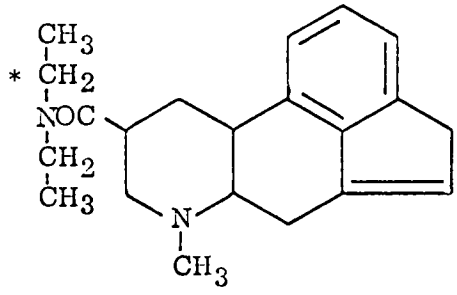
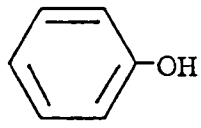
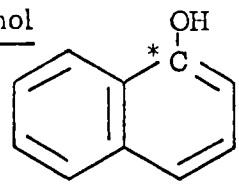
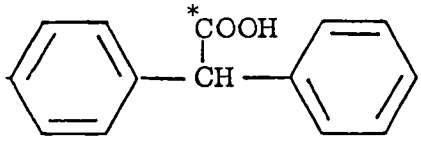
COMPOUND - structure and position of label*	Nature of label	Sp. Act. (μCi / μg)	Source
Phenolphthalein 	[Carbonyl ^{14}C]	0.3	[^{14}C]synthesised in this laboratory from [Carbonyl -^{14}C]-Phthalic anhydride as described.
	[G - ^{3}H]	2.78	[^{3}H] -synthesised in this laboratory from [G -^{3}H]-Phthalic anhydride by method of Capel (1974).
Morphine 	[1(n)-^{3}H]	91200	Radiochemical Centre (RCC), (Amersham, Bucks, England).
	[N-methyl-C]	116	
Lysergic acid diethylamide (LSD) 	[^{14}C - diethyl amide]	13.6	Synthesised in this laboratory from sodium [$^{14}C_1$]-Acetate by Barnes (1974).
Phenol 	U-[^{14}C]	353	RCC (Amersham)
1-Naphthol 	[1(1-^{14}C)]	353	RCC (Amersham)
Diphenylacetic acid 	[carboxyl-^{14}C]	3.0	Synthesised in this laboratory from [^{14}C]-Cyanide by Dixon (1976).

Table 2. 2:Sources of compounds used in this study

Compound	Source
Phenolphthalein (PP)	Sigma Chemical Co., St. Louis, Missouri, USA.
Phenolphthalein β-D glucuronide (Na-salt)	Koch Light Laboratories, Colnebrook, Bucks., England
Morphine hydrochloride	Macarthys Laboratories, Romford, Essex, England.
(+)-Lysergic acid diethylamide ((+)-LSD-tartrate)	Sandoz A. G., Basle, Switzerland.
1-Naphthol	'Analar' - Hopkins and Williams, Chadwell Heath, Essex, England.
1-Naphthyl β-D glucuronide (Na-salt)	Koch-Light
1-Naphthyl sulphate (K-salt)	Sigma
Phenol	B.D.H. Chemicals Ltd., Poole, England.
Phenyl β-D-glucuronide (monohydrate)	Koch-Light
Phenyl sulphate	Synthesised in this laboratory by R. Mehta (1977).
Diphenylacetic acid	Ralph N. Emanuel Ltd., Wembley, England.
Bromosulphophthalein (BSP)	Hynson, Westcott and Dunning Inc., Baltimore, Maryland, USA

containing 1 mg of the labelled or authentic phenolphthalein on a grating infra-red spectrophotometer (Perkin Elmer, Model 157G).

The preparation of 13-hydroxy-[^{14}C]-Lysergic acid diethylamide (LSD)

Pooled bile, collected from five female Wistar rats injected intra-peritoneally (i/p) with [^{14}C]-LSD (a total of 4.85 mg of specific activity 2.2 μCi/mg) was passed through a column (30 cm length x 1 cm diameter) packed with XAD-2 (BDH) and eluted with 100 ml water. The column was dried with nitrogen and finally eluted with 100 ml methanol. The methanol eluate was evaporated to dryness in vacuo at 30 - 40°C and redissolved in 2 - 3 ml of methanol. The concentrated methanol was applied to preparatory thin layer chromatography (t.l.c.) plates that had been pre-eluted with methanol (HF_{254} Silica gel of 0.8 mm thickness on 20 x 20 cm glass plates) and developed in solvent A (see Table 2.3). Viewing under ultra-violet (U.V.) light showed the presence of two fluorescent bands at Rf's 0.30 and 0.34, corresponding to 13 and 14-hydroxy-LSD glucuronides respectively, as shown by Siddik, (1975). The band at Rf 0.30 was scraped off the plate into methanol, filtered to remove the silica and evaporated to dryness in vacuo at 30 - 40°C. The glucuronide was taken up in 1 ml 0.2M sodium acetate buffer (pH5) to which was added 2 ml Ketodase (William R. Warner and Co. Ltd., Eastleigh, Hants, England) and incubated for 7 hours at 37°C in order to hydrolyse the glucuronide. Four hours after the start of hydrolysis, t.l.c. of an aliquot of the incubating mixture indicated that over 60% of the 13-hydroxy-LSD glucuronide had been hydrolysed. After incubation, methanol was added to precipitate the protein present in the mixture which was removed by filtration and the filtrate then evaporated to dryness in vacuo at 30 - 40°C. The residue was taken up in a small volume of methanol, applied to a second preparatory plate and developed in solvent D (see Table 2.3). Viewing under UV light showed a band at the origin representing the unchanged glucuronide and a broad band at Rf 0.39 corresponding to 13-hydroxy-LSD. The latter band was scraped off the plate into methanol, filtered to remove the silica and the filtrate finally evaporated to dryness in vacuo at 30 - 40°C. The compound was taken up in a sample of fresh rat bile and an aliquot applied to a t.l.c. plate (Merck $60F_{254}$) and developed in solvent C

(see Table 2.3). Visualisation of the plate under U.V. light revealed the presence of a single metabolite at Rf 0.79 corresponding to 13-hydroxy-LSD.

Non-labelled compounds

Compounds used in this study are listed in Table 2.2.

Methods

Chromatographic techniques

Thin layer chromatography (t.l.c.)

Identification of all compounds and their metabolites was achieved by t.l.c on silica gel plates of 0.2 mm thickness (Merck $60F_{254}$). Urine (10 - 100μl), bile (50 - 100 μl), samples of perfusion buffer collected following intestinal perfusion (perfusate) (5 - 100 μl), the contents of *in situ* intestinal sacs (5 - 100 μl) and incubation buffer collected from *in vitro* gut sac preparations (5 - 100 μl) was applied directly to t.l.c. plates and developed in the appropriate solvent systems (see Tables 2.3 and 2.4).

Table 2.3 Solvent systems used in t.l.c.

Solvent System	Components
A	Propan-1-ol, ammonia (7:3)
B	Pyridine, pentan-1-ol, water (7:7:6)
C	Chloroform, methanol, water (5:5:1)
D	Chloroform, methanol (4:1)
E	Butan-1-ol, acetic acid, 5% aqueous ammonia, water, acetone (45:10:10:20:15)
F	Butan-1-ol, water, acetic acid (4:5:1)
G	Butan-1-ol, ammonia, water (10:1:1)
H	Acetone, benzene, glacial acetic acid (2:2:1)
I	Benzene, acetone, glacial acetic acid (6:2:1)

Table 2.4 Rf values for compounds used in this study

Compound	System 1 SS	System 1 Rf	System 2 SS	System 2 Rf
Phenolphthalein	A	0.71	B	0.78
Phenolphthalein glucuronide	A	0.23	B	0.44
LSD	C	0.83	D	0.53
13-hydroxy-LSD	C	0.79	D	0.39
13-hydroxy-LSD-glucuronide	C	0.41	D	0.00
14-hydroxy-LSD	C	0.78	D	0.34
14-hydroxy-LSD-glucuronide	C	0.49	D	0.00
Morphine	E	0.42	F	0.44
Morphine sulphate	E	0.33	F	0.23
Morphine-3-glucuronide	E	0.14	F	0.35
Morphine-6-glucuronide	E	0.22	F	0.45
1-Naphthol	H	0.96	-	-
1-Naphthyl sulphate	H	0.77	-	-
1-Naphthyl glucuronide	H	0.35	-	-
Phenol	A	0.76	G	0.82
Phenyl sulphate	A	0.50	G	0.56
Phenyl glucuronide	A	0.28	G	0.36
Diphenylacetic acid	H	0.88	I	0.61
Diphenylacetyl glucuronide	H	0.10	I	0.00

* Each compound and their respective metabolites were chromatographed using two different solvent systems, 1 and 2, the compositions of which (SS), designated A - I, are given in Table 2.3 .

When the authentic compounds were not available, Rf values were obtained from the literature [13 and 14-hydroxy-LSD and their glucuronides - Siddik, (1975), morphine glucuronides and sulphate - Yoshimura et al., (1969) and diphenylacetyl glucuronide - Dixon, (1976)].

Extraction of metabolites

In order to identify compounds and their metabolites in samples of tissue and faecal homogenates and whole blood, these samples were extracted in methanol. Samples were shaken with methanol for 30 minutes in a tube shaking unit (Griffin), followed by centrifugation at 2000 r.p.m. in an MSE 'Major' centrifuge. The supernatant obtained was reduced to a small volume in vacuo and the resulting concentrated methanolic extract could then be applied directly to t.l.c. plates and chromatographed. Aliquots of the sample were counted at each stage of the extraction procedure in order to estimate the percentage of the original material recovered during extraction which was found to range from 70 - 100%.

Development and visualisation of t.l.c.

Chromatograms were developed in solvent systems listed in Table 2.3. Rf values obtained for the compounds studied are given in Table 2.4. All compounds and metabolites, with the exception of phenolphthalein and its metabolites, were visualised under U.V. light (254 nm Hanovia Chromalight, Slough, Bucks., England) appearing as fluorescent and quenching spots or bands. Phenolphthalein and its metabolites were visualised on t.l.c. plates following acid hydrolysis involving the spraying of the plates with 0.1M HCl and heating at 80°C in an oven for 15 minutes. After heating, the plates were sprayed with 0.2 M NaOH, the presence of phenolphthalein or its glucuronide indicated by the appearance of reddish-brown bands or spots.

Hydrolysis of conjugates with β-glucuronidase or sulphatase

In order to confirm the identity of conjugated metabolites, suspected glucuronic acid conjugates and sulphoconjugates were hydrolysed with β-glucuronidase (Ketodase) and sulphatase (Type H-2, Sigma) respectively. The treated material was then compared by t.l.c. with the original samples of conjugated metabolite. Samples of bile (10 - 100 μl), urine (50 - 100 μl), perfusate (50 - 100 μl) and extracts from tissue or blood reduced to dryness in vacuo were adjusted to pH 5 with 0.2 M sodium acetate buffer. 1 ml Ketodase or 0.5 ml Sulphatase was added to the mixture which was then incubated at 37°C for 12 - 24 hours. The presence of β-glucuronidase activity in the commercial preparations of Sulphatase required the addition of 2 mg saccharo-1,4-lactone (Sigma) to the sulphatase incubations in order to inhibit

β-glucuronidase. Controls contained boiled enzyme, saccharo-1,4-lactone or phenolphthalein glucuronide where appropriate. A pink colouration appearing on the addition of alkali to the tube containing phenolphthalein glucuronide served as an indication that the β-glucuronidase in Ketodase incubations was active. Following incubation, methanol was added to precipitate protein and tha latter removed by centrifugation at 2000 r.p.m. in an MSE 'Major' centriguge. The supernatant was reduced in volume in vacuo and an aliquot chromatographed.

Radiochemical techniques

Liquid scintillation counting

Levels of [^{14}C] or [^{3}H] in labelled samples were determined by liquid scintillation counting in either a dioxan based scintillation fluid (Bridges, 1967) for [^{14}C] samples or a toluene/dioxan based fluid (Capel, 1974) for tritium samples and counted in a Packard Tri-Carb scintillation spectrometer (models 3214 and 3320). In later studies both [^{14}C] and [^{3}H] labelled samples were counted in a toluene /Triton X-100 (Koch-light laboratories) based scintillant consisting of 12.5g 2,5-Diphenyloxazole, 0.75g 1,4-di-2(5-phenyloxazolyl)-benzene, per 2.5l of toluene, two volumes of which were added to one volume of Triton X-100. Urine (0.1 - 1 ml), bile (10 - 100 μl), perfusate (0.1 - 1 ml) and [^{14}C] labelled whole blood samples (50 μl) were counted directly in the appropriate scintillant. Counting efficiencies, determined by the Channels-ratio method, were typically 19 - 28% for titrium and 70 - 90% for [^{14}C] labelled samples.Samples of tissue and faeces were homogenised prior to counting using an 'Ultra-Turrax' homogeniser. Aliquots of homogenates (0.1 - 1 ml) were counted in scintillant continaing a thioxtropic gel[Cab-O-Sil (Koch-Light Laboratories)] 5% w/v.

Radiochromatographic techniques

Samples of labelled compounds containing approximately 1×10^{4} d.p.m. [^{14}C] or 1×10^{6} d.p.m. [^{3}H] were applied as a band to t.l.c. plates and, following development, were scanned on a Packard Radiochromatogram Scanner (Model 7200). From the resulting scanner tracing, a qualitative estimate of the activity present could be obtained, peaks of activity corresponding to the position of labelled compounds on the radiochromatogram. A rough quantitative

estimate of the relative proportions of each compound present on the plate could be obtained by comparing the peak heights. Accurate quantitative analysis was achieved by cutting the radiochromatogram into 1 cm sections and counting each individual section by liquid scintillation counting (efficiencies typically 70 - 85% for [^{14}C] and 20 - 28% for [^{3}H].)

In vivo studies

Animals

Female Wistar albino rats (150 - 220 g) were used exclusively throughout this study and were supplied by Allington Farms (Porton Down, Salisbury, Wiltshire, England) or Anglia Laboratory Animals (Alconbury, Huntingdon, Cambridgeshire, England). Rats were fed on 41.b nuts supplied by Labsure Animal Diets (Poole, Dorset, England).

Administration of compounds to rats

Compounds were generally administered intravenously (i/v) via the femoral or jugular vein or intraperitoneally (i/p) in various injection vehicles according to the compound used: -

Phenolphthalein - dimethyl sulphoxide (DMSO) max. vol. / rat 0.1 ml or propylene glycol (max. vol. /rat 0. 2 ml).

Morphine - saline (max. vol. /rat 0. 5 ml).

Diphenylacetic acid - propylene glycol (max. vol. /rat 0. 2 ml).

LSD - aqueous solution containing a small amount of tartaric acid (max. vol. /rat 0. 2 ml).

Phenol and 1-Naphthol - aqueous solution (max. vol. /rat 0. 5 ml).

In certain experiments, compounds were administered intraduodenally (i/d), in which case compounds were dissolved in fresh rat bile.

Antibiotics - Antibiotics were suspended in an aqueous mixture of Tween 80, 5% v/v (polyoxyethylene sorbitan mono-oleate - BDH) and administered orally by passing a dosing needle (size 19G x 80 mm) down the oesophagus of the rat for a distance of about 5 cms.

Collection of urine and faeces in intact rats

Rats were housed in glass metabolism cages ('Metabowls' - Jencons, Hemel Hempstead, Herts., England), enabling urine and faeces to be collected

separately. Urine and faeces were collected at daily intervals during which time the rats were allowed free access to food and water.

Surgical Procedures

Anaesthesia

Rats were anaesthetised either with pentobarbitone sodium, 60mg/kg - i/p ('Nembutal' - Abbott Laboratories Ltd., Queensborough, Kent, England, or 'Sagatal' - May and Baker Ltd., Dagenham, Essex, England) or diethyl ether by inhalation. Diethyl ether was used prior to short operative procedures such as i/v injection, while for longer procedures such as bile duct cannulation, the longer acting barbiturates were found to be more convenient.

Bile duct and duodenal cannulation

The rats were anaesthetised with pentobarbitone sodium and the abdomen opened approximately 2 cm below the sternum. The duodenum was located and exteriorised, allowing location of the bile duct. A cannula, consisting of a length of polypropylene tubing (Portex PP 25- internal diameter 0.4 mm x external diameter 0.8 mm), was inserted into the bile duct at a point approximately 2 cm from its junction with the duodenum. The cannula was passed into the duct, towards the liver, for a distance of about 2 cm from its point of entry and secured with a ligature.

In studies requiring intraduodenal infusion, a second cannula was inserted into the bile duct distally to the first, so that the tip just entered the duodenum and was ligatured in place. Following cannulation, the abdominal wall was closed around the cannula with the aid of sutures and secured with suture clips. When bile was collected for periods of time extending beyond the anaesthetic range of pentobarbitone sodium, bile duct cannulated rats were housed in restraining cages, kept warm with a heating lamp and allowed free access to water.

In biliary excretion studies following i/p or i/d administration of compounds, bile was collected at hourly intervals for 24 hours by connecting the cannula of the bile duct cannulated rat to a fraction collector (Towers Automatic Fraction Collector, Model A). Urine was collected in a tray positioned beneath the restraining cage.

Intraduodenal infusion

In EHC studies, bile mixed with free compounds or bile containing their respective biliary metabolites (donor bile - collected from a 'donor' rat receiving an i/p dose of the free compound), was infused into the duodenum of a 'recipient' bile duct cannulated rat via an i/d cannula, fitted as previously described. Infusion was achieved by the use of an infusion pump (Palmer Slow Injection Apparatus) at rates of 0.64 and 1.28 ml/h. Recipient bile was collected at hourly intervals for 24 hours as described for the biliary excretion studies.

In the so called 'linked animals' studies, bile was allowed to drain directly from donor rats, into the duodenum of the recipient rats, via a 'linking'cannula (Portex PP25) connecting the bile duct of the donor with the duodenum of the recipient. The restraining cage housing the donor rat, was positioned approximately 20 cm above the cage housing the recipient so as to gain a 'gravity assisted' flow of bile from donor to recipient.

Antibiotic pretreatment

In certain studies rats were pretreated orally with antibiotics prior to bile duct cannulation and i/d infusion. An antibiotic dose consisted of a suspension of 100 mg neomycin sulphate (Upjohn Ltd., Crawley, Sussex, England), 50 mg tetracycline hydrochloride ('Achromycin' - Lederle Laboratories, Gosport, Hants., England) and 50 mg bacitracin (Burroughs, Wellcome and Co., London, England). The dosing regime used in the pretreatment entailed single daily doses for two days, followed on the third day by a dose 4 hours prior to and 4 hours after biliary cannulation and i/d infusion of the pretreated animal.

Cannulation of blood vessels

These operative procedures were carried out under barbiturate anaesthesia and for long term serial blood sampling necessitating a prolonged sedation, several doses of pentobarbitone sodium (20 - 40 mg/kg) were administed at approximately 5 hourly intervals following an initial anaesthetic dose (60 mg/kg).

The blood vessels employed in this study for the intravenous administration of compounds or withdrawal of blood were the femoral, jugular and pancreatico-dudoenal veins or carotid artery. Having located the appropriate blood vessel it was cleared of surrounding connective tissue and loose ties were made around either end of the exposed section of the vessel. From this point onwards, the

procedure varied depending on the particular blood vessel.

Veins

a) femoral and jugular veins

The venous end of the vein was ligatured with ties and a cannula, consisting of a length of polypropylene tubing (Portex PP25), filled with heparinised isotonic saline (500 I.U. Heparin / ml saline - Heparin Mucous B.P. - Paines and Byrne Ltd., Greenford, Middx., England) was inserted towards the arterial end of the exposed section of the vein and finally secured with a ligature.

b) pancreatico-duodenal vein

This vessel was used as a means of entry to the hepatic portal vein, enabling the collection of portal blood. A cannula, identical to that described for cannulation of the femoral and jugular veins, was inserted into the pancreatico-duodenal vein towards the junction of the latter with the hepatic portal vein. The cannula was secured with a ligature in such a position that the cannula tip just entered the portal vein, allowing the withdrawal of portal blood without interrupting the portal blood flow.

c) The carotid artery

The vessel was ligatured at the venous end and an artery clip placed across the artery, in order to temporarily occlude the arterial end during cannulation. The venous end was ligatured and a cannula, identical to that described for venous cannulation was inserted into the vessel between the artery clip and the venous ligature. The cannulae was finally secured with a ligature allowing the removal of the artery clip.

Serial blood sampling

Blood was withdrawn from the cannulae described previously via a luer fitting syringe adapter (Racath-LL: - C. R. Bard Inc., Billerica, Mass., U.S.A.) attached to each cannula. Samples were taken at 10, 20, 30, 45 and 60 minutes after administration of labelled compounds, followed by hourly samples for up to 14 hours. Samples (50 - 100 μl) withdrawn at each sampling interval were replaced with an equal volume of heparinised saline.

During long term blood sampling, rats were ventilated mechanically, (Small Animal Ventilator, Model 5056 - Scientific Research Instruments Ltd.,

Edenbridge, Kent, England) via a length of polypropylene tubing (Portex PP270 - internal diameter 2 mm x external diameter 3 mm) inserted into the trachea through a small incision and ligatured in place. Body temperature was maintained at 39°C (anal temperature) by placing the rats on a heated table, their temperature being regulated automatically via an anal tele-thermometer probe linked to a thermostatic control unit (Electrothermal Engineering Ltd., London, England).

Blood sampling from the tail

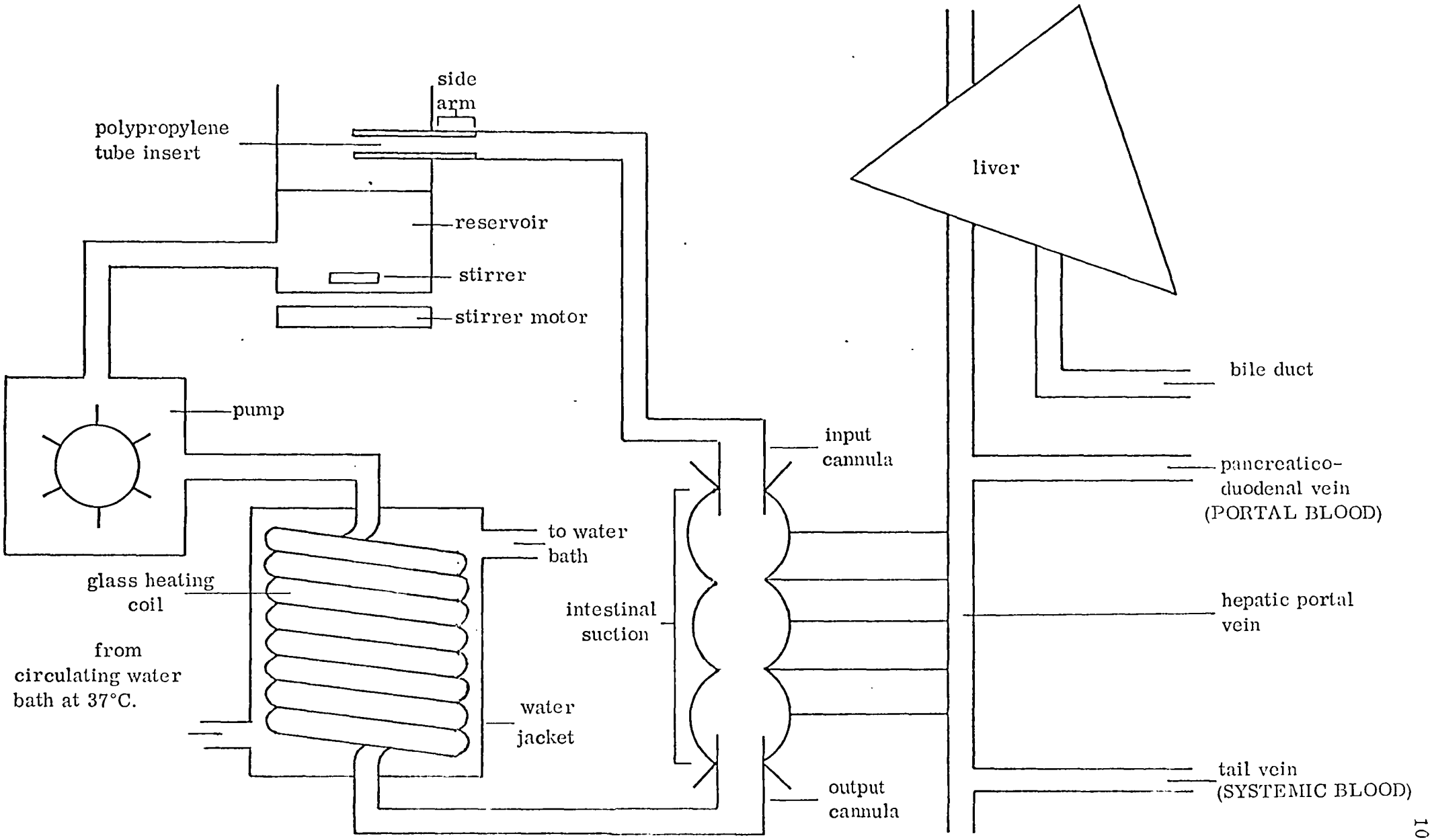
During intestinal perfusion studies, systemic blood was sampled from the blood vessels in the tail. Using a scalpel blade, the ventral side of the proximal portion of the tail was punctured to induce bleeding. A blood sample (50 μl) was taken using a micro pipette and the bleeding stopped by application of tape to the site of the wound. Removal of the tape allowed subsequent samples to be taken.

Intestinal Perfusion

Perfusion apparatus

The apparatus, as shown in Fig 2.2, consisted essentially of a pump, heating coil and a reservoir interconnected by glass tubing (internal diameter 3mm, x external diameter 5mm) with silicone rubber joints (internal diameter 3mm x external diameter 7mm - 'Silescol' - Esco (Rubber)Ltd., London, England). A peristaltic roller pump (Metaloglass Inc., Boston, Mass., U.S.A.) operating at a constant speed of 50 rev./min. and providing 300 pulsations/min., propelled perfusate draining from the reservoir, through the glass spiral heating coil. The coil was enclosed within a perspex water jacket connected to an externally sited circulating water bath ('Tecam' - tempunit) maintained at 37°C. Perfusate passed from the coil into the intestinal input cannula, through the intestinal section and out via the intestinal output cannula, draining into the top of the reservoir. The reservoir consisted of a glass tube (diameter 16 mm x length 115 mm) with a side arm (3.5 cm from the top) and a port at the base. Perfusate entered the reservoir via the side arm and was drained by the port. The insertion of a 1 cm length of polypropylene tubing (Portex PP55, internal diameter 0.75 mm x1.45 mm external diameter) into the side arm of the reservoir (see Fig. 2.2) allowed the perfusate to form droplets, the rate of

Fig 2.2 Diagram of the intestinal perfusion preparation



which was used as an indication of the flow rate through the system. Samples of perfusate were taken via a sampling port situated at the top of the reservoir. The contents of the reservoir were continuously mixed by a magnetic stirrer (6 x 12 mm). The entire perfusion apparatus was housed in a perspex cabinet (42 x 44 x 44 cm) with hinged doors allowing access and fitted with a thermostatically controlled fan heater which maintained a temperature of 37°C. The circulating water bath and stirrer motor (Monostir - Raven Scientific Instruments, Haverhill, Suffolk, England) were sited outside the cabinet.

Operative procedure

This procedure was carried out under anaesthesia (pentobarbitone sodium) and involved the initial cannulation of the bile duct and pancreatico-duodenal vein, by methods previously described, prior to intestinal cannulation. A section of the small intestine, extending from 2 cm below the duodeno-jejunal flexure to 30 cm distal to this point, was exteriorised and ligatured at either end. A cannula, consisting of a luer syringe filling needle (size 13g - Holborn Surgical Instruments Co. Ltd., London) 2 cm in length attached to a 2cm section of polythene tubing (Portex P55) was inserted at either end of the intestinal section just inside the ligatures and secured with ties. The entire intestinal section was flushed out with 50 -100 ml of water and replaced in the abdomen, leaving the cannulae protruding from the incision. The abdominal walls were closed around the cannulae with sutures and suture clips.

Perfusion

The apparatus was filled with 35 ml of a perfusion medium, consisting of an aqueous solution of sodium chloride (7.84g /L), potassium chloride (0.82 g/L) and sodium dihydrogen orthophosphate (0.96 g/L) adjusted to pH 7.4 to which had been added the labelled compound being studied. Having attained a constant temperature of 37°C within the apparatus and taken a control sample of the perfusate, the prepared rat was linked into the apparatus via its inflow and outflow cannulae and the perfusion started. Flow rate was adjusted to approximately 2 ml/minute and the volume of the perfusate in the reservoir noted.

Sampling

At intervals of 10, 20, 30, 40, 60 and 90 minutes after commencing perfusion, samples of perfusate (100 μl), portal blood (50 μl from the pancreatico-duodenal vein), systemic blood (50μl from the tail vein) and bile (collected continuously) were collected, aliquots of which were counted and chromatographed. At these sampling intervals, the volume of the perfusate in the reservoir was noted, enabling the concentration of the labelled compound in the perfusate to be estimated.

After 90 minutes, the perfusion was stopped, the perfusate drained from the system and the entire circuit, including the intestinal section, flushed with approximately 50 ml of water. The intestinal cannulae were disconnected from the apparatus and the rat was killed by a blow to the head. The intestinal section and liver of the rat were removed, homogenised and aliquots of the homogenates counted. Portal and systemic blood, bile and the homogenate of the intestinal section were extracted in methanol where appropriate and chromatographed.

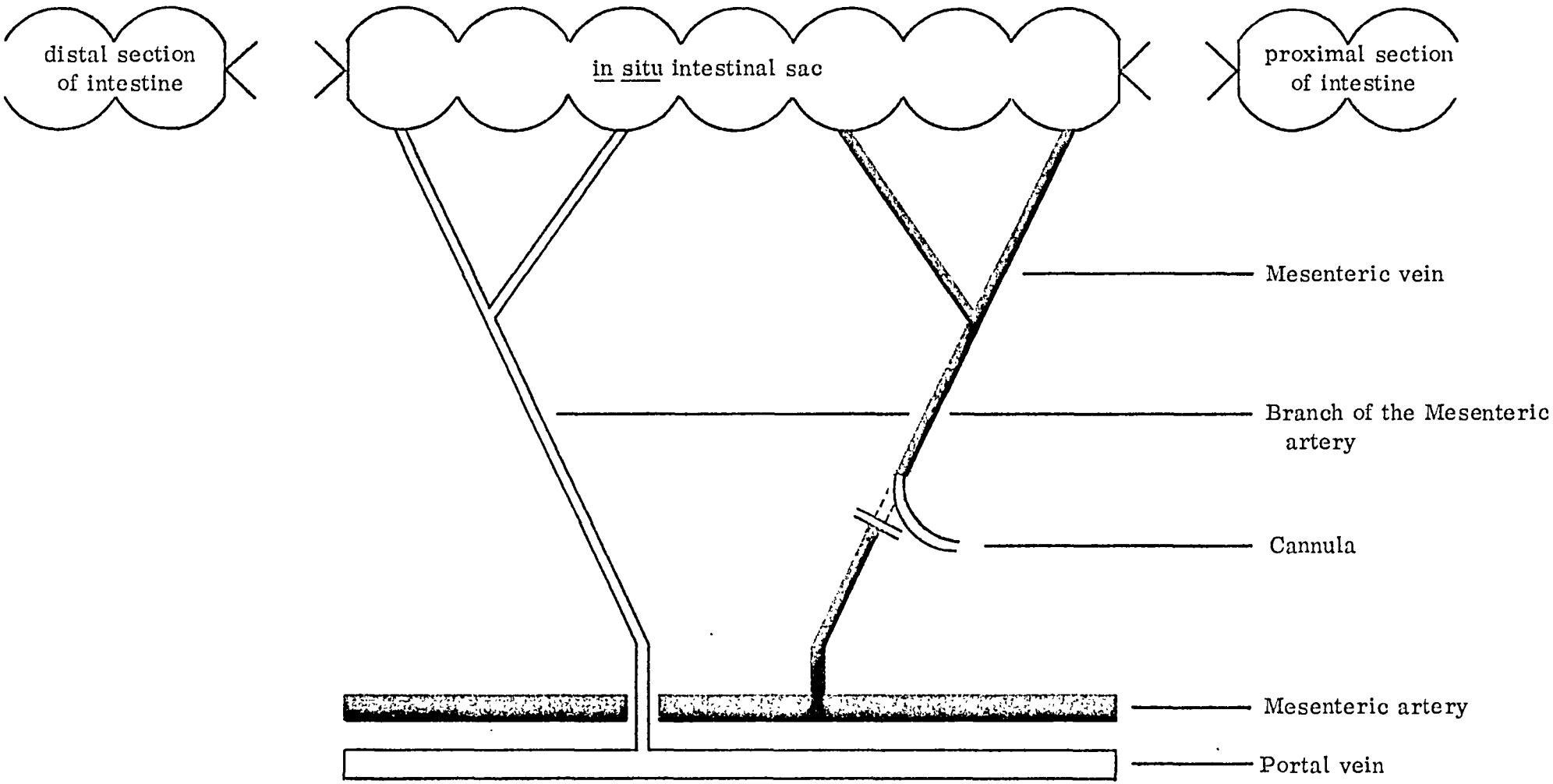
The in situ intestinal sac preparation

The preparation consisted of a semi-isolated intestinal sac connected to the animal solely by a branch of the mesenteric artery, as illustrated in Fig 2.3.

Operative procedure

Rats were anaesthetised with pentobarbitone sodium, an abdominal incision made and a loop of intestine about 5 cm in length was exteriorised approximately 10 cm from the duodenojejunal flexure. Two ligatures were made in the loop, about 3 cm apart, to form a sac of intestine. The sac was isolated from the remainder of the intestine by making two further ligatures close to those existing and cutting between each pair of ligatures. At this point, the sac was attached to the rat by a branch of the mesenteric artery and a vein draining blood from the sac into the portal vein. This vein was cannulated with polypropylene tubing (Portex PP25) so as to drain the sac (see Fig 2.3); the cannulation procedure being similar to that described for other veins. Blood was allowed to flow freely from this cannula and was replaced by infusion of heparinised rat blood (500 I.U. Heparin / ml) into the jugular vein via a

Fig 2.3 Diagrammatic representation of the in situ intestinal sac preparation



polypropylene cannula(Portex PP 25) using a syringe pump (Model 5200, Scientific Research Instruments) at a rate corresponding to the rate of blood loss from the cannulated mesenteric vein which varied from approximately 0.3 - 0.5 ml/minute.

Administration of labelled compounds

The labelled compound under study dissolved in 0.5 ml of isotonic salt solution (composition identical to that used in intestinal perfusion), was introduced into the lumen of the intestinal sac using a syringe fitted with a size 27G (0.4 mm x 15.5 mm) needle.

Sampling

Blood was collected at 5 minute intervals for 30 minutes from the cannulated mesenteric vein following the administration of the labelled compound to the sac. After 30 minutes the rat was killed by a blow to the head and the intestinal sac removed. The contents of the sac were collected and aliquots counted and chromatographed while the sac itself was weighed and homogenised. Aliquots of blood samples and the homogenate of the sac were counted, extracted in methanol and chromatographed.

In Vitro Techniques

Preparation of everted gut sacs

Rats were killed by a blow to the head, and the entire intestine, extending distally from the stomach to the ileocaecal junction, was removed and flushed with about 100 ml of water. The isolated intestine was divided into three sections. Using a glass rod (3 mm diameter), each section was everted then ligatured at one end and filled with a buffer saturated with 95% O_2 / 5% CO_2 (the serosal buffer) consisting of NaCl 0.134 M, KCl 0.008 M, NaH_2PO_4 0.008 M, $CaCl_2$ 0.001 M and glucose 0.02 M adjusted to pH 7.4 as described by Smith et al., (1963). From the middle of each intestinal section a sac 3 cm in length was made between two ligatures and cut away from the remaining tissue.

In vitro gut sac incubation

Each gut sac was placed in a small conical flask containing 15 ml of a buffer (the mucosal buffer) identical in composition to the serosal buffer, but to which had been added the labelled compound being studied. Each flask was supplied with a constant stream of 95% O_2 / 5% CO_2 and incubated in a

shaking water bath (H. Hickle, Gromshall, Surrey, England) and maintained at 37°C. After 90 minutes incubation, the sacs were removed from their mucosal bathing buffer, drained of serosal buffer and the lumen rinsed in water. Each sac was weighed, homogenised and an aliquot of the homogenate counted. Aliquots of both mucosal and serosal buffers were counted and chromatographed. The homogenate of the gut sac was extracted in methanol and an aliquot of the extract chromatographed.

Binding studies of compounds to hepatic and intestinal mucosal cytoplasmic proteins

The binding of compounds in vivo and in vitro to cytoplasmic proteins of 110,000 g supernatants of liver and intestinal mucosal homogenates was studied by gel filtration using similar methods to those described by Levi et al., (1969) and Takada et al., (1974).

It is well known that a mixture of proteins loaded onto a gel filtration column passes through the gel at a rate dependent upon their molecular size when eluted with a suitable buffer (Sephadex - Gel filtration in theory and practice, 1976). High molecular weight proteins pass through the gel more rapidly than those of a lower molecular weight and thus the former appear in the eluate from the column prior to the latter.

Preparation of the 110,000 g supernatants from rat liver and intestinal mucosal homogenates

Rats were killed by a blow to the head and the liver and intestine rapidly removed and placed in ice.

a) Liver

The organ was perfused via the hepatic veins with cooled (5°C) isotonic saline using a cannula of polypropylene tubing (Portex PP 270) attached to a peristaltic pump (Watson-Marlow MHRE-200) in order to remove all traces of blood from the liver. The organ was homogenised to a 25% homogenate (w/v) in 0.25 M sucrose / 0.01 M phosphate buffer pH 7.4 at 5°C.

b) Intestinal mucosa

The lumen of the intestine was flushed with cooled (5°C) isotonic saline and the mucosa scraped away from the underlying muscular tissue using a glass microscope slide. The mucosal tissue was homogenised to a 25% w/v homogenate in a buffer of identical composition to that used to prepare the liver homogenate.

The liver and intestinal mucosal homogenates were centrifuged at 110,000g for 2 hours at 5°C in an MSE 'High Speed 65' centrifuge. Following centrifugation the supernatant was separated from the pellet and aliquots of each were counted. The pellet was then discarded and the supernatant stored at -70°C. The protein concentration of supernatants was estimated by the Biuret method (Robinson, 1940) using bovine serum albumin (Sigma) as a standard.

In vivo and in vitro studies

a) In vivo

Rats were killed by a blow to the head and the liver and intestine removed at 5 or 10 minutes after i/v administration of the labelled compounds being studied. 110,000g supernatants were prepared as previously described and stored at -70°C until analysed by gel filtration. Aliquots of the homogenates and supernatants were counted or analysed spectrophotometrically to estimate the % of the administered dose recovered at each stage of the procedure.

b) In vitro

The labelled compounds being studied were incubated with aliquots of liver or intestinal mucosal 110,000g supernatants containing 25 - 50 mg protein for 1 hour at 5°C prior to gel filtration.

Gel filtration

Aliquots of 110,000g supernatants of liver and intestinal mucosa, containing 25 - 50 mg protein, derived from in vitro incubations or from in vivo studies, were loaded onto a column 2.2 cm diameter x 30 cm long (bed volume = 115 ml Wright Scientific, London, England) packed with Sephadex G75 'superfine' (bead size 10 - 40 μ - Pharmacia, Sweden). Samples were eluted from the column with 0.01 M phosphate buffer containing 0.02% w/v sodium azide (BDH) by an upward flow of 12 - 30 ml / hour using a peristaltic pump (LKB 2120 Varioperspex II). Eluate emerging from the top of the column passed directly through a U.V. detector (LKB Uvicord II / 8300 U.V. analyser) linked to a chart recorder (Servoscribe (1s) Model RE S40.20) set at 280 nm in order to monitor protein concentration. Eluate passed from the U.V. detector and was collected as timed fractions (60 - 100 fractions of 2 - 4 ml) on a fraction collector (LKB Ultrorac 7000). The entire procedure was carried out at 5°C. The concentration of the various compounds studied in each fraction was determined either by counting or spectrophotometrically in a U.V.

spectrophotometer (Model CE 595, Cecil Instruments, Cambridge, England) at 580 nm for bromosulphophthalein (BSP - Hynson, Westcott and Dunning Inc., Baltimore, Maryland, U.S.A.) following the addition of 0.01 M NaOH. When labelled compounds were present aliquots of each fraction were counted in order to estimate the concentration of compounds in each individual fraction.

Association of a compound with protein in the same fraction was taken as indicative of binding; unbound compounds were found to be associated with the latter fractions (approximate fraction nos. 60 - 100) in which protein was absent.

Estimation of partition ratio

The partition ratios of radiolabelled phenolphthalein, morphine, LSD, 13-hydroxy-LSD, diphenylacetic acid and their respective glucuronides were determined between octan-1-ol and 0.1 M phosphate buffer pH 7.4 as used by Leo et al., (1971), Tute (1971), Hirom et al., (1974) and Houston, (1974). The glucuronides were obtained by scraping the glucuronide containing bands from the t.l.c. plates on which samples of bile, collected from rats receiving the [^{14}C] parent compounds, had been chromatographed. Each compound was dissolved in 10 ml of the phosphate buffer and 10 ml of octan-1-ol added (the octan-1-ol and phosphate buffer had been equilibrated together prior to addition of compounds). The mixture was shaken in a shaking unit (Griffin) for 30 minutes followed by centrifugation at 2000 rpm for 10 minutes. Aliquots (50 μl) of both the organic and aqueous layer were counted. From the % of the total activity present in each layer the partition ratio was calculated.

CHAPTER THREE

The Enterohepatic Circulation of Phenolphthalein

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Introduction

Pharmacological properties

Phenolphthalein belongs to the group of drugs known as stimulant cathartics which are characterised by their ability to induce defaecation by increasing the motor activity of the gut. The precise mechanism of action of stimulant cathartics is not known, but is generally thought to be a stimulation of peristalsis either by a selective action on the intermural plexus or a local irritation of the intestinal mucosa (Travell, 1954). More recently stimulant cathartics have been shown to act on electrolyte transport such that the reabsorption of water and electrolytes from the gut is reduced, however the relative importance of this particular action to the overall cathartic effect of these drugs is unclear, (Forth et al., 1966). The pharmacological activity of phenolphthalein was first studied by Vamossy (1936) who showed that the drug exerted its greatest effect on the colon, but also increased the motor activity of the small intestine. In man, the onset of action has been shown to occur at 6 - 8 hours following oral administration (Fantus and Steigman, 1938).

Metabolism and disposition

Early studies in the dog indicated that phenolphthalein was excreted in the bile as the glucuronic acid conjugate and was subsequently reabsorbed from the gut (Abel and Rowntree, 1909). Later quantitative work in dogs treated orally with phenolphthalein showed that 40% of the dose was excreted in the bile, 35% in the urine and 11% in the faeces over 3 days (Visek, 1956). A species difference as to the extent of biliary excretion of phenolphthalein has been reported, the rat excreting 85% of a parenteral dose over 3 hours, the guinea pig 22%, the rabbit 25%, the dog 57%, the hen 43% and the cat 13% of the dose over a similar time period, mainly as a glucuronic acid conjugate (Abou-El-Makarem,1967). Limited evidence in man suggests that phenolphthalein is excreted in the bile as a glucuronic acid conjugate. In one patient 26% of an oral dose was excreted in the bile, of which 21% was found to be conjugated, the remainder as the free drug (Pekanmaki and Salmi, 1961). A relationship between the EHC of phenolphthalein and

its cathartic activity has been suggested (Pekanmaki and Salmi, 1961), which may explain the absence of cathartic responses to the drug in cats with obstructive jaundice (Steigmann and Barnard, 1938). This condition has been shown to give rise to a reduced biliary excretion of phenolphthalein and a complementary increase in urinary output and abnormally high plasma levels of the drug (Fantus and Steigmann, 1941).

Methods

1. Excretion of [^{3}H]-Phenolphthalein in the urine and faeces of intact rats

Urine and faeces were collected at daily intervals for 4 days from rats dosed with 25 mg/kg i/p [^{3}H]-Phenolphthalein. Aliquots of urine and faecal homogenates were counted and subjected to t. l. c. using solvents A and B (see Table 2. 3).

2. Estimation of the biliary excretion of [^{3}H]-Phenolphthalein

Rats were bile duct cannulated in the manner described in Chapter 2 and dosed with 25 mg/kg i/p [^{3}H]-Phenolphthalein. Bile was collected continuously at intervals of 1, 2, 3 and 24 hours and urine collected for the duration of the experiment. Aliquots of bile and urine were counted and chromatographed on t. l. c. using solvents A and B (see Table 2. 3).

3. Intraduodenal infusion

Bile was collected continuously for 3 hours from bile duct cannulated rats receiving doses of 25 mg/kg i/p [^{3}H]-Phenolphthalein. This donor bile was then infused into the duodenums of a second group of recipient bile duct cannulated rats at a rate of 0. 64 or 1. 28 ml/hour, the bile from which was collected at hourly intervals for 24 hours. Urine was collected for the duration of the experiment, aliquots of both urine and bile were counted and chromatographed as previously described.

In a second series of experiments, the donor bile infused into the recipient animals was substituted with bile containing [^{3}H]-Phenolphthalein at a dose equivalent to the amount of [^{3}H] present in a dose of donor bile.

4. 'Linked'animal method

Donor rats receiving 25 mg/kg i/p [^{3}H]-Phenolphthalein were linked to recipient rats as described in Chapter 2. Bile and urine from the recipients was collected and analysed as described for the i/d infused recipient.

5. Interruption of EHC

a) Treatment with saccharo-1,4-lactone

Saccharo-1,4-lactone was prepared by the reflux of 2 g potassium hydrogen saccharate (BDH) with 20 ml water at pH 3. 5 for 30 minutes as described by Levvy (1952). 5 mls of the resulting solution, containing approximately 500 mg saccharo-1,4-lactone, was infused via the intra-duodenal cannula of the recipient bile duct cannulated rat over a period of

5 minutes. 30 minutes after administration of saccharo-1, 4-lactone, donor bile or bile containing [^{3}H]-Phenolphthalein was infused as described in previous studies.

b) Antibiotic pretreatment

Rats were pretreated with a combination of antibiotics as described in Chapter 2 prior to intraduodenal infusion of donor bile or bile containing [^{3}H]-Phenolphthalein into recipient rats as described above.

6. Intestinal perfusion

Following procedures described earlier for intestinal perfusion, rats were perfused with buffer containing either 5 mg [^{3}H]-Phenolphthalein or an equivalent amount of 'donor' bile containing [^{3}H]-Phenolphthalein biliary metabolites on the basis of [^{3}H] activity. Aliquots of bile, perfusate, urine, gut homogenate, liver homogenate, portal and systemic blood samples collected as previously described, were counted and subjected to t. l. c. using solvents A and B (see Table 2. 3).

7. Absorption of [^{3}H]-Phenolphthalein from gut sacs.

Gut sacs were prepared as described in Chapter 2 and each sac incubated in a mucosal buffer solution containing 0. 5 mg [^{3}H]-Phenolphthalein or bile containing an equivalent amount of [^{3}H]-Phenolphthalein metabolites collected from 'donor' rats as described earlier. Following incubation, aliquots of the gut sac homogenate, mucosal and serosal fluids were counted and subjected to t. l. c. using solvents A and B (see Table 2. 3).

8. Estimation of systemic blood levels in rats receiving intravenous doses of [^{3}H]-Phenolphthalein.

The carotid artery was cannulated in intact and bile duct cannulated rats using the procedure described in Chapter 2. The animals were injected intravenously with 25 mg/kg [^{3}H]-Phenolphthalein and blood samples withdrawn at intervals , aliquots of which were prepared for counting and t. l.c.. using solvents A and B, (see Table 2. 3). In bile duct cannulated animals, bile was collected continuously and samples collected at the same intervals as blood samples were withdrawn. Aliquots of bile samples were counted and subjected to t. l. c. using solvents A and B, (see Table 2. 3).

9. Protein bindings studies with [^{14}C]-Phenolphthalein and bromosulphophthalein (BSP)

a) In vivo studies

Rats were killed at 5 or 10 minutes following i/v administration of [^{14}C]-Phenolphthalein (25mg/kg) or BSP (50mg/kg), the livers were removed and 110,000g supernatants of the liver homogenate prepared as described in Chapter 2. Samples of the supernatants (equivalent to 75 mg protein) were applied to gel filtration columns and eluted at a rate of 12 - 25 ml/h as described in Chapter 2.

b) In vitro studies

Aliquots (1 - 4mls) of 100,000g supernatants equivalent to either 75 or 25 mg protein, prepared from liver and intestinal mucosal homogenates respectively, were incubated for 2 hours at 4°C with 250μg (0.8 μM) [^{14}C]-Phenolphthalein or 794 μg (10μM) BSP. Following incubation, samples were subjected to gel filtration at elution rates of 12 - 25 ml/h as described in Chapter 2.

Results and Discussion

1) The excretion of [^{3}H]-Phenolphthalein in the urine and faeces of intact rats

Over a period of 4 days following dosing, 96.5% of the dose was recovered of which 86.5% appeared in the faeces and 10.0% in the urine. The daily excretion levels shown in Table 3.1 indicate that while the overall excretion of the drug was delayed with only 26% of the dose appearing in the first 24 hours, the bulk (78%) was recovered within 48 hours.

T.l.c. and radiochromatogram scanning of urine samples collected 24 hours after dosing revealed the presence of a single metabolite chromatographically identical to phenolphthalein β-D-glucuronide. T.l.c. and radiochromatogram scanning of aliquots of a homogenate of faeces collected 24 hours after dosing showed the presence of two metabolites, one chromatographically identical to phenolphthalein and the other to phenolphthalein glucuronide. The relative proportions of the two metabolites present in the faeces was shown to be 83.3% phenolphthalein and 16.7% phenolphthalein glucuronide. The identity of the metabolites thought to be glucuronides was confirmed following treatment with β-glucuronidase whereby t.l.c. of the β-glucuronidase treated urine and faecal homogenates revaled the complete hydrolysis of the glucuronide to yield the free drug.

2) Extent of biliary excretion of [^{3}H]-Phenolphthalein

Over a period of 24 hours following dosing with [^{3}H]-Phenolphthalein, 94.6% of the dose appeared in the bile, while only 0.2% appeared in the urine, (Table 3.2).

T.l.c. and radiochromatogram scanning of bile samples showed the presence of a single metabolite chromatographically identical to phenolphthalein glucuronide. Identity of the biliary metabolite as phenolphthalein glucuronide was confirmed following treatment with β-glucuronidase. Comparing the excretion data from bile duct cannulated rats to that in intact rats, it can be seen that at 24 hours after dosing, biliary excretion (94.6%) exceeds the faecal excretion (20%) suggesting that the difference (74.6%) is reabsorbed from the gut to enter EHC. The urinary excretion in intact rats at 24 hours after dosing was found to exceed that in bile duct cannulated rats, the difference of 5.8% of the dose thought to represent that portion of the dose reabsorbed from the gut which escapes hepatic uptake and enters systemic circulation.

Table 3.1 Excretion of [^{3}H]-Phenolphthalein in the urine and faeces of intact rats

Day	% [^{3}H] in the urine	% [^{3}H] in the faeces	Total [^{3}H] in the urine and faeces
1	6.0 (0.5)	20.0 (9.5)	26.0
2	2.6 (0.8)	49.4 (4.5)	52.0
3	0.8 (0.2)	9.8 (3.1)	10.6
4	0.6 (0.2)	7.3 (3.0)	7.9
1 - 4 (TOTAL)	10.0 (1.0)	86.5 (3.9)	96.5

* (% [^{3}H] = % of [^{3}H] present in the injected dose (i/p) of [^{3}H]-Phenolphthalein - 25 mg/kg) Values quoted are the mean of three animals and those in brackets, the standard error of the mean.

Table 3.2 The biliary excretion of [^{3}H]-Phenolphthalein

Time	Sample	% [^{3}H] of the dose
1	Bile	60.0 (1.7)
2	Bile	20.6 (2.0)
3	Bile	8.5 (1.8)
24	Bile	5.5 (2.5)
24	Urine	0.2 (0.01)
0 - 24	Bile and Urine	94.8 -

* % [^{3}H] = % of the [^{3}H] present in the injected dose (i/p) ([^{3}H]-Phenolphthalein 25 mg/kg) appearing in the bile. Values quoted are the mean of three animals and those in brackets, the standard error of the mean.

The data suggests an extensive EHC of phenolphthalein operates in the rat, whereby 75% of the dose appearing in the bile is reabsorbed from the gut of which about 6% is lost to the systemic circulation and ultimately to the urine. From this data it could be predicted from the difference between the amount of drug reabsorbed from the gut and that lost to the urine, that about 70% of the dose would be re-excreted in the bile.

The bile has been shown to contain phenolphthalein glucuronide as the sole metabolite and thus the identification of free phenolphthalein as a major component of metabolites in the faeces suggests that a portion of the biliary conjugates are hydrolysed in the gut lumen to yield the free drug.

3. Intraduodenal infusion studies

In order to confirm and quantify the EHC of phenolphthalein on the basis of biliary re-excretion of the drug, donor bile containing [^{3}H]-Phenolphthalein glucuronide was infused intraduodenally (i/d) into recipients and the re-excretion of the drug in recipient bile estimated. To ascertain the contribution of the proposed luminal hydrolysis of biliary conjugates of glucuronide to

to the EHC of phenolphthalein, phenolphthalein was infused in place of the glucuronide in later experiments.

a) Infusion of donor biliary metabolites

Bile collected from donor rats was found to contain 80 - 90% of the i/p dose of [^{3}H]-Phenolphthalein, in the form of phenolphthalein glucuronide as the sole biliary metabolite. On the i/d infusion of donor bile, which is essentially a dose of phenolphthalein glucuronide, into recipients at a rate of 1.28 ml/hour, 62.4% of the infused dose of [^{3}H] was recovered in the bile of the recipient over 24 hours. On infusion at a lower rate of 0.64 ml/hour, 85.3% of the dose was recovered in recipient bile over 24 hours. The remainder of the infused dose was recovered mainly in the faeces and gut contents and a small amount in the urine (see Table 3.3). This data confirms the conclusions drawn from previous studies in intact and bile duct cannulated rats that phenolphthalein undergoes a considerable EHC in the rat.

The amount of donor metabolites appearing in recipient bile following infusion at 1.28 ml/hour was found to be significantly different ($P < 0.02$) from that following infusion at the lower rate of 0.64 ml/hour (see Fig 3.1). This difference suggests that the extent of absorption of donor metabolites from the gut is in some way dependent upon the rate at which donor bile is delivered to the duodenum of the recipient. It seems probable that at high rates of infusion, the concentration of donor metabolites arriving in the gut is sufficiently high to saturate some mechanism involved in their absorption from the gut and re-excretion in recipient bile. It may be significant that infusion rates of 1.28 ml/hour would represent up to twice the normal physiological rate at which bile enters the duodenum which, in our studies, averaged 0.7 $\pm$ 0.2 ml/hour in bile duct cannulated rats.

Chromatographically, bile collected from recipient rats was shown to be identical to donor bile in that it contained phenolphthalein glucuronide as the sole metabolite.

b) Infusion of bile mixed with [^{3}H]-Phenolphthalein

On i/d infusion of bile mixed with [^{3}H]-Phenolphthalein into recipeints, 72 - 77% of the infused dose was recovered in the recipient bile within 24 hours.

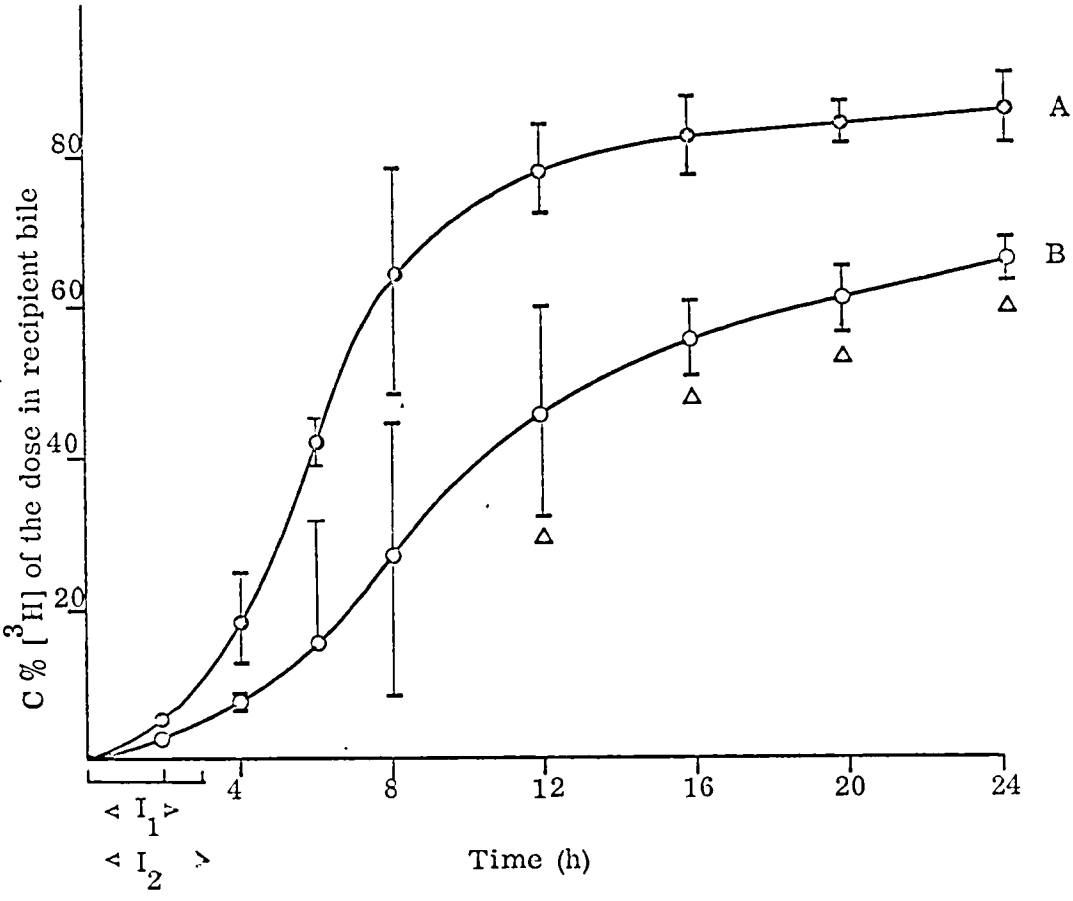


Fig 3.1 The rate of excretion of [^{3}H] in the bile of recipient rats infused (i/d) with [^{3}H]-Phenolphthalein glucuronide at various infusion rates.

% [^{3}H] = % of the [^{3}H] infused (i/d) dose appearing in the bile of the recipient. A = recipients infused at rate of 0.64 ml/h. B = recipients infused at rate of 1.28 ml/h. I_1 and I_2 = duration of i/d infusion at rates of 1.28 and 0.64 ml/h respectively. Each point represents the mean of three animals and the vertical bars, the standard error of the mean. Δ = the points at which A differs significantly ($P < 0.02$) from B. (C= Cumulative)

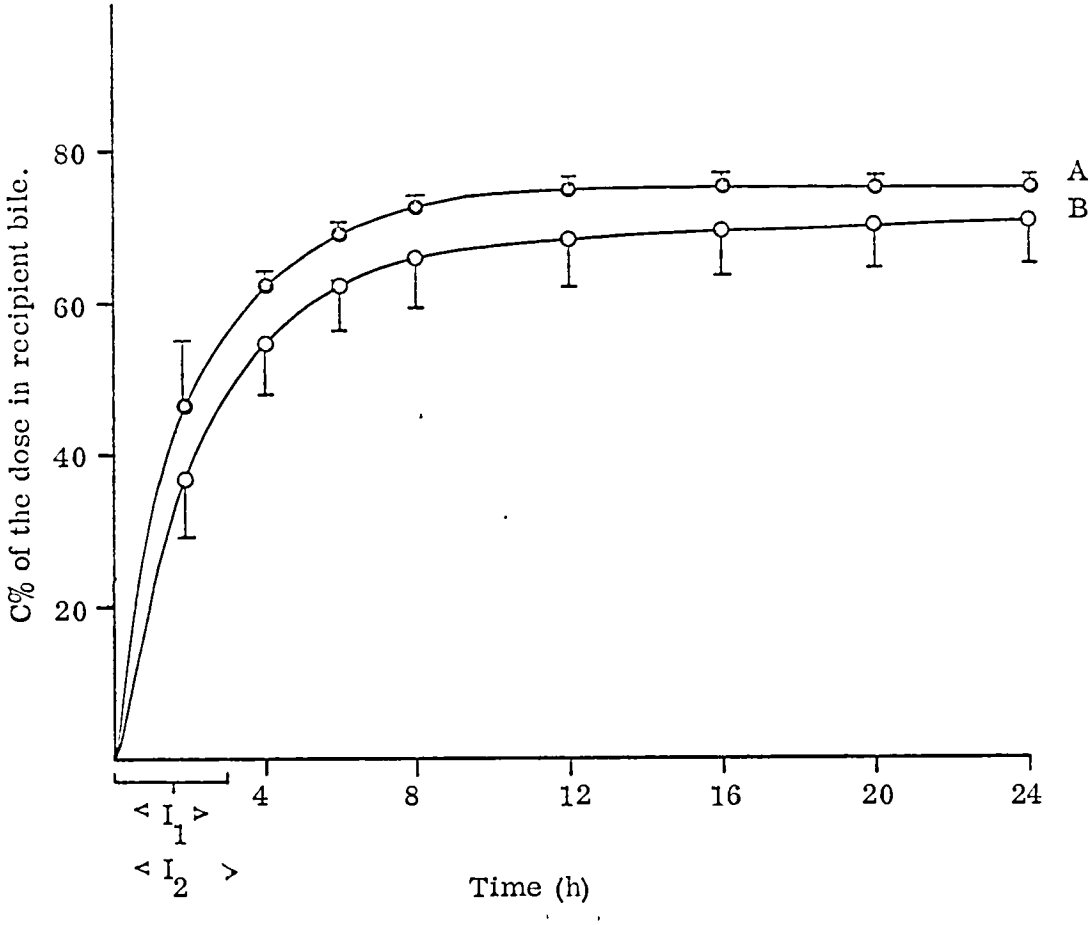


Fig 3.2 The rate of excretion of [^{3}H] in the bile of recipient rats infused (i/d) with [^{3}H]-Phenolphthalein at various infusion rates.

% [^{3}H] = % of the [^{3}H] infused (i/d) dose appearing in the bile of the recipient. A = recipients infused at rate of 0.64 ml/h. B = recipients infused at rate of 1.28 ml/h. I_1 and I_2 = duration of i/d infusion at rates of 1.28 and 0.64 ml/h respectively. Each point represents the mean of three animals and the vertical bars, the standard error of the mean. No significant difference ($P > 0.05$) was observed at any point between A and B. (C = cumulative)

Table 3.3. The excretion of [^{3}H]-Phenolphthalein glucuronide in recipient bile duct cannulated rats following administration of donor bile by 'link' or intraduodenal infusion

Sample	Time	i/d infused (1.28 ml/h) C %[^{3}H] in bile	i/d infused (0.64 ml/h) C % [^{3}H] in bile	linked animals C % [^{3}H] in bile
Bile	1	0.6 (0.2)	- -	0.6 (0.6)
	2	1.3 (0.1)	4.4 (1.3)	1.7 (1.4)
	4	4.2 (0.1)	15.9 (6.1)	9.9 (8.7)
	6	12.1 (3.4)	42.4 (16.3)	14.3 (11.8)
	8	27.2 (18.0)	64.9 (16.8)	21.1 (12.0)
	12	43.4 (14.0)	80.7 (6.5)	32.9 (7.8)
	16	56.4 (5.3)	83.7 (5.1)	42.0 (3.2)
	20	60.6 (3.3)	84.6 (4.5)	50.1 (2.0)
	24	62.4 (2.5)	85.3 (4.9)	54.2 (4.4)
Urine	24	0.5 (0.1)	0.3 (0.1)	0.8 (0.1)
Faeces and gut content	24	14.0 (2.2)	1.5 (0.2)	5.4 (3.7)
Total	24	79.0 (6.0)	87.1 (4.8)	60.6 (4.2)

* % [^{3}H] = % of the infused dose present in the recipient bile, urine or gut contents at the time indicated. Values quoted are the mean of three animals and figures in brackets the standard errors of the means. (C = cumulative).

No significant difference in the recoveries of [^{3}H] in recipient bile was observed between rats infused at 1.24 ml/hour and thoseinfused at 0.64 ml/hour (see Fig 3.2). T.l.c. and radiochromatogram scanning of recipient bile again showed the presence of phenolphthalein glucuronide as the only biliary metabolite.

Comparing the rate and extent of [^{3}H] excretion in recipient bile following infusion of donor bile (phenolphthalein glucuronide) with that following infusion of the bile mixed with phenolphthalein, major differences were observed. Firstly, that over the first 8 hours following infusion, there was significantly lower excretion of [^{3}H] in the bile of recipients infused with glucuronide, when compared with recipients infused with phenolphthalein (see Fig 3.3). This result may be explained if phenolphthalein were absorbed by the gut preferentially to its glucuronide, as will be shown later and that phenolphthalein glucuronide excreted in the bile is hydrolysed in the gut lumen by the gut flora, as suggested by earlier experiments. The slow excretion of [^{3}H] in recipient bile over the first 8 hours following infusion of the glucuronide may be a delay arising from the time required for the microfloral hydrolysis of the poorly absorbed glucuronide to yield the readily absorbed phenolphthalein. Secondly, differences in the overall [^{3}H] recovered in the bile of recipients following infusion, at varying rates, of the glucuronide were not observed when repeated with phenolphthalein itself under similar conditions. This may suggest that the hydrolytic capacity of the gut microflora may be the rate limiting saturatable step in the overall process of the biliary re-excretion of phenolphthalein glucuronide.

4) Linked animals method

Intraduodenal infusion of donor bile involves the delivery of [^{3}H] - Phenolphthalein biliary metabolites at a fixed concentration to the duodenum of the recipient. In the physiological situation, we have shown from biliary excretion studies that the concentration of phenolphthalein biliary metabolites that would be delivered to the duodenum, varies according to the time after dosing, (Table 3.3). As donor bile passes directly from the bile duct to the duodenum of the recipient in the linked animal technique, the normal physiological delivery is retained. Following the administration of 25 mg/kg i/p [^{3}H]-Phenolphthalein to the donor, it was assumed from earlier studies that

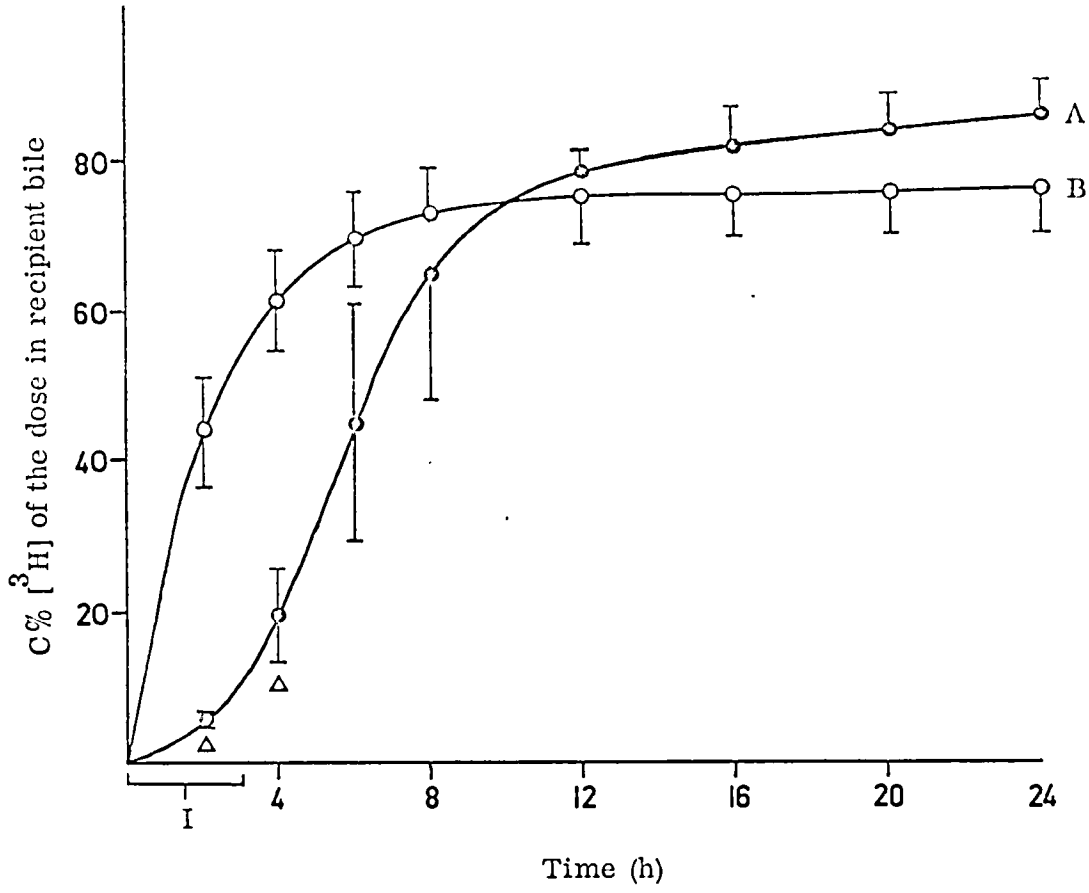


Fig 3.3 Comparison of the rates of [^{3}H] excreted in the bile of recipient rats between infusion (i/d) with (A) [^{3}H]-Phenolphthalein glucuronide and (B) [^{3}H]-Phenolphthalein.

% [^{3}H] = % of the [^{3}H] infused (i/d) dose appearing in the bile of the recipient. I = the duration of the i/d infusion (0.64 ml/h). Each point represents the mean of three animals and the vertical bars, the standard error of the mean. Δ = the points at which A differs significantly ($P < 0.05$) from B. (C = cumulative)

approximately 80 - 90% of the injected dose would appear in the bile of the donor passing into the recipient via the 'link'. Over 24 hours, only 54% of the dose assumed to have reached the duodenum of the recipient, was recovered in the recipient bile, as compared with 85% recovered following intraduodenal infusion, (Table 3. 3).

It is suggested from these results that either the delivery of bile from donor to recipient was restricted or that the extent of biliary excretion of the drug by the donor was lower than was recovered in the bile in previous experiments (see Table 3. 2). The inability to assess the extent of biliary excretion of the drug in the donor as a result of the link is a major criticism of the technique. All further studies requiring intraduodenal delivery of a drug to a recipient were made by infusion at the physiological rate of 0. 64 ml/hour.

5) Effect of interruption of phenolphthalein EHC

In order to assess the role of luminal hydrolysis of biliary metabolites in the intestinal phase of phenolphthalein EHC, the intraduodenal infusion of phenolphthalein or its glucuronide to recipients and the subsequent biliary excretion from recipient bile was studied in rats in which the hydrolytic capacity of the gut microflora was depressed. A reduction in hydrolytic activity of the gut microorganisms was achieved either by inhibition of bacterial β-glucuronidase using the inhibitor saccharo-1,4-lactone, or a reduction in the gut microfloral population following antibiotic treatment.

a) Pretreatment with saccharo-1,4-lactone

The extent of excretion of [^{3}H] over 24 hours in the bile of recipients infused with phenolphthalein glucuronide at varying concentrations, and receiving saccharo-1,4-lactone, ranged from 14 - 67% of the infused dose over 24 hours. The bulk of the infused dose not appearing in the recipient bile was recovered in the gut contents. Controls not receiving the inhibitor gave recipient biliary recoveries of 79 - 95% suggesting that the inhibitor was reducing the extent of [^{3}H] excreted in recipient bile. The wide variation in the biliary recoveries in the group receiving the inhibitor was such that no statistical difference could be shown between this group and the control group. It is thought that the variation in biliary recovery in the group receiving the inhibitor may be due to inhibitory concentrations of saccharo-

1,4-lactone not being reached in the gut lumen of certain rats.

b) Antibiotic pretreatment

Over 24 hours only 22% of an i/d dose of phenolphthalein glucuronide was recovered in the bile of recipient rats pretreated with antibiotics, the remainder recovered in the gut contents (67.4 ± 3.2%) and urine (0.3 ±0.1%). In similarly treated rats receiving phenolphthalein in place of its glucuronide, 84% of the infused dose was recovered in the bile of the recipient and the remainder in the gut contents (11.3 ± 4.7%) and urine (0.3 ± 0.1%). The wide variations in biliary recoveries observed following saccharo-1,4-lactone pretreatment were not seen following antibiotic pretreatment. Comparison of the rate of biliary excretion of [^{3}H] in recipients receiving phenolphthalein with that in animals receiving its glucuronide showed a significant difference ($P < 0.001$) between the groups at all times up to 24 hours (see Fig 3.4).

These findings confirm the earlier studies that suggested the hydrolysis of the biliary glucuronide was a requirement for the extensive absorption of the biliary metabolites from the gut. The particular combination of antibiotics and dosing regime used in this study has previously been shown to markedly reduce the number of viable organisms present in the caecal contents of rats. At 4 hours following the last dose of antibiotic in the regime, the number of viable organisms present in the caecal contents has been estimated to be less than 50% of the levels present in controls rats not receiving antibiotics. Regrowth of microflora following cessation of the antibiotic treatment was found to be fairly rapid, returning to control levels 48 hours after the last dose of antibiotic (Gingell, 1970).

The individual antibiotics which made up the combination used in this study are known to possess bacteriostatic properties capable of eliminating the majority of gut microfloral species, including those having high β-glucuronidase activity.

Neomycin sulphate

Neomycin has a broad bacteriostatic spectrum which includes staphylococci, the major enterobacteria and mycobacteria, but excluding anaerobic baccilli (Waksman and Lechevalier, 1950). When administered orally, neomycin is

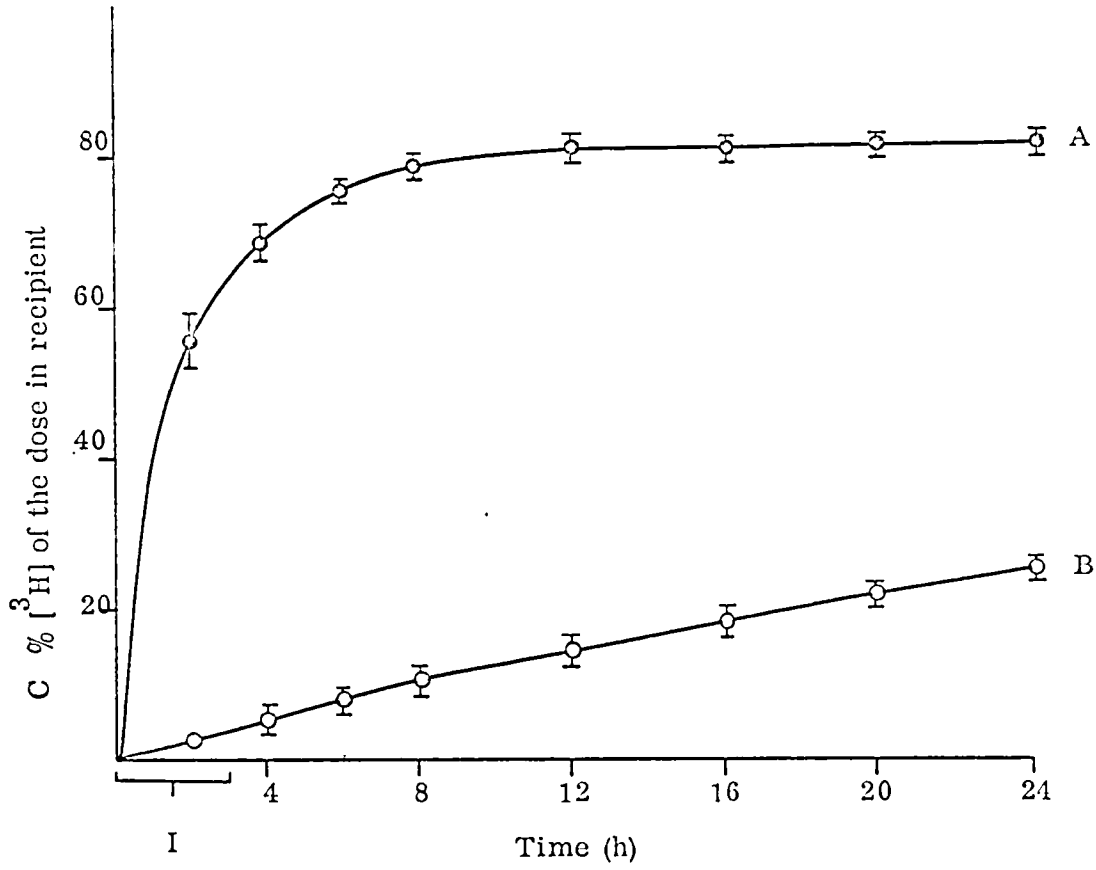


Fig 3.4 A comparison of the rate of [^{3}H] excreted in the bile of antibiotic pretreated recipient rats receiving A) [^{3}H]-Phenolphthalein and B) [^{3}H]-Phenolphthalein glucuronide by i/d infusion.

% [^{3}H] = % of the [^{3}H] infused (i/d) dose appearing in the bile of the recipient. I = the duration of the infusion (0.64 ml/h). Each point represents the mean of three animals and the vertical bars, the standard error of the means.
C = cumulative

poorly absorbed from the gut and its onset of antibiotic activity develops slowly (Poth et al., 1950). Neomycin has been shown to induce a malabsorption syndrome in man at oral doses of about 3 - 12 g / day, (Jacobson et al., 1960). The malabsorption syndrome is manifested as increases in the faecal excretion of fat, nitrogen, sodium, potassium, a lowering of serum cholesterol levels and malabsorption of vitamin B_{12}, iron, d-xylose and lactose (Jacobson et al., 1960, Faloon et al., 1964 and Cain et al., 1968). This neomycin induced malabsorption was shown to be associated with morphological changes in the ileal mucosa which included a shortening of the villi, an increased mitotic count and infiltration of the lamina propria with plasma cells, eosinophils and pigment containing macrophages in man and rats at doses of 3 - 12 g/day (Dobbins, 1968, Ksiazkiewicz-Scapiro et al., 1969 and Keusch et al., 1970).

Tetracycline hydrochloride

This antibiotic has a particularly wide spectrum of antibiotic activity which includes staphylococci, clostridia, bacteroides and lactobacilli. On oral administration of tetracycline, a portion of the dose is absorbed from the gut and undergoes EHC (Adir, 1975). Reports of an intestinal irritation associated with oral tetracycline treatment exist and are thought to be a direct effect of the drug on the intestinal mucosa (Myler and Herxheimer, 1972).

Bacitracin

This drug is antibiotically active against gram-positive microorganisms and is particularly effective in combination with neomycin in preventing overgrowth of neomycin-resistant staphylococci and pseudomonads (Wilson et al., 1968, Gingell, 1970). When administered orally, bacitracin is poorly absorbed from the gut which may, in part, explain its extremely low toxicity, which in mice is reflected by an LD 50 of 8.5 g/kg (Myler and Herxheimer, 1972).

Despite the possibility that the particular dose and dosing regime for antibiotic pretreatment used in our studies could conceivably have induced a malabsorption syndrome, the absorptional capacity of the gut for phenolphthalein in antibiotic pretreated rats appeared normal. This could be shown by comparing the recoveries of [^{3}H] in the bile of antibiotic pretreated

recipients receiving [^{3}H]-Phenolphthalein (i/d) with normal recipients receiving [^{3}H]-Phenolphthalein (i/d). No significant difference in the biliary recovery was observed between normal recipients (75.0 ± 5.1%) and antibiotic pretreated recipients (81.7 ± 1.6%).

A reduction in the gut microfloral hydrolysis of biliary glucuronides, associated with a reduction in the extent of EHC, has been shown for several drugs in rats pretreated orally with certain antibiotics. Neomycin pretreatment was shown to reduce the extent of the EHC of stilboestrol (Clark et al., 1969) and mestranol (Brewster et al., 1976) while ampicillin has been found to limit the EHC of etorphine (Dobbs and Hall, 1968). In the latter study, a direct relationship between ampicillin pretreatment and a reduction in gut luminal β-glucuronidase activity was established in vitro. Caecal enzyme activity was found to vary considerably at 1900 - 5000 enzyme units in control animals compared with 60 - 2800 in ampicillin treated rats. β-glucuronidase activity in the small intestine and colon was found to be less variable at 1800 - 2000 units in controls and 0 - 100 in treated rats (Dobbs and Hall, 1968).

Gut microfloral hydrolysis of biliary glucuronides followed by the absorption of the free drug has been reported for several drugs known to undergo EHC including chloramphenicol (Glazko et al., 1952), indomethacin (Yesair, et al., 1970 and Duggan et al., 1975), morphine (Walsh and Levine, 1975) and hexachlorophene (Gandolfi and Buhler, 1974).

6) Intestinal Perfusion Studies

a) Perfusion with [^{14}C]-Phenolphthalein

At the end of perfusion (90 minutes) with buffer containing [^{14}C]-Phenolphthalein (5 mg) (the perfusate), 61% of the [^{14}C] present at the start of perfusion was lost from the perfusate (see Figure 3.5). The fraction of the drug lost from the perfusate was taken as having been absorbed from the intestinal section, the majority of which was recovered in the bile (38%), liver (7%) and the tissue of the intestinal section (16%).

T.l.c. of bile, liver and systemic blood samples, taken at the end of the perfusion (90 minutes), showed the presence of phenolphthalein glucuronide

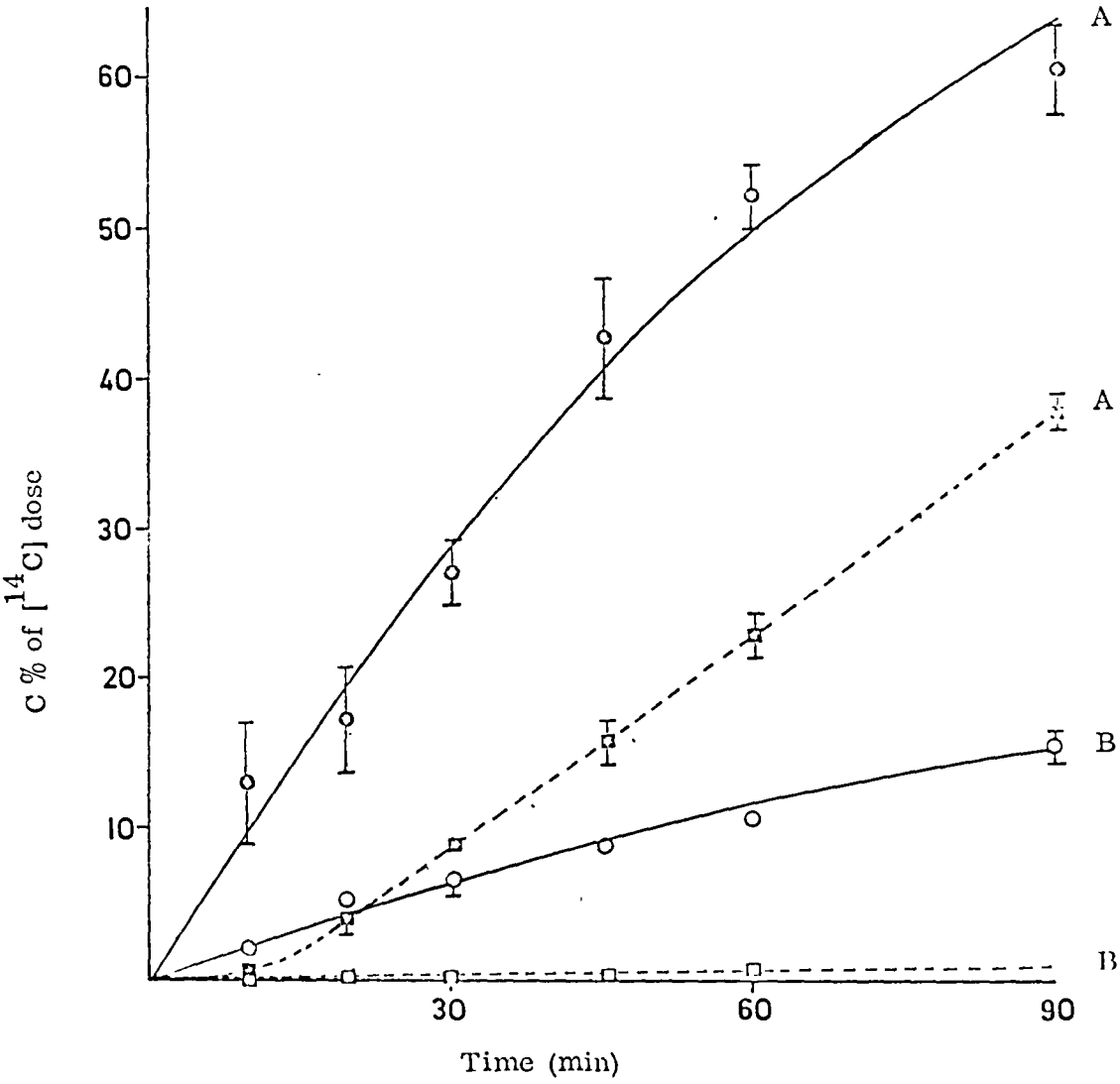


Fig 3.5 - The rate of intestinal absorption and appearance in the bile of [^{14}C] following intestinal perfusion with buffer containing A) [^{14}C]-Phenolphthalein and B) [^{14}C]-Phenolphthalein glucuronide.

% of [^{14}C] dose = the % of the dose of [^{14}C]-Phenolphthalein or [^{14}C]-Phenolphthalein glucuronide, present in the perfusate at the start of perfusion, absorbed from the perfused intestinal section (_____) and appearing in the bile (------). Each point represents the mean of three animals and the vertical bars, the standard errors of the means.
C = cumulative

only, presumably resulting from the hepatic conjugation of the phenolphthalein absorbed from the gut. T. l. c. of samples of a homogenate of the perfused intestinal section and portal blood, taken at the end of perfusion (90 minutes), revealed the presence of phenolphthalein (95% and 64% respectively), and its glucuronide (5% and 36% respectively). The occurence of both phenolphthalein and its glucuronide in the portal blood is thought to be due to mixing of systemic blood, previously shown to contain phenolphthalein glucuronide only, with phenolphthalein absorbed from the perfused section of the gut. The presence of glucuronide in the homogenate of the intestinal section may arise either as a result of intestinal mucosal conjugation of phenolphthalein with glucuronic acid, or may be of hepatic origin arriving in the intestinal tissue via the portal or systemic blood.

A net increase in portal [^{14}C] levels over systemic levels were observed during perfusion (see Fig 3. 6), which may be indicative of an extensive hepatic uptake of phenolphthalein, following its absorption from the gut. An extensive hepatic uptake of phenolphthalein would explain the absence of phenolphthalein in the systemic blood while present in portal blood. The absence of free phenolphthalein in the bile, liver and systemic blood suggests that the absorbed drug, on reaching the liver, is completely metabolised to form the glucuronic acid conjugate.

b) Perfusion with [^{14}C]-Phenolphthalein glucuronide

After 90 minutes of perfusion, 15% of the dose present at the start of perfusion was lost from the perfusate. This suggests that only 15% of the glucuronide present in the perfusate was absorbed, against 61% following perfusion with phenolphthalein itself (see Fig 3. 5). This confirms that the glucuronide is less readily absorbed from the intestine than is phenolphthalein, as was suggested from the results of previous studies.

The majority (11%) of the glucuronide absorbed (15%) from the intestinal section, was recovered in the homogenate of the intestinal section and the remainder in the liver (2. 6%) and bile (0. 8%). A difference in [^{14}C] levels between portal and systemic blood observed during perfusion with phenolphthalein was not seen during perfusion with phenolphthalein glucuronide. Portal and systemic blood levels of [^{14}C] during intestinal perfusion with the glucuronide were found to be considerably lower than observed during perfusion with

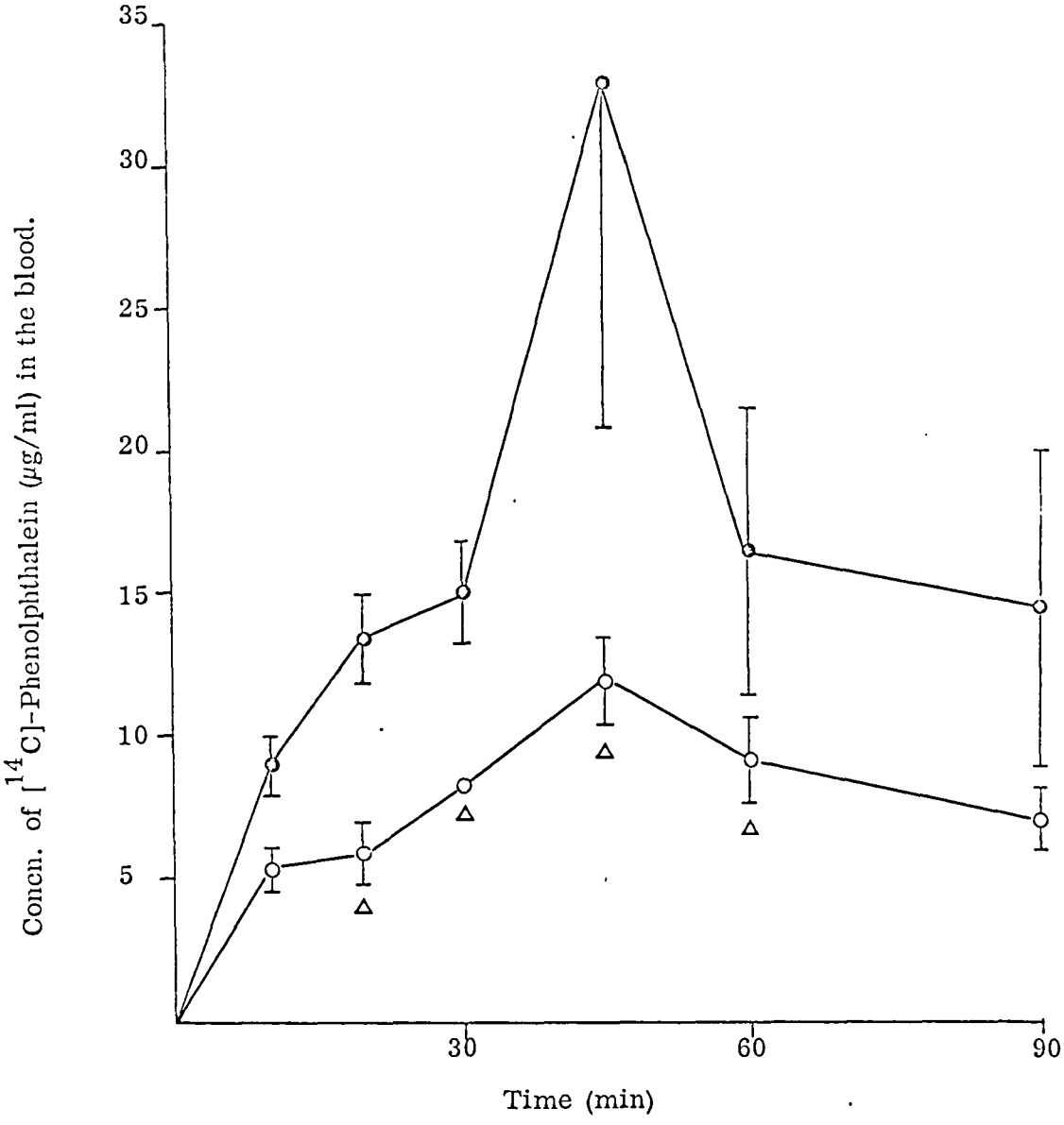


Fig 3.6 Systemic and portal blood concentration of [^{14}C] in rats, following intestinal perfusion with perfusate containing [^{14}C]-Phenolphthalein (5 mg).

-o- portal blood, -o- systemic blood. Each point represents the mean of 3 animals and the vertical bars, the standard errors of the means.

Δ = the points at which portal and systemic blood levels are significantly different ($P < 0.01$). The levels of [^{14}C] measured in portal blood are expressed here as µg/ml of [^{14}C[-Phenolphthalein.

phenolphthalein, as would be expected from the relatively small amount of glucuronide absorbed from the intestine. The nature of metabolites occuring in the systemic and portal blood could not be determined due to the relatively low [^{14}C] levels present.

T.l.c. and radiochromatographic analysis of a sample of the homogenate of the intestinal section indicated that besides the expected glucuronide, most of the [^{14}C] present was in the form of the free drug (93%) while the perfusate itself contained only the glucuronide. The presence of free phenolphthalein in the gut wall indicated that a considerable hydrolysis of the glucuronide, had occured. Hydrolysis could have resulted from either β-glucuronidase activity that may have been present in the lumen despite a thorough flushing of the section with buffer prior to perfusion, or by β-glucuronidase in the tissue of the intestinal section itself. β-glucuronidase activity in the intestinal tissue is known to exist, however, the activity has been shown to be low in comparison with microfloral β-glucuronidase. The low activity of β-glucuronidase in a tissue environment is thought to be due to the intracellular pH (7), at which the enzymes activity has been shown to be approximately 10 times lower than occurs at its pH optimum of 5, (Aitio, 1973).

7) Absorption of [^{14}C]-Phenolphthalein and [^{14}C]-Phenolphthalein Glucuronide in Everted Gut Sacs.

a) Incubation with [^{14}C]-Phenolphthalein

Following incubation, the gut sacs and the serosal buffers were found to contain 19.6 - 25.3% and 1.2 - 2.3% respectively of the [^{14}C] present in the mucosal buffers prior to incubation. Taking the percentage loss from the mucosal buffer as the percentage absorbed by the gut sac, this represents a total absorption by the sac of approximately 25% of the mucosal dose of [^{14}C]-Phenolphthalein. Absorption was found to vary slightly depending upon the particular region of the intestine from which the gut sac was prepared (see Table 3.4), but was not found to be a statistically significant variation. T.l.c. and radiochromatogram scanning of sample of mucosal buffers and homogenates of gut sacs indicated the presence of phenolphthalein only while serosal fluid was found to contain small amounts (2 - 7%) of phenolphthalein glucuronide in addition to phenolphthalein (93 - 98%). The presence of

Table 3.4 The distribution of [^{14}C] in everted gut sac preparations after incubation in mucosal buffer containing [^{14}C]-Phenolphthalein and [^{14}C]-Phenolphthalein glucuronide

Section of Intestine	Phenolphthalein			Phenolphthalein glucuronide		
	MUCOSAL % [^{14}C]	SEROSAL % [^{14}C]	SAC % [^{14}C]	MUCOSAL % [^{14}C]	SEROSAL % [^{14}C]	SAC % [^{14}C]
Proximal	74.8 (2.8)	1.2 (0.4)	19.6 (0.2)	94.7 (2.9)	0.9 (0.4)	3.7 (0.6)
Medial	74.0 (0.7)	2.3 (0.4)	25.3 (1.7)	99.2 (2.3)	0.9 (0.2)	3.4 (0.7)
Distal	73.0 (2.3)	1.7 (0.4)	23.0 (1.4)	95.6 (1.4)	1.2 (0.1)	2.5 (0.3)

* % [^{14}C] = the % of [^{14}C] in the pre-incubation dose (mucosal dose) present in the mucosal and serosal buffers and sac tissue after incubation. Proximal, medial and distal refers to the particular area of the intestine from which the gut sacs were prepared (see Chapter 2). Values are the means of three animals and figures in brackets represent the standard errors of the means.

phenolphthalein glucuronide in the serosal buffer suggests that the gut wall is capable of conjugating phenolphthalein with glucuronic acid at a rate calculated to be 0.003 - 0.007 ng/mg tissue/min.

b) Incubation with [^{14}C]-Phenolphthalein glucuronide

Following incubation, gut sacs and serosal buffers were found to contain only 2.5 - 3.7 % and 0.9 - 1.2 % respectively of the [^{14}C] present in the mucosal buffers prior to incubation (see Table 3.4). These values represent an absorption by the gut sacs of 4.1% of the mucosal dose of [^{14}C]-Phenolphthalein glucuronide as compared with approximately 25% absorbed in gut sacs incubated with [^{14}C]-Phenolphthalein.

T.l.c. and radiochromatography of samples of mucosal buffers indicated the presence of phenolphthalein glucuronide only, whereas serosal buffers and homogenates of the gut sacs contained free phenolphthalein (2 - 48% and 6 - 52% respectively) in addition to its glucuronide.

Our studies in everted gut sacs have shown:-

i) Phenolphthalein glucuronide is less readily absorbed from everted gut sacs than is phenolphthalein itself which confirms previous studies that the glucuronide is poorly absorbed from the gut relative to phenolphthalein.

ii) Phenolphthalein can undergo conjugation with glucuronic acid within the gut wall, but to a limited extent and could provide only a minor fraction of the total glucuronide shown to be present in portal blood following intestinal perfusion studies with phenolphthalein.

iii) The presence of phenolphthalein in the gut sac and serosal buffer following incubation with phenolphthalein glucuronide suggests that the gut wall, in addition to conjugation, is capable of deconjugation and may possess β-glucuronidase activity as previously suggested by intestinal perfusion studies with phenolphthalein glucuronide. The extent of phenolphthalein glucuronide deconjugation in everted gut sac preparations was found to vary between preparations, but the variation could not be related to the area of the intestine from which the gut sac was prepared. The identification of a deconjugation mechanism for phenolphthalein glucuronide in the gut wall may in part be responsible for the low rate of conjugation observed with phenolphthalein in the gut wall.

8. Systemic Blood Levels of [^{3}H]-Phenolphthalein in Intact and Bile Duct Cannulated Rats.

The levels of [^{3}H] in the systemic blood of bile duct cannulated rats receiving [^{3}H]-Phenolphthalein (25 mg/kg, i/v), when plotted in a semi-log fashion vs. time, showed a biphasic profile characteristic of a two compartment system. In contrast, the profile obtained for intact rats, showed a more complex pattern resulting from the presence of a secondary peak in the elimination phase of the profile, (see Fig. 3.7). The secondary peak is thought to be derived from that fraction of the drug involved in EHC which escapes biliary re-excretion as this peak coincides with the time (6 hours) at which the bulk of the biliary phenolphthalein metabolites have been shown to be absorbed from the gut and enter portal circulation. The absence of such a secondary peak in the systemic [^{3}H] profile in bile duct cannulated rats in which an EHC of the drug is absent, would confirm that the secondary peak is a result of EHC.

Calculation of the pharmacokinetic parameters for the drug, from the [^{3}H] systemic blood levels vs. time plots, gave a higher value for the area under the curve (AUC - area under the [^{3}H] systemic blood level vs. time curve) for intact rats (5694 μg/ml/min) than in bile duct cannulated rats (2925 μg/ml/min). The $T_{\frac{1}{2}}$ (α) and $T_{\frac{1}{2}}$ (β) were calculated to be 117 and 162 minutes respectively for phenolphthalein in bile duct cannulated rats. The presence of a secondary peak in the systemic blood level vs. time profile observed with intact rats prevented the determination of the half lives for both α and β phases ($T_{\frac{1}{2}}(\alpha)$ and $T_{\frac{1}{2}}(\beta)$ respectively) by conventional methods of calculating these parameters. Although the values for $T_{\frac{1}{2}}$ (α) and (β) could not be compared for intact and bile duct cannulated rats, it appears from the profiles (see Fig 3.5), that the $T_{\frac{1}{2}}$ (α) is similar for both intact and bile duct cannulated rats, while the $T_{\frac{1}{2}}$ (β) may be longer in intact than bile duct cannulated rats. These results suggest that the EHC of phenolphthalein may be increasing the systemic availability of the drug. T.l.c. and radiochromatogram scanning of methanolic extracts of blood showed that systemic blood sampled 6 hours after injection, contained a single metabolite, phenolphthalein glucuronide in both intact and bile duct cannulated rats.

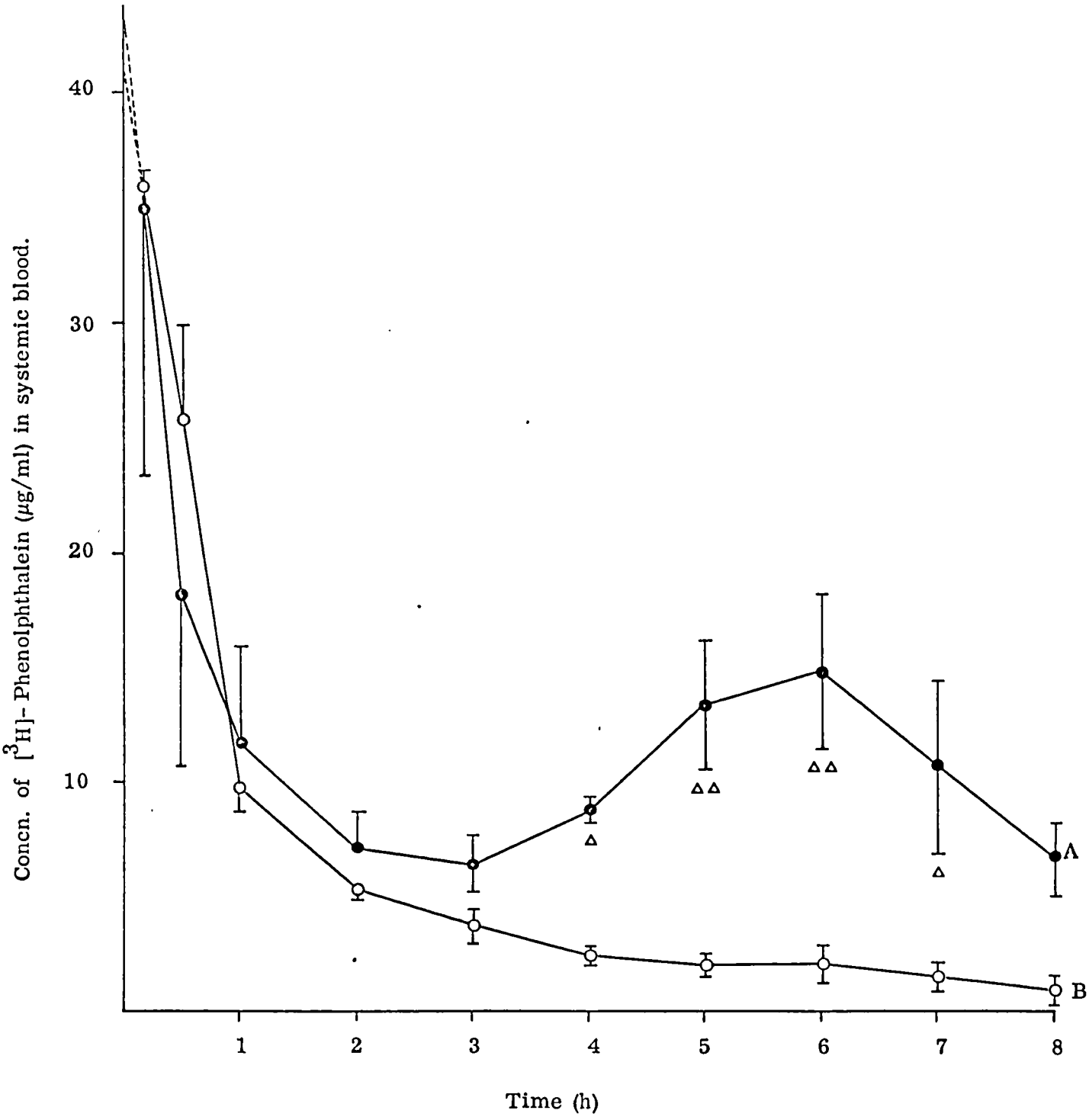


Fig 3.7 A comparison of the systemic blood levels of [^{3}H]-Phenolphthalein between intact and bile duct cannulated rats receiving the drug (25 mg/kg) intravenously.

A (-●-) intact rats, B (-○-) bile duct cannulated rats. Each point represents the mean of 6 - 8 rats and the vertical bars the standard errors of the means. Points at which a significant difference between A and B was observed are indicated by
Δ = $P < 0.01$ and ΔΔ = $P < 0.001$.

Portal blood sampled at 6 hours after injection from intact rats was found to contain both free phenolphthalein (32%) and its glucuronide (68%), while portal blood sampled from bile duct cannulated rats was found to contain the glucuronide only. These results suggest that free phenolphthalein appearing in the portal blood of intact rats is derived from EHC i.e. that fraction undergoing absorption from the gut.

9) Cytoplasmic Protein Binding Studies

The separation of the cytoplasmic proteins present in the 110,000 g supernatant of rat liver and intestinal mucosa on gel filtration, showed the presence of at least three distinct proteins. The bulk of cytoplasmic protein appeared at fraction nos. 20 - 28, while a smaller peak occured at fraction no. 30 and a larger peak, consisting of two smaller peaks, was present at fraction nos. 40 - 60, (see Fig 3.8) designated X, Y and Z respectively. The proportion of Z protein in relation to the total cytoplasmic protein was found to be approximately 2 - 3 times greater in supernatant derived from the intestinal mucosa in comparison with that from the liver (see Figs. 3.9 and 3.10). Differences in the degree of protein separation were noted between supernatant derived from liver and that from intestinal mucosa which is thought to be due to differences in the volumes of supernatants from liver (1 - 2 ml) and intestinal mucosa (4 ml) applied to the gel filtration column. Despite differences in the degree of separation, all three major proteins X, Y and Z, were identified in both liver and intestinal mucosa, (see Figs. 3.9 and 3.10). The separation and proteins identified compared well with that reported by other workers using similar procedures, (Levi et al., 1969, Takada et al., 1974 and Matsushita et al., 1976).

Analysis of the data obtained following gel filtration of liver supernatants from in vivo procedures with BSP indicated that this compound was bound mainly to the Y protein and to a lesser extent the Z protein, (see Fig. 3.8). The binding of BSP to Y and Z protein has been reported previously (Levi et al., 1969 and Matsushita et al., 1975) which confirmed that our gel filtration system was operating in a manner similar to that reported by other workers. An in vitro binding of BSP to Y and Z proteins in 110,000 g supernatants of liver was demonstrated which agreed with similar studies

carried out by Levi et al., (1969) and Matsushita et al., (1975).

In vivo studies with [^{14}C]-Phenolphthalein showed that when the liver was removed at 5 and 10 minutes after i/v administration of 25 mg/kg [^{14}C]-Phenolphthalein, it contained 28% and 18% of the dose respectively. On preparing the 110,000 supernatant of liver homogenates removed at 5 minutes after i/v administration, the supernatant was found to contain 48.3% of the [^{14}C] present in the homogenate while the pellet contained the remaining 51.7%. Similarly with livers removed at 10 minutes following i/v injection, the supernatant was found to contain 55.3% of the [^{14}C] present in the homogenate, while the pellet contained the remainder.

Application of these 110,000 g supernatants to gel filtration procedures showed that phenolphthalein was absent in fractions containing cytoplasmic proteins, (see Fig. 3.9) suggesting that the drug is present in the supernatant in solution and not bound to proteins. In vitro incubations of [^{14}C]-Phenolphthalein with 110,000 g supernatants from both liver and intestinal mucosa, for 1 and 2 hours at 5°C followed by gel filtration, again indicated that phenolphthalein was not capable of binding to cytoplasmic proteins either in vivo or in vitro, (see Fig. 3.10).

It has been suggested that the hepatic uptake of certain compounds such as BSP, bilirubin and thyroxine is influenced by their binding to hepatic cytoplasmic proteins (Arias et al., 1976). The inability of phenolphthalein to bind to such proteins suggests that the mechanism involved in its hepatic uptake is not dependent upon its binding to cytoplasmic protein.

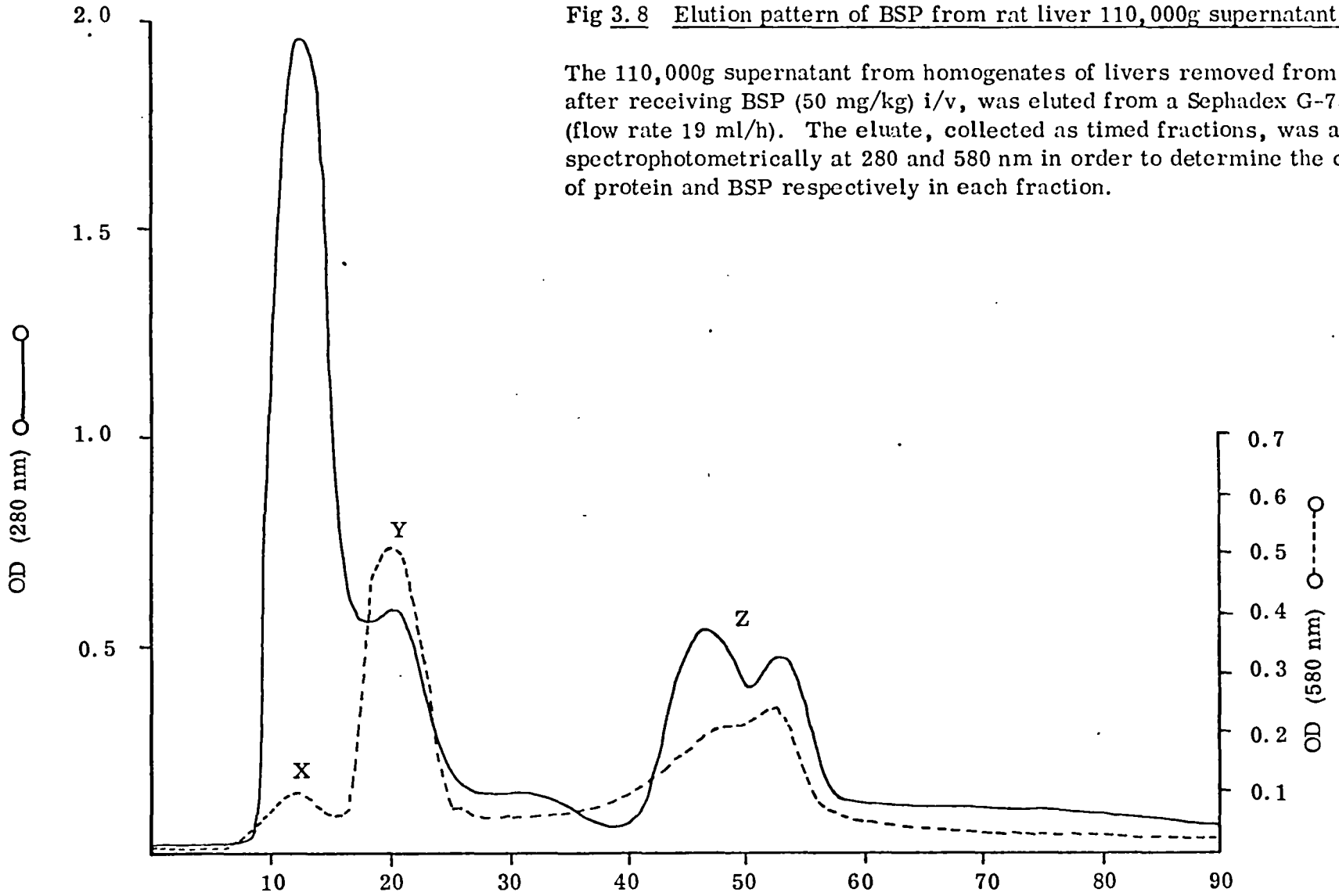


Fig 3.8 Elution pattern of BSP from rat liver 110,000g supernatant (in vivo).

The 110,000g supernatant from homogenates of livers removed from rats 5 min. after receiving BSP (50 mg/kg) i/v, was eluted from a Sephadex G-75S column (flow rate 19 ml/h). The eluate, collected as timed fractions, was analysed spectrophotometrically at 280 and 580 nm in order to determine the concentration of protein and BSP respectively in each fraction.

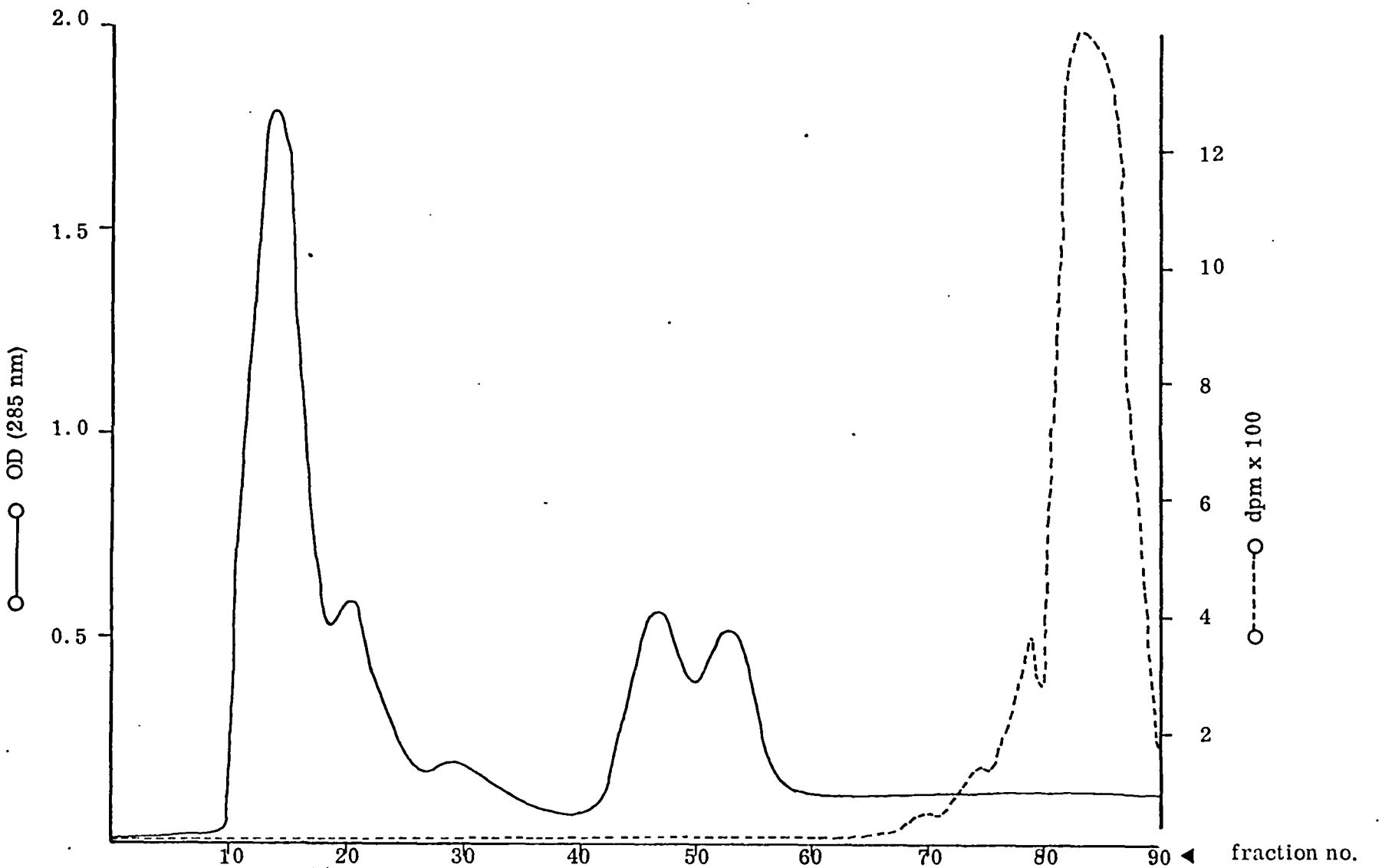
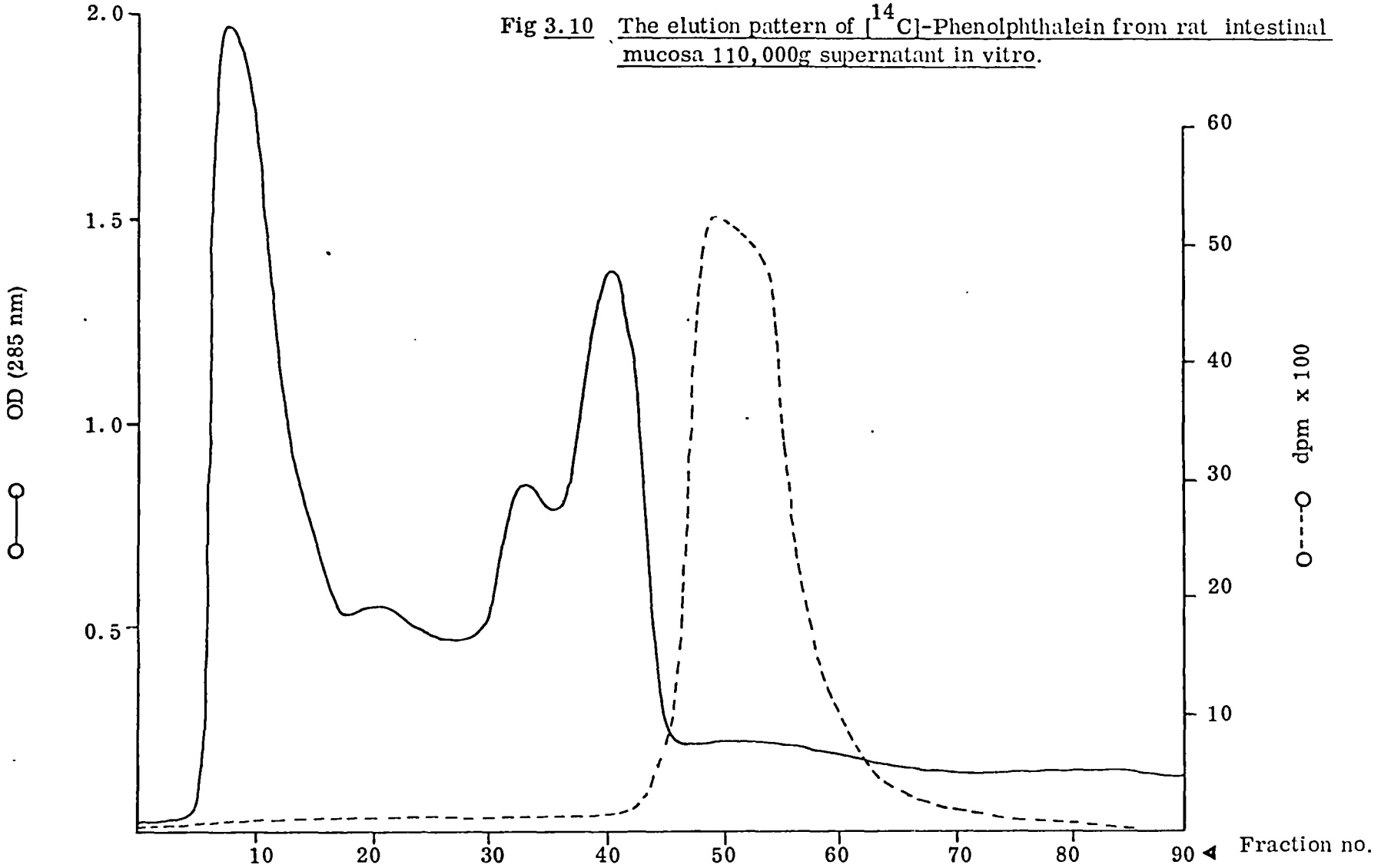


Fig 3.9 Elution pattern of [^{14}C]-Phenolphthalein from rat liver 110,000g supernatant (in vivo).

The 110,000g supernatant from homogenates of livers removed from rats 5 min after i/v administration of [^{14}C]-Phenolphthalein (25 mg/kg), was eluted from a Sephedex G75S column (flow rate 19 ml/h). The eluate, collected as timed fractions, was analysed spectrophotometrically at 280 nm and aliquots counted in order to estimate protein concentration and [^{14}C]-Phenolphthalein concentration respectively, in each fraction.

Fig 3.10 The elution pattern of [^{14}C]-Phenolphthalein from rat intestinal mucosa 110,000g supernatant in vitro.



Aliquots of supernatant from 110,000g homogenates of rat intestinal mucosa containing 25 mg protein, were incubated at 5°C for 2 h with 0.8 μM [^{14}C]-Phenolphthalein, then eluted on a Sephedex G-75S column (flow rate 24 ml/h). The eluate , collected as time fractions, was analysed spectrophotometrically at 285 nm and aliquots counted, in order to determine the concentrations of protein and [^{14}C]-Phenolphthalein respectively in each fraction.

Summary

Our studies have shown that the following events are involved in the EHC of phenolphthalein:-

i) On parenteral administration, phenolphthalein is quantitatively excreted in the bile as it's glucuronic acid conjugate (95% of the dose in 24 hours).

ii) The biliary glucuronide, on arrival to the gut, is totally hydrolysed by the gut microflora to liberate phenolphthalein, this hydrolysis being the rate limiting step in the subsequent absorption and biliary re-excretion of the conjugate.

iii) The free drug is absorbed from the gut more rapidly than is its conjugate, such that virtually none of the drug is absorbed as the conjugate. Of the parenteral dose reaching the gut in the bile (95%), the majority (82%) is absorbed while the remainder (13%) accumulates in the gut lumen and ultimately appears in the faeces.

iv) During the passage of the drug from the gut lumen to the portal circulation, a minor fraction (>1%) of the total drug absorbed is reconjugated in the intestinal mucosa.

v) The free drug appearing in the portal blood is completely extracted by the liver and undergoes a total hepatic reconjugation with glucuronic acid.

vi) Most (81%) of the drug absorbed from the gut and reconjugated in the liver is re-excreted in the bile although a small amount (1%) escapes to the systemic circulation, giving rise to a secondary peak in systemic blood levels of phenolphthalein glucuronide, ultimately appearing in the urine.

These events are represented diagrammatically in Fig. 3.11. The values quoted above for the extent of absorption from the gut, biliary re-excretion and loss from EHC to the urine and faeces, were obtained from biliary excretion and i/d infusion studies and refer to a single enterohepatic cycle. If it is assumed that these values remain constant with subsequent enterohepatic cycles, it can be calculated that phenolphthalein would undergo 7 complete cycles before it is totally lost to the urine and faeces. If phenolphthalein undergoes 7 complete cycles, each estimated to be of 6 - 8 h duration, it can be calculated that the drug would persist in the rat for 42 - 56 hours which agrees with the value of about 48 hours, over which time 96% of an i/p dose of the drug was shown to be lost to the urine and faeces in intact rats.

The characteristics which have shown phenolphthalein to be most suitable as a model for studying EHC of xenobiotics are as follows:-

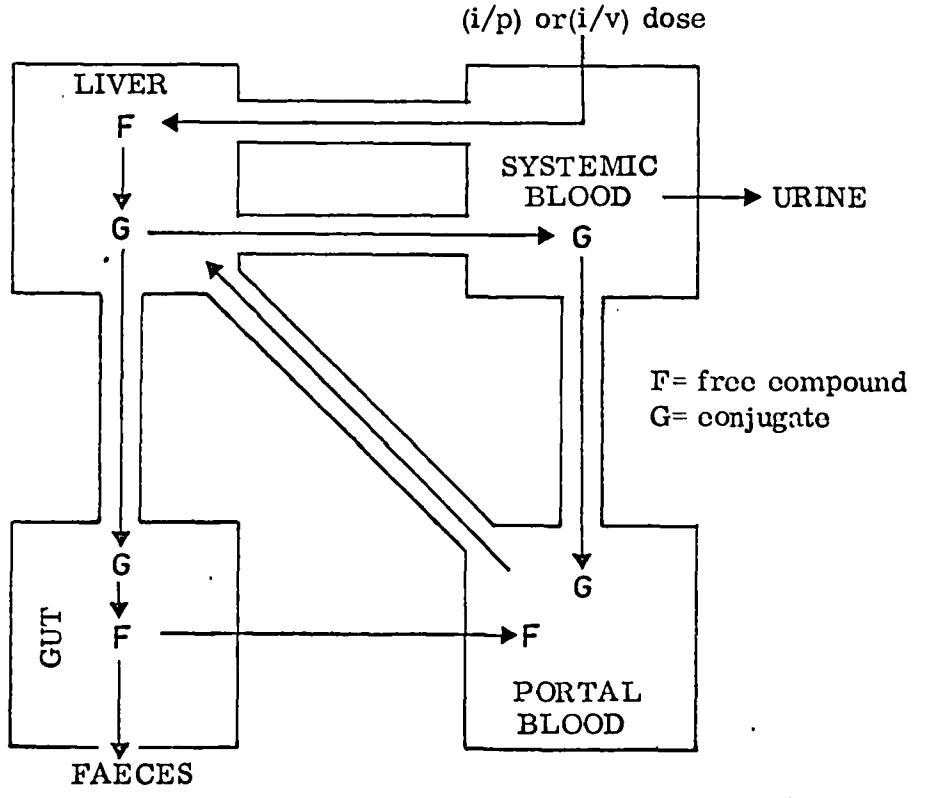
a) Phenolphthalein shows a straight forward metabolism in the rat forming a single metabolite, phenolphthalein glucuronide, which is extensively excreted in bile. Phenolphthalein and its glucuronide are readily analysed both quantitatively and qualitatively in biological material. The most frequently occuring form in which drugs undergoing EHC appear in the bile, is as their glucuronic acid conjugates, (see Table 1.12).

b) The individual steps involved in the EHC of phenolphthalein are relatively efficient and typically show recoveries of 70 - 80%.

c) Phenolphthalein provides an example of a compound which is lost from EHC predominantly to the faeces.

d) The low toxicity of phenolphthalein enables the use of a wide range of doses to be administered without the risk of toxic effects. The procedures used in the study of phenolphthalein EHC and in particular i/d infusion, were optimised to give the most physiological model for EHC of the drug. Using identical procedures, the EHC of morphine, LSD and diphenylacetic acid was subsequently studied in order to assess the value of phenolphthalein as a model compound.

Fig. 3.11 Diagrammatic representation of the EHC of phenolphthalein



CHAPTER FOUR

The Enterohepatic Circulation of Morphine, Lysergic acid diethylamide(LSD) and Diphenylacetic acid.

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Introduction

a) Morphine

The extent to which morphine is excreted in the bile has been found to vary considerably with species. Up to 60% of a dose of morphine can be recovered in the bile of the rat (March and Elliott, 1954, Walsh and Levine, 1975), 25 - 30% in the dog (Woods, 1954), 20% in the rhesus monkey (Mellett and Woods, 1956), 14% in the cat (Yeh and Woods, 1971, Smith and Peterson, 1973) and 7% in man (Elliott et al., 1954).

The major biliary metabolite of morphine in most of the species studied has been identified as morphine-3-glucuronide except in the cat where morphine-3-ethereal sulphate appears to be the major biliary metabolite (Yeh and Woods, 1971). Minor biliary metabolites of morphine, amounting to only about 5% of the total biliary metabolites, have been shown to be unchanged morphine and morphine-6-glucuronide (Fujimoto and Way, 1957, Yoshimura et al., 1969).

In several species including the dog, rhesus monkey and rat, the biliary excretion of morphine has been shown to exceed the faecal excretion of the drug which may suggest that morphine undergoes EHC in these particular species, (Cochin et al., 1954, Elliott et al., 1954, March and Elliott, 1954).

b) LSD

LSD is known to be excreted extensively in the bile of mice, (Idanpaan-Hiekkila and Schoolar, 1969), rats (68% of the dose - Boyd, 1959, Siddik, 1975) and guinea pigs (52% of the dose - Siddik, 1975). The major biliary metabolites of LSD, occuring in bile collected from isolated rat livers perfused with the drug, have been identified as glucuronides of 13 and 14-hydroxy LSD. These glucuronides account for about 80% of the total biliary metabolites of LSD, the remainder shown to be 2-oxo-LSD (8%), 13-hydroxy-LSD (2%) and unidentified compounds (12%).

The biliary metabolites of LSD occuring in the guinea pig, differ from those in the rat, in that 14-hydroxy-LSD glucuronide (33%) and 2-oxo-LSD (31%), have been identified as the major metabolites while 13-hydroxy-LSD glucuronide accounts for only 6% of the total biliary metabolites, (Siddik, 1975). As the major faecal metabolites of LSD have been identified as decomposition products of 14-hydroxy-LSD, 13-hydroxy LSD and unconjugated

unknown metabolites, it is probable that the bulk of the LSD biliary glucuronides undergo microfloral hydrolysis to form the unconjugated faecal metabolites (Siddik, 1975).

c) Diphenylacetic acid

Early metabolic studies with this compound indicated that it was excreted in man, dog and rabbit predominantly as an ester glucuronide (Miriam et al., 1927). Recent studies in man, certain sub-human primates and several non-primate species have shown that diphenylacetic acid was excreted to the extent of 20 - 100% in the urine over 48 hours mainly as the ester glucuronide and up to 10% as the unchanged acid.

Biliary excretion studies in the rat, following parenteral administration of diphenylacetic acid have found up to 72% of the dose recovered in the bile over 3 hours, predominantly as the glucuronic acid conjugate (84%) and as the free acid (16%), (Dixon, 1976).

The choice of compounds

Consideration of the biliary, urinary and faecal excretion data for morphine, LSD and diphenylacetic acid, (see Table 4.1) suggested that these compounds may undergo EHC in the rat, however, no direct demonstration of their undergoing EHC has been reported.

Table 4.1 The biliary, faecal and urinary excretion of morphine, LSD and diphenylacetic acid in the rat.

Compound	Bile Duct Cannulated		Intact	
	% bile	% urine	% faeces	% urine
Morphine	62.6*	18.1*	24.0	52.1
LSD	70.9	7.4	62.2	12.7
Diphenylacetic acid	73▲	12▲	-	48△

% = % of parenteral dose excreted in the bile, urine and faeces in intact and bile duct cannulated rats over 24 hours. Data was obtained from March and Elliott (1954), Siddik, (1975) and Dixon (1976). (▲, * and △, = values for samples collected -▲3hours, *6 hours and △48 hours after dosing).

Morphine, a clinically important drug, has been suggested to undergo EHC by several workers (Cochin et al., 1954, Elliott et al., 1954). At the time our studies were carried out, no direct demonstration of an EHC occuring with morphine had been reported, however more recently, an EHC of morphine has been described in the rat (Walsh and Levine, 1975).

LSD was chosen as an example of a drug which is metabolised both by Phase I and II reactions, these being hydroxylation and glucuronic acid conjugation respectively, appearing in the bile as glucuronic acid conjugates of 13 and 14-hydroxy-LSD (Siddik, 1975).

Diphenylacetic acid was chosen firstly as an example of a compound which appears in the bile both as its glucuronic acid conjugate and as the free unchanged acid. Secondly this compound forms an ester glucuronic acid conjugate (Dixon, 1976), unlike the other compounds we have studied which form ether glucuronic acid conjugates.

In addition to the reasons stated above, these compounds were chosen as being xenobiotics whose metabolism had been well documented in the rat, were available as labelled compounds and readily analysed both quantitatively and qualitatively in biological material.

Methods

1) Biliary excretion and collection of donor bile

Bile was collected hourly for 24 hours from bile duct cannulated rats receiving either [^{3}H]-Morphine (5mg/kg), [^{14}C]-LSD (1mg/kg) or [^{14}C]-Diphenylacetic acid (25mg/kg) i/p. Urine was collected over this period and an aliquot counted. Aliquots of bile samples collected were counted and analysed by tlc. as described in Chapter 2. Donor bile, for use in i/d infusion studies, was collected for 3 hours in bile duct cannulated rats receiving morphine, LSD or diphenylacetic acid (i/p) at the doses stated above.

2) Intraduodenal infusion studies

i) In normal rats. Donor bile, collected as described above, was infused (i/d) into bile duct cannulated recipient rats from which recipient bile was collected at hourly intervals for 24 hours. Urine was collected over this period and an aliquot counted. In a second series of experiments, the free drugs and 13-hydroxy-LSD were infused (i/d) into bile duct cannulated recipients, at a dose equivalent to the infused dose of their respective biliary metabolites. Aliquots of recipient bile samples were counted and analysed by t.l.c. as described in Chapter 2.

ii) Intraduodenal infusion in antibiotic pretreated rats. Rats, pretreated with antibiotics as described in Chapter 2, were infused (i/d) with [^{14}C]-Morphine, [^{14}C]-Diphenylacetic acid or their respective biliary metabolites as described above.

3) Intestinal perfusion studies

Using the technique described in Chapter 2, rats were perfused with a buffer containing either 5 mg [^{14}C]-Morphine or 5 mg [^{14}C]-Diphenylacetic acid. Aliquots of perfusate, bile, systemic and portal blood and a homogenate of the intestinal section were counted and analysed by t.l.c. using methods as described in Chapter 2.

4) Comparison of systemic blood levels of [^{14}C]-Morphine, [^{14}C]-Diphenylacetic acid between intact and bile duct cannulated rats.

[^{14}C]-Morphine (1.2 and 5 mg/kg), [^{14}C]-LSD (1 mg/kg) and [^{14}C]-Diphenylacetic acid (25 mg/kg) were administered (i/v) to intact and bile duct cannulated rats, blood samples were collected and aliquots counted and chromatographed as described in Chapter 2.

Results and Discussion

1) The biliary and urinary excretion of [^{3}H]-Morphine, [^{14}C]-LSD and [^{14}C]-Diphenylacetic acid in bile duct cannulated rats.

a) Morphine

Following administration of [^{3}H]-Morphine (5mg/kg, i/p) to bile duct cannulated rats, 48.7 ± 4.8% and 26.8 ± 3.9% of the dose was recovered in the bile and urine respectively over 24 hours (see Table 4.2). T.l.c. of bile samples, collected at 3 hours after dosing, revealed the presence of a single metabolite at Rf 0.14 and 0.35, in solvents E and F respectively. These Rf values were found to correspond with those quoted in the literature for morphine-3-glucuronide (Yoshimura et al., 1969). Hydrolysis of an aliquot of 3 hour bile with β-glucuronidase, followed by t.l.c. of a sample of the treated bile, confirmed the identity of the biliary metabolite as a glucuronic acid conjugate. Morphine-3-glucuronide was found to account for approximately 97% of the total [^{3}H] present in 3 hour bile, while the unchanged drug accounted for less than 1%. The recovery of morphine in the bile and urine of bile duct cannulated rats was similar to that reported by March and Elliott (1954) and Walsh and Levine (1975).

b) LSD

In bile duct cannulated rats receiving [^{14}C]-LSD (1 mg/kg, i/p), 79.3 ± 3.0% and 5.7 ± 0.1% of the dose were recovered in the bile and urine respectively over 24 hours, (see Table 4.2). Similar recoveries in the bile and urine have been reported by Siddik (1975) for LSD in rats. Two major metabolites were identified in 3 hour bile using t.l.c., corresponding in Rf values to those quoted in the literature with 13 and 14-hydroxy-LSD glucuronides (Siddik, 1975) and accounting for 83% of the [^{14}C] present in 3 hour bile. The remainder of the metabolites in 3 hour bile were found to be resistant to treatment with β-glucuronidase and were not identified, corresponding in Rf value to those quoted by Siddik (1975) with 'unknown' metabolites.

c) Diphenylacetic acid

Following the administration of [^{14}C]-Diphenylacetic acid (25 mg/kg, i/p) to bile duct cannulated rats, 68.7 ± 3.1% and 9.8 ± 6.8% of the dose was recovered in the bile and urine respectively over 24 hours (see Table 4.2) and was similar

Table 4.2 The biliary and urinary excretion of morphine, LSD and diphenylacetic acid in the rat

Sample	Time after dosing (h)	Morphine % [^{3}H]	LSD % [^{14}C]	Diphenylacetic acid % [^{14}C]
Bile	0 - 1	31.2 (2.9)	56.3 (3.9)	27.1 (5.9)
	1 - 2	8.6 (3.1)	14.2 (3.8)	21.1 (2.1)
	2 - 3	5.3 (2.2)	4.6 (5.2)	8.5 (1.3)
	3 - 24	3.6 (2.7)	4.2 (3.0)	12.0 (5.4)
Total Bile	0 - 24	48.7 (4.8)	79.3 (3.0)	68.7 (3.1)
Urine	0 - 24	26.8 (3.9)	5.7 (0.1)	9.8 (6.8)
Total Recovered	0 - 24	75.5 (2.8)	85.0 (2.7)	78.5 (4.3)

% [^{14}C] / [^{3}H] = % of the dose (i/p) of [^{3}H]-Morphine (5 mg/kg), [^{14}C[-LSD (1 mg/kg) and [^{14}C]-Diphenylacetic acid (25 mg/kg) administered to bile duct cannulated rats appearing in the urine and bile. Each value represents the mean of three animals and the figures in brackets, the standard errors of the means.

to that reported by Dixon (1976). T.l.c. of an aliquot of 3 hour bile revealed two metabolites, one chromatographically identical to the free acid (13.1%) and the other major metabolite (86.9%) corresponding in Rf value to that quoted in the literature with diphenylacetyl glucuronide (Dixon, 1976). Treatment of an aliquot of 3 hour bile with β-glucuronidase confirmed the identity of the two biliary metabolites as the free unchanged acid and its glucuronic acid conjugate.

2. Intraduodenal infusion studies with [^{3}H]-Morphine, [^{14}C]-LSD, [^{14}C]-Diphenylacetic acid, their respective biliary metabolites and 13-hydroxy-[^{14}C] -LSD in normal and antibiotic pre-treated rats.

a) Morphine and its biliary metabolite

i) Studies in normal rats - On infusion (i/d) of donor bile containing the biliary metabolite of [^{3}H]-Morphine (essentially morphine-3-glucuronide), into bile duct cannulated rats, 41.3 ± 4.7% of the infused dose appeared in the bile over 24 hours, while the remainder of the dose was recovered in the urine (20.3 ± 4.0%) and gut contents (11.2 ± 3.4%). Morphine-3- glucuronide was identified by t.l.c. as the major metabolite (98%) in a sample of 3 hour bile.

These finding suggest that morphine undergoes EHC in the rat, but unlike phenolphthalein where the major loss from EHC is via the faeces, morphine appears to be lost predominantly in the urine. The latter agrees with studies in intact rats (March and Elliott, 1954), where it was found that 52.1% and 24.0% of a subcutaneous dose of morphine (5 mg/kg) were recovered in the urine and faeces respectively over 24 hours.

In a second series of experiments, where [^{3}H]-Morphine was infused (i/d) into bile duct cannulated recipient rats, 39.5 ± 1.0% of the infused dose appeared in the bile of the recipient over 24 hours and the remainder of the dose was recovered in the urine (25.3 ± 4.9%) and gut contents (13.8 ± 5.3%). Morphine-3-glucuronide was identified by t.l.c. as the major metabolite (approximately 98% of the total [^{3}H] present) in 3 hour bile.

Comparing the results of the two series of experiments (see Fig 4.1), it was found that although the overall extent to which the infused doses were recovered in recipient bile did not differ significantly, the initial rate of recovery in the

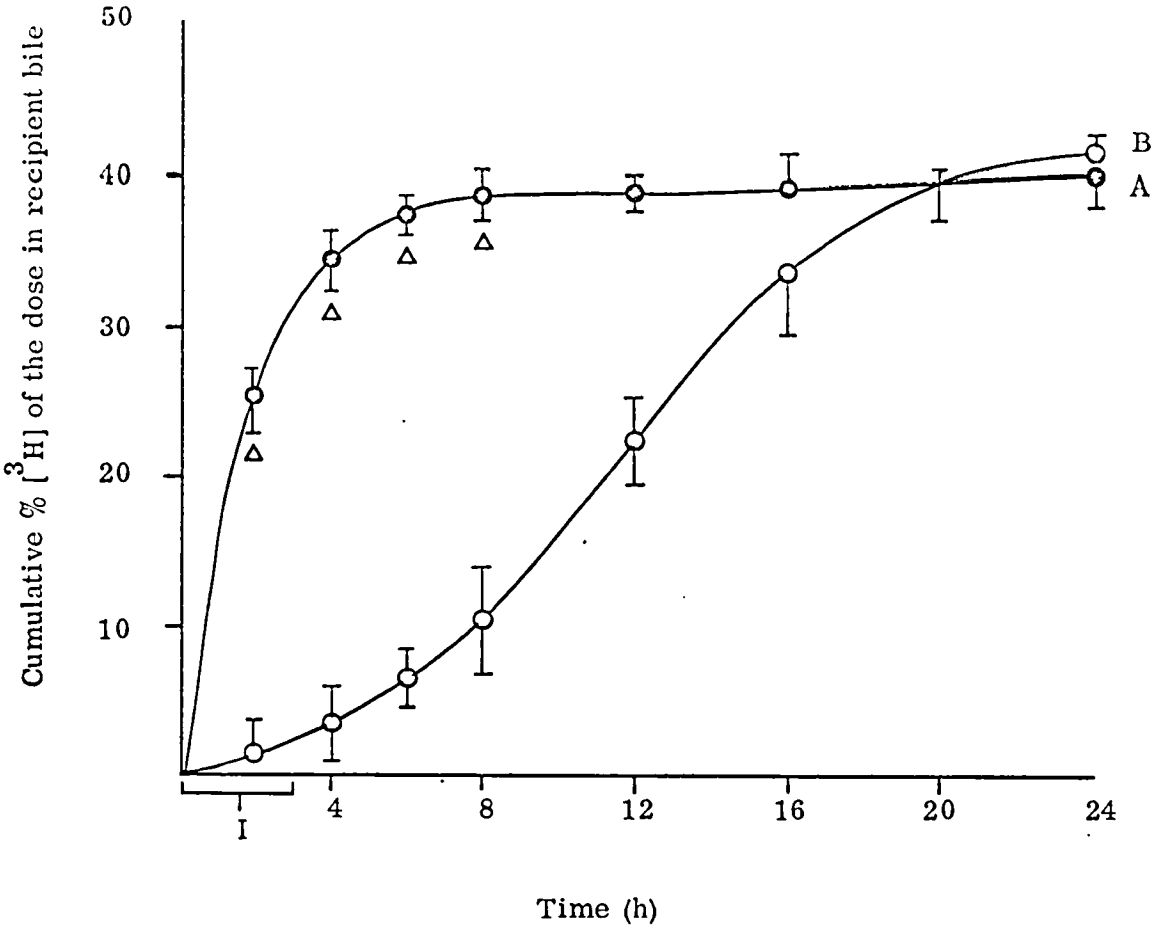

Fig 4.1 Comparison of the rates of [^{3}H] excreted in the bile of recipient rats infused (i/d) with A) [^{3}H]-Morphine and B) [^{3}H]-Morphine-3- glucuronide.

% [^{3}H] = % of the [^{3}H] infused (i/d) dose appearing in the bile of the recipient. I = the duration of i/d infusion (0.64 ml/h). Each point represents the mean of three animals and the vertical bars, the standard errors of the means. The points at which A differs significantly from B are denoted by Δ ($P < 0.01$).

bile was found to be significantly greater in recipients infused with morphine than those infused with its glucuronide. A similar situation was encountered with phenolphthalein and its glucuronide, which was explained by there being a requirement for the glucuronide to undergo hydrolysis in the gut lumen as a prerequisite to absorption from the gut and subsequent re-excretion in the bile. The delay in the appearance of the infused dose of the glucuronide in recipient bile, was suggested to be the time required for hydrolysis of the glucuronide to liberate the more readily absorbed free drug. In order to confirm that gut luminal hydrolysis was involved in the absorption of the biliary metabolites of morphine, the infusion studies were repeated in antibiotic pre-treated rats in which β-glucuronidase levels and hence hydrolytic activity has been shown to be reduced (Gingell, 1970; Dobbs and Hall, 1968).

ii) Studies in antibiotic pre-treated rats - On infusion (i/d) of [^{3}H]-Morphine into antibiotic pre-treated bile duct cannulated rats, 44.1 ± 2.7% of the infused dose appeared in the bile over 24 hours and the remainder of the dose was recovered in the urine (21.3 ± 4.9%) and gut contents (19.6 ± 4.7%). In contrast, only 8.6 ± 3.0% of an infused dose of [^{3}H]-Morphine-3-glucuronide appeared in the bile of antibiotic pre-treated recipient rats over 24 hours; the remainder of the dose being recovered in the urine (11.7 ± 3.1%) and gut contents (51.3 ± 6.2%). Morphine-3- glucuronide was identified by t.l.c. as the sole metabolite appearing in the bile of both recipients infused with [^{3}H]-Morphine and those receiving [^{3}H]-Morphine-3-glucuronide.

These findings suggest that antibiotic pre-treatment can significantly reduce the extent to which morphine-3-glucuronide is absorbed from the gut, observed as a reduction in both the rate and extent of recovery of an infused dose of the glucuronide in the bile of antibiotic pre-treated recipient rats relative to the recovery in normal rats (see Figs. 4.1 and 4.2). The studies in antibiotic pre-treated rats confirm the suggestion made earlier that the gut luminal hydrolysis of morphine biliary metabolites is a requirement for their rapid absorption from the gut as the free drug. This would infer that the glucuronide is less readily absorbed from the gut than is morphine itself; this possibility will be discussed later.

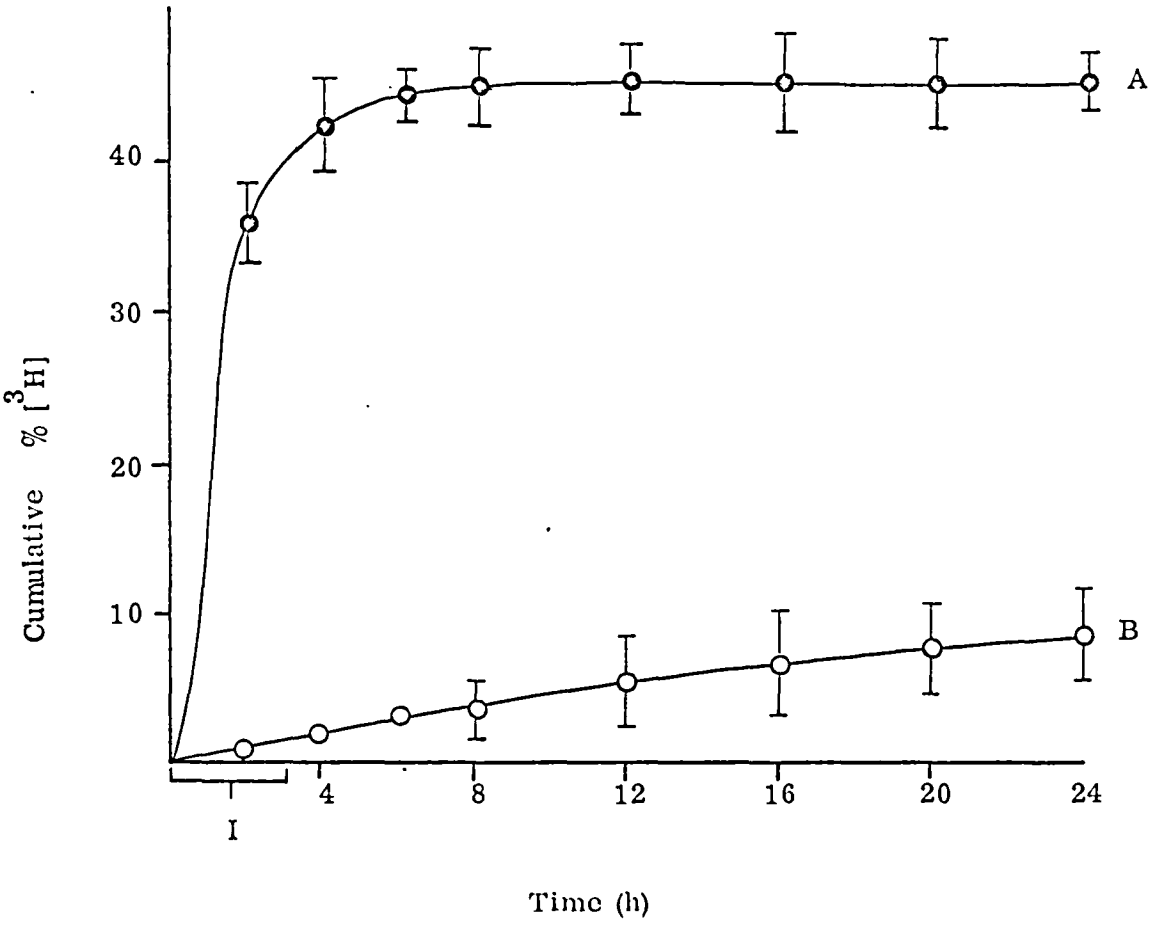

Fig 4.2 Comparison of the rates of [^{3}H] excreted in the bile of antibiotic pre-treated recipient rats receiving A) [^{3}H]-Morphine and B) [^{3}H]-Morphine-3-glucuronide by infusion (i/d).

% [^{3}H] = % of the [^{3}H] infused (i/d) dose appearing in the bile of the recipient. I = the duration of infusion (0.64 ml/h). Each point represents the mean of three animals and the vertical bars, the standard errors of the means. A significant difference ($P < 0.001$) was observed between (A) and (B) at all points.

b) LSD, it's biliary metabolites and 13-hydroxy-LSD

Intraduodenal infusion studies with this drug were carried out in normal rats only.

i) Infusion of the biliary metabolites of LSD - 28.2 ± 4.0% of an infused dose of [^{14}C]-LSD biliary metabolites appeared in the bile of recipient rats over 24 hours, the remainder of the dose being recovered in the urine (6.7 ± 3.2%) and gut contents (45.0 ± 5.5%). 13 and 14-hydroxy-LSD glucuronides were identified using t.l.c. as the major metabolites present in 3 hour recipient bile accounting for 83% of the total metabolites present, the remainder of the metabolites being resistant to β-glucuronidase treatment and were not identified.

These findings suggest that LSD undergoes EHC in the rat. However the extent of EHC appears relatively limited as compared with the other compounds studied. Loss from EHC was found to occur predominantly via the faeces, although 10% of the infused dose was lost in the urine. An extensive loss of LSD from EHC to the faeces is supported by studies in intact rats receiving LSD parenterally (Siddik, 1975) where it was found that 66.2% and 12.7% of the dose was recovered in the faeces and urine respectively over 24 hours. As 45.0 - 5.5% of the infused dose remained in the gut at the end of the experiment, it is suggested that the biliary metabolites of LSD or their deconjugation products are poorly absorbed from the gut.

ii) Infusion of 13-hydroxy LSD - In a second series of experiments, 13-hydroxy LSD, the aglycone of 13-hydroxy-LSD glucuronide, was infused (i/d) into bile duct cannulated recipient rats. After 24 hours, 33.5 ± 1.1% of the infused dose had appeared in the bile while the remainder of the dose was recovered in the urine (5.0 ± 3.5%) and gut contents (48.1 ± 5.5%). 13-hydroxy-LSD glucuronide was identified in 3 hour recipient bile as the major metabolite (88.2% of the total metabolites present), the remainder being resistant to β-glucuronidase treatment and were not identified.

Comparing the rate and extent of recovery in the bile of recipients receiving LSD biliary metabolites with that in recipients infused with 13-hydroxy-LSD (see Fig 4.3), it was found that a significant difference between the two groups existed only for the initial rates of the biliary recoveries, while the extent of the biliary recoveries were not found to differ significantly.

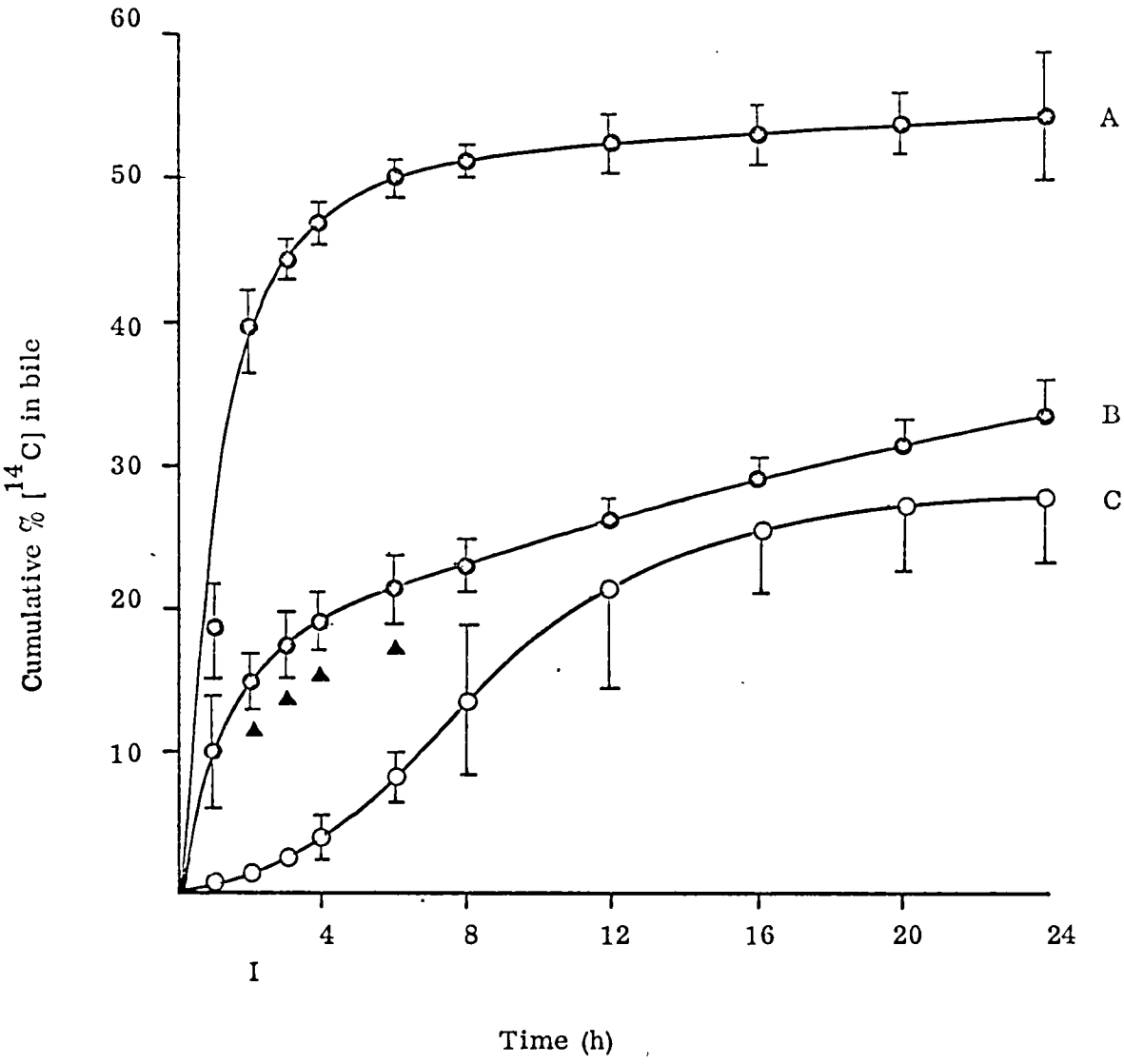

Fig 4.3 Comparison of the rates of [^{14}C] excreted in the bile of recipient bile duct cannulated rats receiving A) [^{14}C]-LSD, B) [^{14}C]-13-hydroxy-LSD and C) biliary metabolites of [^{14}C]-LSD, by intraduodenal infusion.

% [^{3}H] = % of the [^{14}C]'infused (i/d) dose appearing in the bile of recipients. I = the duration of infusion (0.64 ml/h). Each point represents the mean of three animals and the vertical bars, the standard errors of the means. Points at which (C) differs significantly from (B) are denoted by ▲ ($P < 0.01$).

Examination of the gut contents of recipient rats infused with LSD biliary metabolites at 24 hours revealed that none of the remaining [^{14}C] was present as glucuronic acid conjugates, but as the aglycones 13 and 14-hydroxy-LSD and several unidentified metabolites. This suggests that the LSD biliary glucuronides were hydrolysed in the gut and may have been absorbed as their respective aglycones in a manner similar to that shown to occur with morphine and phenolphthalein. The time required for the hydrolysis of the biliary glucuronides, could explain the delay observed in the initial rate of biliary recovery in recipients receiving LSD biliary glucuronides relative to the recovery in recipients receiving 13-hydroxy-LSD.

iii) Infusion of LSD - In a third series of experiments, [^{14}C]-LSD was infused into bile duct cannulated rats, 54.4 ± 1.8% of the infused dose appearing in the bile over 24 hours, the remainder being recovered in the urine (6.4 ± 1.6%) and the gut contents (24.9 ± 2.7%). T.l.c. of 3 hour bile revealed the presence of two major metabolites, accounting for 87% of the total metabolites present. These two metabolites were identified as 13 and 14-hydroxy-LSD glucuronides; the remainder of the biliary metabolites found to be resistant to β-glucuronidase treatment and were not identified.

Comparing the results of these three experiments, (see Fig 4.3) it was found that the rate and extent to which the infused dose was recovered in the bile was significantly greater following the infusion of LSD itself than after infusion of LSD biliary metabolites or 13-hydroxy-LSD. It is suggested from this finding that LSD is absorbed more rapidly and extensively from the gut than either 13-hydroxy-LSD or the biliary metabolites of LSD. In addition this finding suggests that had LSD undergone only conjugation with glucuronic acid and not been metabolised by Phase I reactions, LSD may have shown a more extensive EHC as the product of hydrolysis would have been LSD itself rather than the apparently less readily absorbed 13 and 14-hydroxy-LSD.

Phase I reactions generally increase the polarity of a compound (Williams, 1959) while increasing polarity is usually associated with a corresponding reduction in ability to undergo absorption from the gut (Schanker, 1960). It could be predicted from our studies with LSD, that compounds undergoing EHC, excreted in the bile as the glucuronic acid conjugates of their Phase I metabolites, would be likely to undergo a less extensive EHC than compounds

excreted as the glucuronic acid conjugate of the unchanged compound. The proviso to such a prediction would be that (1) the parent compounds were of a similar polarity and (2) that any gut microfloral metabolism of the compounds, other than hydrolysis of their glucuronic acid conjugates, did not result in the formation of metabolites that were more polar than the parent compounds.

C) Diphenylacetic acid and its biliary metabolites

i) Studies in normal rats - 66.6 ± 3.6% of an infused (i/d) dose of the biliary metabolites of [^{14}C]-Diphenylacetic acid appeared in the bile of recipient rats over 24 hours, the remainder of the dose being recovered in the urine (7.3 ± 2.1%) and gut contents (15.1 ± 4.3%). Diphenylacetyl glucuronide was identified by t.l.c. as being the major metabolite in 3 hour bile accounting for 98.4 % of the total [^{14}C] present in the bile sample. No unchanged diphenylacetic acid was detected in recipient bile.

These findings suggest that this compound undergoes an extensive EHC in the rat, loss from the cycle occuring both in the faeces and urine. Excretion studies in the intact rat, following administration of diphenylacetic acid (114 mg/kg) have shown that over 48 hours, 48% of the dose was recovered in the urine (Dixon, 1976) which confirms that a considerable loss from EHC must occur via the urine.

In a second series of experiments, diphenylacetic acid was infused (i/d) into bile duct cannulated recipient rats, 74.5 ± 5% of the infused dose was recovered in recipient bile over 24 hours, while the remainder of the dose was found in the urine (8.7 ± 1.8%) and gut contents (14.4 ± 6.1%).

Comparing the results of the two series of experiments (see Fig 4.4), it was again observed that although there was no significant difference between the overall extent to which the infused doses were recovered in the bile of recipients, the initial rate of recovery was found to be greater in recipients infused with the free acid than those infused with its biliary metabolites despite the fact that the biliary metabolites of diphenylacetic acid have been shown to contain up to 13% as the free acid. The delayed excretion of the infused dose in recipient bile following infusion of the biliary

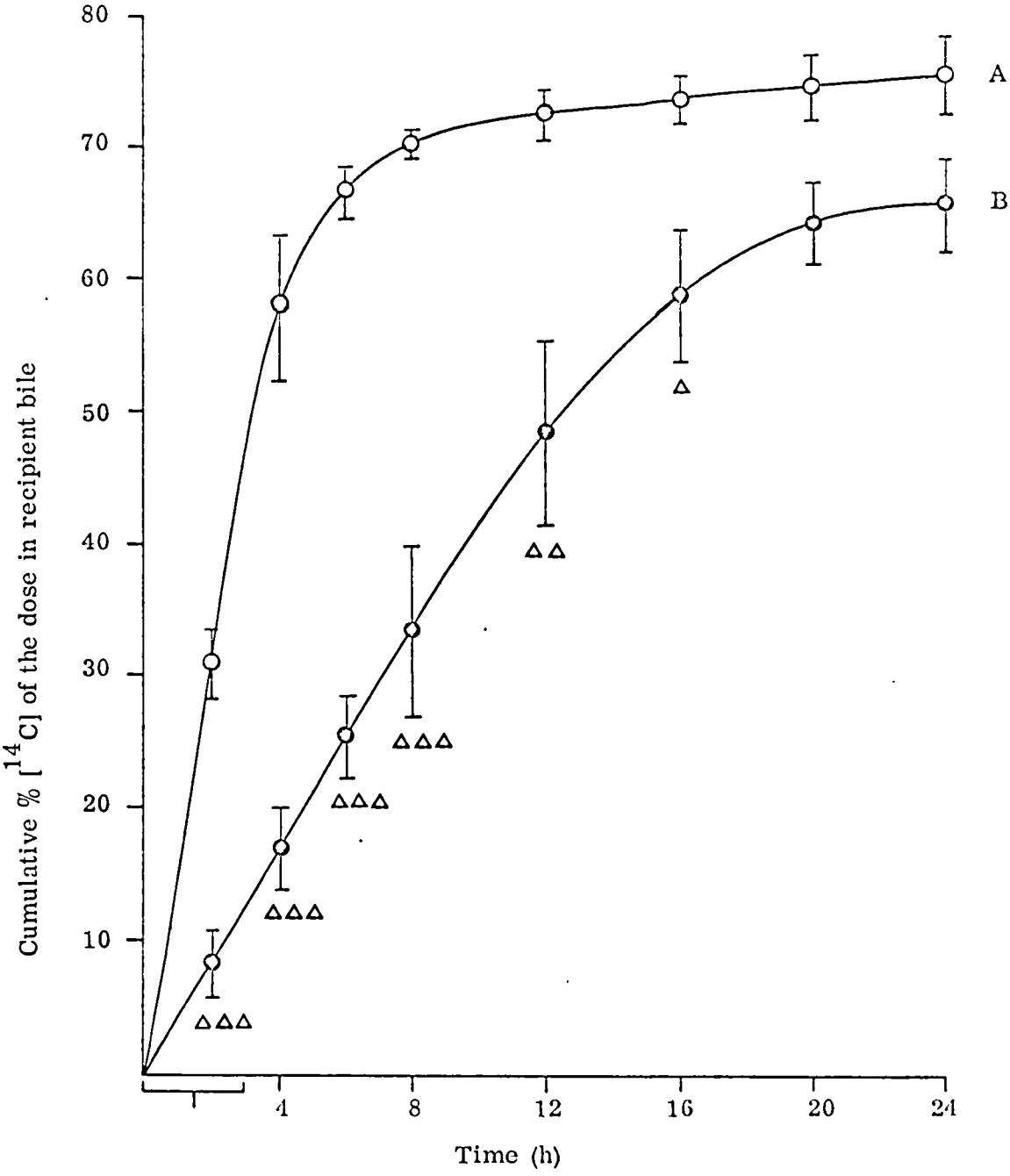

Fig 4.4 Comparison of the rates of [^{14}C] excreted in the bile of recipient rats infused (i/d) with A) [^{14}C]-Diphenylacetic acid and B) the biliary metabolites of [^{14}C]-Diphenylacetic acid.

% [^{14}C] = % of the [^{14}C] infused (i/d) dose appearing in the bile of the recipient. I = the duration of infusion (0.64 ml/h) and the vertical bars, the standard errors of the means. The points at which A differs significantly from B are denoted by Δ , ΔΔ and ΔΔΔ ($P < 0.02$, 0.01 and 0.001 respectively).

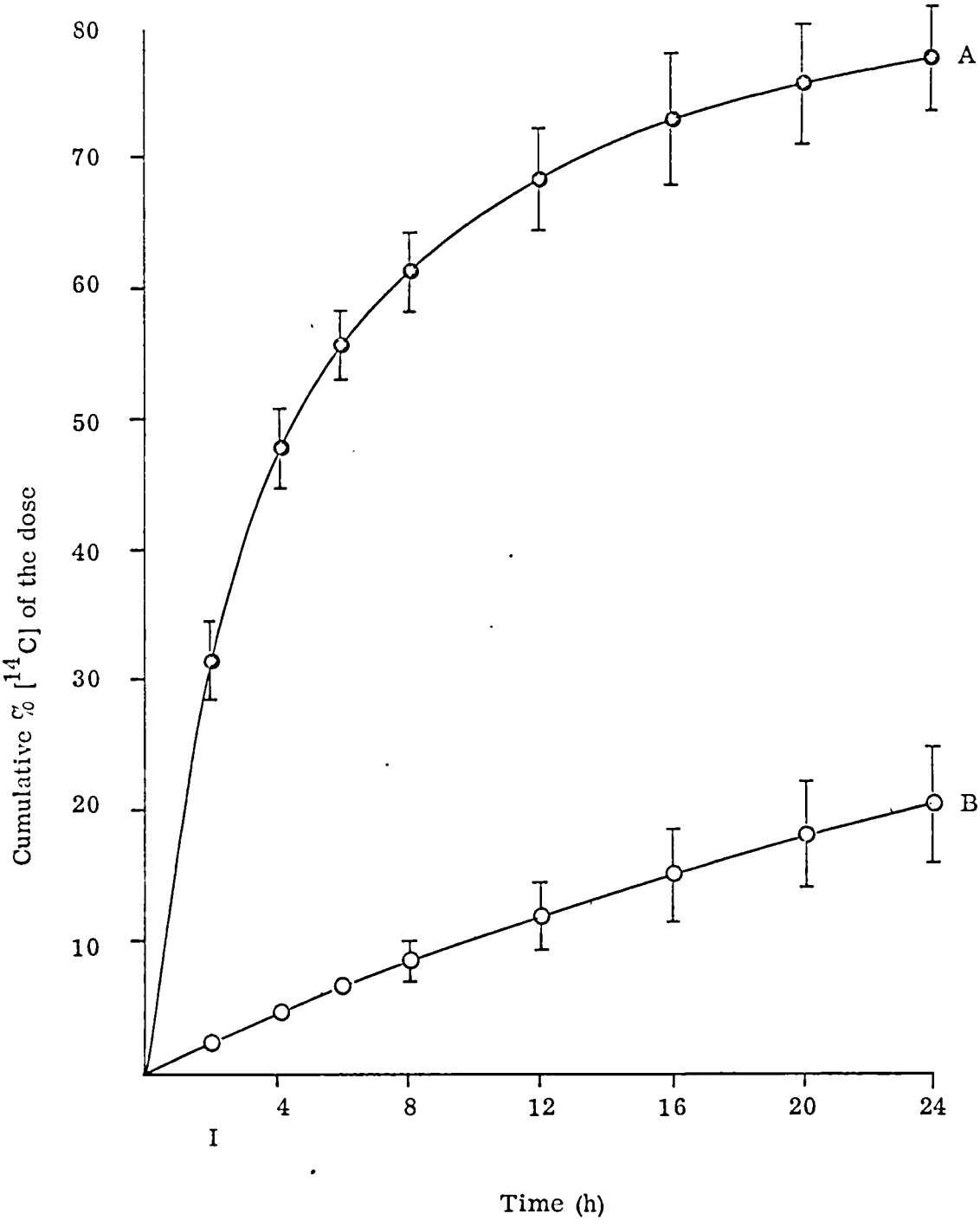

Fig 4.5 Comparison of the rate of biliary excretion of [^{14}C] in antibiotic pre-treated recipient rats, receiving A) [^{14}C]-Diphenylacetic acid and B) biliary metabolites of [^{14}C]-Diphenylacetic acid, by intraduodenal infusion.

% [^{14}C] = % of the [^{14}C] infused (i/d) dose appearing in the bile of the recipient rats. I = the duration of the infusions (0.64 ml/h). Each point represents the mean of three animals and the vertical bars, the standard error of the means. A (-O-) = infused with [^{14}C]-Diphenyl acetic acid and B (-O-) = infused with the biliary metabolites of [^{14}C]-Diphenylacetic acid. A significant difference ($P < 0.001$) was obtained between (A) and (B) at all points.

metabolites is comparable with the situation observed for all compounds we have studied.

ii) Studies in antibiotic pre-treated rats - 78.0 ± 3.6% of an infused (i/d) dose of diphenylacetic acid appeared in the bile of antibiotic pre-treated rats, as compared with 22.1 ± 4.3% following the infusion of its biliary metabolites (see Fig. 4.5). The remainder of the doses were recovered in the urine (6.3 - 1.3% and 2.8 ± 4.7%) and gut contents (10.1 ± 1.3% and 58.3 ± 4.7%) for recipients infused with the free acid and those infused with its biliary metabolites respectively. T.l.c. of bile collected 3 hours after infusion of the biliary metabolites of diphenylacetic acid was found to contain diphenylacetyl glucuronide exclusively, while bile collected following infusion of the free acid contained both the free acid (10.7%) and its glucuronide (89.1%).

These studies suggest that the hydrolysis of the biliary glucuronide of this compound is necessary for an extensive absorption of the biliary metabolites from the gut.

Summary of the intraduodenal infusion studies

During these studies, one particular situation was observed that appeared to be common to all the compounds studied, this being the greater initial rate of recovery in recipient bile following the infusion of the corresponding biliary metabolites (glucuronic acid conjugates). It was suggested that the delayed recovery following infusion of the biliary glucuronides, was due to the time required for the gut luminal hydrolysis of the biliary glucuronides to liberate the corresponding free compounds or aglycones. The basis of this proposal would be that the biliary glucuronides are less readily absorbed from the gut than are their respective free forms (the unchanged compound in the case of phenolphthalein, morphine and diphenylacetic acid) or aglycones (13 and 14-hydroxy-LSD).

We have shown by direct means (absorption in everted gut sacs and in intestinal perfusion studies) that phenolphthalein is more readily absorbed from the gut than is its glucuronide and have inferred that a similar situation exists for morphine, diphenylacetic acid and their corresponding glucuronides, from studies in antibiotic pre-treated rats.

Other compounds including stilboestrol (Fischer and Millburn, 1970), testosterone (Kreek, 1962), salicylic acid (Schachter et al., 1959) and TRIAC (Hertz et al., 1961) have been shown to be more readily absorbed from everted gut sacs than their corresponding glucuronic acid conjugates.

The mechanism by which most xenobiotics are absorbed from the gut is generally accepted to be a diffusion process (Levine 1970, Bates and Gibaldi, 1970); the rate of diffusion dependent upon the degree of lipophilicity exhibited by compounds undergoing absorption (Schanker, 1960). It is generally considered that there exists a relationship between the lipid solubility of a compound and its ability to undergo absorption from the gut, such that compounds of high lipid solubility are more readily absorbed than compounds of lower lipid solubility (Kakemi et al., 1967; Kurtz 1971; Houston et al., 1974). The partition ratio of a compound between an organic solvent and water has been shown to be an acceptable estimate of the lipid solubility of a compound (Leo et al., 1971; Tute, 1971) and although some workers have expressed reservations (Kurtz, 1971), others report good correlations, (Hogben et al., 1959; Houston et al., 1974).

When the partition ratios for the compounds we have studied were compared with those of their corresponding glucuronic acid conjugates, the log P values for the aglycones lay within a range of +0.90 to - 0.09, whereas those for their corresponding glucuronides were significantly lower at - 0.42 to - 1.34, (see Table 4.3). These findings suggest that the lipid solubilities of the glucuronic acid conjugates are significantly lower than those of their corresponding free compounds which supports the hypothesis that the conjugates are less readily absorbed from the gut than their free forms.

Variations between the individual compounds studied as to their extent and rate of recovery of the infused dose in recipient bile were observed. The biliary metabolites of phenolphthalein and diphenylacetic acid on infusion (i/d) were found to be recovered in recipient bile to a greater extent and at a faster rate than following infusion (i/d) of the biliary metabolites of morphine or LSD. The possible explanations for this finding are:-

i) Differences in the rate and the extent of gut luminal hydrolysis between the various biliary glucuronides.

Table 4.3 The partition ratios for the compounds studied and their corresponding metabolites.

Compound	P	log P
Phenolphthalein	3.64	+ 0.56
Phenolphthalein glucuronide	0.19	- 0.72
Morphine	0.81	- 0.09
Morphine-3-glucuronide	0.11	- 0.98
LSD	7.07	+ 0.85
13-hydroxy-LSD	0.46	- 0.34
13-hydroxy-LSD glucuronide	0.046	- 1.34
Diphenylacetic acid	7.93	+ 0.90
Diphenylacetyl glucuronide	0.38	- 0.42
Phenol	19.41	+ 1.29
1-Naphthol	42.50	+ 1.62

P = the partition ratio between 1-octanol and 0.1M phosphate buffer, pH 7.4, log P = the logarithm of the partition ration (P).

ii) Variation in the rate and extent to which the products of the gut luminal hydrolysis undergo absorption from the gut.
iii) The extent to which the compound, subsequently absorbed from the gut, is lost to the urine rather than undergoing biliary excretion.

Although the rate of gut luminal hydrolysis was not investigated, examination of the gut contents at the end of each (i/d) infusion experiment revealed that no glucuronic acid conjugates were present, suggesting that the hydrolysis of biliary metabolites was complete for all the compounds studied.

Explanation of the differences in (i/d) infusion biliary recovery on the basis of variation in the extent to which the deconjugation products are absorbed from the gut, would appear to fit the observations if lipid solubility is accepted as a reliable measure of ability to undergo absorption from the gut [Phenolphthalein (log P + 0.56) and diphenylacetic acid (log P + 0.90) being more lipid soluble than morphine (log P + 0.09) or 13-hydroxy-LSD (log P -1.34)].

Explanation of the differences in (i/d) biliary recovery on the basis of the extent to which the compounds are lost to the urine can also be shown to fit the observations to some extent. The % of the infused (i/d) doses of phenolphthalein (>1%) and diphenylacetic acid (7 - 8%) appearing in the urine being slightly lower than for LSD (approx. 12%) and considerably lower than morphine (approx. 20%). On balance it appears that although the differences in the extent to which these compounds are re-excreted in the bile may be a combination of all the three factors considered, the exceptionally high urinary excretion of morphine and low lipid solubility of 13-hydroxy-LSD relative to the other compounds, would suggest that these are the major factors responsible for their low recovery in recipient bile.

3) Intestinal perfusion studies

a) Morphine

20.6 ± 1.0% of the morphine present at the start of perfusion was lost from the perfusate after 90 minutes of perfusion, suggesting that this amount was absorbed from the perfused intestinal section, (see Table 4.4). Of the material absorbed, 6.4 ± 1.1% was recovered in the bile, 6.2 ± 0.8% in the

Table 4.4 The intestinal absorption, biliary excretion, systemic and portal blood levels of [^{14}C]-Morphine following its intestinal perfusion in rats.

Time (min)	% of dose absorbed	% of dose absorbed appearing in bile	Portal blood (μg/ml)	Systemic blood (μg/ml)	P < (Systemic/ portal)
10	3.1 (1.3)	0.1 (0.01)	1.8 (0.6)	0.2 (0.1)	0.001
20	8.3 (2.3)	0.7 (0.03)	0.6 (0.1)	0.6 (0.1)	0.05
30	10.01 (1.4)	1.7 (0.3)	0.6 (0.04)	0.4 (0.1)	0.001
45	12.2 (1.7)	3.1 (0.3)	0.5 (0.03)	0.2 (0.1)	0.001
60	16.9 (1.2)	4.4 (0.3)	1.6 (0.3)	0.4 (0.1)	0.001
90	20.6 (1.0)	6.4 (0.2)	3.6 (0.4)	1.1 (0.1)	0.001

% dose = % of the dose of [^{14}C]-Morphine (5mg) present at the start of perfusion, lost from the perfusate (% dose absorbed) and appearing in the bile, on a cumulative basis. Portal and systemic blood levels of [C] are expressed as μg/ml of morphine. Each value represents the mean of three animals and those in brackets, the standard errors of the means. The degrees of significant difference between systemic and portal blood levels are expressed as P values.

tissue of the intestinal section 0.7 ± 0.3% in the urine and 0.6 ± 0.2% in the liver.

Identification of the metabolites present in various samples collected at the termination of perfusion are listed in Table 4.5. Morphine-3-glucuronide, detected in the perfusate and tissue of the perfused intestinal section is thought to originate either from the conjugation of morphine with glucuronic acid by the intestinal mucosa or from the liver, appearing in the intestinal tissue via the systemic and portal blood and diffusing into the perfusate.

Table 4.5 The nature of the metabolites present in the various samples collected at 90 min after the start of intestinal perfusion with buffer containing [^{14}C]-Morphine.

Sample	% of total metabolites present in sample as:-	
	Morphine	Morphine-3-glucuronide
Bile	ND	96.3
Urine	ND	95.8
Perfusate	63.6	36.4
Gut (tissue)	94.1	5.2
Systemic blood	ND	97.3
Portal blood	1.2	93.7

ND = not detected. Morphine and morphine-3-glucuronide were analysed by t.l.c. using solvents E and F (see Chapter 2).

The appearance of free morphine in the portal blood is thought to represent the free drug which is undergoing absorption from the intestinal section.

A significant difference between portal and systemic levels of [^{14}C] during perfusion, (see Table 4.4) may be indicative of the hepatic uptake of the free

Table 4.6 Intestinal absorption, biliary excretion and blood levels of [^{14}C]-Diphenylacetic acid following its intestinal perfusion in rats.

Time (min)	% dose absorbed	% dose absorbed appearing in bile	Portal blood μg / ml	Systemic blood μg / ml	P (systemic/ portal blood)
10	16.6 (1.1)	0.4 (0.03)	22.1 (1.9)	17.4 (0.9)	0.05
20	28.0 (1.1)	3.2 (0.7)	23.0 (1.1)	20.4 (1.5)	NS
30	39.5 (2.3)	8.2 (1.7)	26.8 (3.7)	20.9 (0.6)	0.05
45	53.3 (2.6)	17.1 (2.8)	21.4 (1.3)	21.0 (1.6)	NS
60	62.7 (2.7)	27.1 (2.9)	21.6 (1.5)	19.4 (1.2)	NS
90	78.7 (0.9)	44.9 (3.0)	19.4 (1.2)	19.7 (0.6)	NS

% dose = % of [^{14}C]-Diphenylacetic acid (5mg) present at the start of perfusion, lost from the perfusate (% dose absorbed) and appearing in the bile, on a cumulative basis. Portal and systemic blood levels of [^{14}C] are expressed as μg/ml of diphenylacetic acid. Each value represents the mean of three animals and those in brackets, the standard errors of the means. The degrees of significant difference between systemic and portal blood levels are expressed as P values (NS = no significant difference).

morphine entering the portal blood following its absorption, as no free morphine was detected in the systemic blood. Other workers have reported the appearance of free morphine in the systemic blood of rats administered orally at a dose of 5 mg/kg (Iwamoto and Klaassen, 1977), however, the concentrations at which they detected morphine (30 - 80 ng/ml) were below the limit at which we were able to detect the drug, (> 100 ng/ml).

b) Diphenylacetic acid

78.7 ± 0.9% of the diphenylacetic acid present at the start of perfusion was lost from the perfusate after 90 minutes of perfusion and was recovered in the bile (44.9 ± 3.0%), urine (9.8 ± 2.7%), tissue of the perfused section of intestine (7.7 ± 3.1%) and the liver (2.2 ± 0.9%), (see Table 4.6).

The nature of the metabolites collected at the termination of perfusion is shown in Table 4.7.

Table 4.7 Nature of the metabolites present in the various samples collected at 90 min after the start of intestinal perfusion with buffer containing [C]-Diphenylacetic acid

Sample	% of the total metabolites present in the sample as:	
	diphenylacetic acid	diphenylacetyl glucuronide
Bile	11.5	88.5
Urine	ND	100.0
Perfusate	100.0	ND
Gut (tissue)	100.0	ND
Systemic blood	ND	97.8
Portal blood	1.3	97.3

ND = not detected. Diphenylacetic acid and diphenylacetyl glucuronide were identified in samples by t.l.c. using solvents H and I (see Chapter 2).

The absence of diphenylacetyl glucuronide in the tissue of the intestinal mucosa and the perfusate, suggests that the conjugation of the free acid with glucuronic acid in the intestinal mucosa is unlikely for this compound as was confirmed by later studies (see Chapter 5). The absence of the free acid in systemic blood suggests that the free acid absorbed from the intestinal section and shown to present in the portal blood, is extracted by the liver where it undergoes partial conjugation with glucuronic acid. Most of the compound absorbed is excreted in the bile although its presence in the urine suggests that a significant amount reaches the systemic circulation, but only as the glucuronic acid conjugate. This is reflected in the absence of a significant difference between portal and systemic blood levels at certain points during the perfusion, (see Fig 4.6). If the absence of a significant difference between portal and systemic blood levels is taken as representing a relatively poor hepatic uptake, then this must apply to the conjugate rather than the free compound as the latter was not detected in systemic blood.

4) Systemic blood levels of morphine, LSD and diphenylacetic acid in intact and bile duct cannulated rats.

a) Morphine

Following intravenous administration of [^{3}H]-Morphine at doses of 1.2 mg/kg and 5 mg/kg, no significant difference was observed between systemic blood levels of the drug in intact rats and those in bile duct cannulated rats (see Fig 4.6). T.l.c. of a sample of systemic blood collected 6 hours after dosing (5 mg/kg) revealed the presence of morphine-3-glucuronide, no free morphine being detected. A sample of portal blood taken at the same time (6 hours) was shown to contain predominantly morphine conjugate although a small amount (3 -.4% of the total [^{3}H] present in the sample) was present as the unchanged drug.

b) LSD

Over 6 hours, no significant difference was observed between systemic blood levels of LSD in intact rats and those in bile duct cannulated rats following intravenous administration of [^{14}C]-LSD (1 mg/kg), (see Fig 4.7). As a result of the relatively low specific activity of the labelled drug and the low concentration of the drug in the blood, identification of metabolites

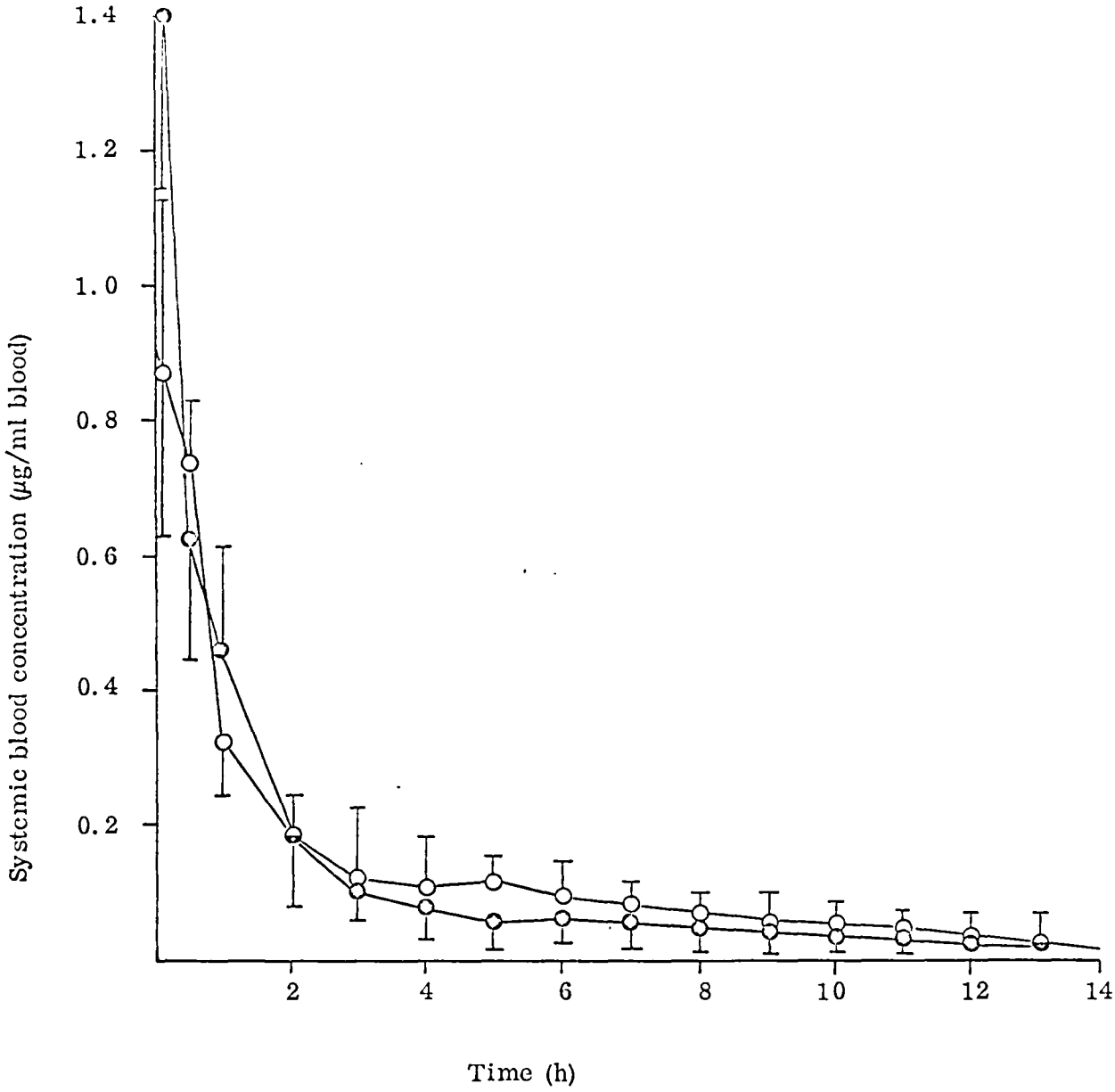

Fig. 4.6 Comparison of systemic blood levels of [^{3}H] between intact and bile duct cannulated rats receiving [^{3}H]-Morphine intravenously (1.2 mg/kg).

(-○-) bile duct cannulated rats, (-○-) intact rats. Each point represents the mean of 6 rats and the vertical bars the standard errors of the means. The [^{3}H] levels measured in the systemic blood are expressed here as μg/ml blood of [^{3}H]-Morphine.

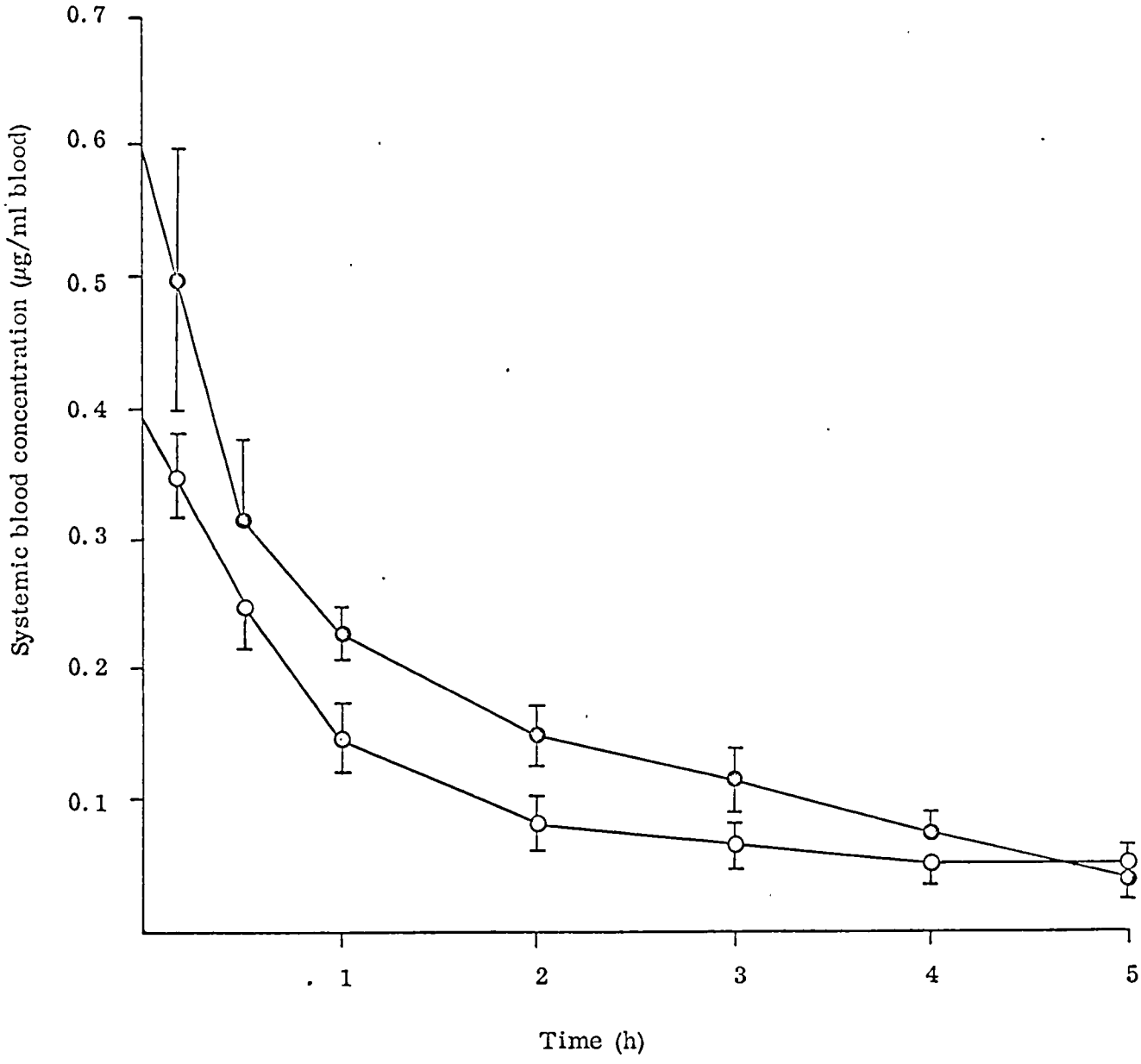

Fig 4.7 Comparison of systemic blood levels of [^{14}C]-LSD between intact and bile duct cannulated rats receiving the drug intravenously.

o intact rats, O bile duct cannulated rats. Each point reperesents the mean of six rats and the vertical bars the standard errors of the means. The [^{14}C] levels measured in the systemic blood are expressed here as µg/ml blood [^{14}C]-LSD.

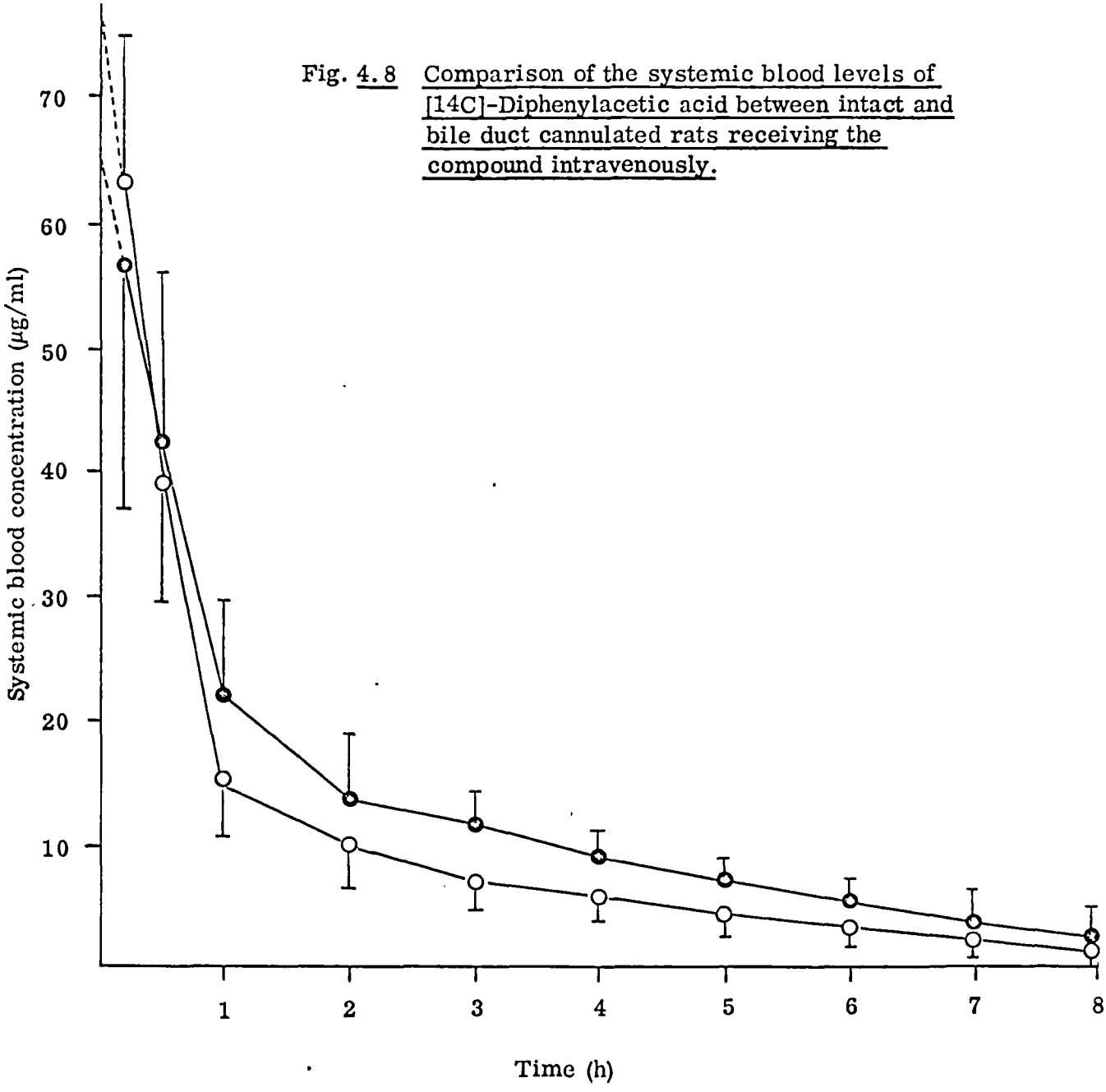

Fig. 4.8 Comparison of the systemic blood levels of [14C]-Diphenylacetic acid between intact and bile duct cannulated rats receiving the compound intravenously.

● intact rats ○ bile duct cannulated rats. Each point represents the mean of six rats and the vertical bars the standard errors of the means. The [^{14}C] levels measured in the systemic blood of rats receiving [14C]-Diphenylacetic acid (25 mg/Kg) are expressed here as µg's diphenylacetic acid/ml blood.

present in systemic and portal blood was not possible.

c) Diphenylacetic acid

Following the administration of [^{14}C]-Diphenylacetic acid (25mg/kg,i/v), no significant difference was obtained between systemic blood levels of the drug in intact rats and those in bile duct cannulated rats (see Fig 4.8). T.l.c. of a sample of systemic blood collected at 6 hours after dosing showed that diphenylacetyl glucuronide was the predominant metabolite, (97% of the [^{14}C] present), no free drug being detected. T.l.c. of a sample of portal blood withdrawn 6 hours after dosing showed that although the glucuronide was the major metabolite present (91% of the [^{14}C]present), the free acid was detected, (6 - 8%).

In contrast to phenolphthalein, the systemic blood vessels of morphine, LSD and diphenylacetic acid were found to show no secondary peak in intact rats or a difference in the levels between intact rats and those in bile duct cannulated rats. On the basis of the systemic blood levels alone, it could have been interpreted that morphine, LSD and diphenylacetic acid were not systemically available from EHC. It has been suggested that the apparent systemic availability of a drug from EHC can be determined by the difference in the urinary excretion of the drug between the extent in bile duct cannulated and that in intact rats (Smith and Millburn, 1975). From the data available for the urinary excretion of these compounds in intact and bile duct cannulated rats over a similar period of time (see Table 4.1), only the data for LSD could be interpreted in such a way as to predict the extent to which a drug is lost from EHC to the urine, arriving at a value of 5.5% of the dose. A more reliable estimate of the loss of a drug from EHC to the urine was obtained from i/d infusion studies following infusion of the biliary metabolites of the compounds studied. On this basis, it was estimated that over a single enterohepatic cycle, less than 1% of an infused dose of phenolphthalein was lost to the urine, morphine (20%), LSD (7%) and diphenylacetic acid (7%). Comparing the systemic blood level data with the values obtained for loss from EHC to the urine, the situation appears contradictory. Phenolphthalein, the only compound shown to give rise to a secondary peak in systemic blood levels of the drug in intact rats, was found to give the lowest loss from EHC to the urine of compounds studied. In contrast, morphine, which showed a 20% loss to the urine from EHC, was not found to

give rise to a secondary peak in systemic blood levels of the drug. This suggests that differences in the systemic blood level profiles between intact and bile duct cannulated rats are not necessarily indicative of systemic availability of a drug from EHC. The extent to which a drug can become systemically available from EHC will depend primarily on the degree to which the drug is extracted by the liver from the portal blood following its intestinal absorption (Smith and Millburn, 1975). Having reached the systemic circulation, the concentration of the drug in the systemic blood will depend upon a variety of factors such as its distribution between the blood and tissue compartments and the rate and extent to which the drug is cleared from the blood by the kidneys. One possible interpretation of the absence of a secondary peak in the blood levels of morphine and LSD in intact rats is the relatively slow rate of entry to the systemic circulation for these particular drugs. Our studies have indicated that during EHC, morphine and LSD are absorbed from the gut slowly relative to phenolphthalein and diphenylacetic acid and thus would be expected to enter systemic circulation at a similarly slow rate. Under such circumstances, these drugs would be less likely to give a noticeable secondary rise in their systemic blood levels than had they entered the systemic circulation from EHC at a faster rate. Following this argument, it may have been expected that diphenylacetic acid would have shown a secondary peak in the systemic blood levels of the drug in intact rats, as it has been found to be absorbed from the gut at a rate comparable with that of phenolphthalein. The absence of such a secondary peak for diphenylacetic acid would suggest that either this compound is more extensively extracted by the liver than phenolphthalein or, on entry to the systemic circulation, is more extensively taken up into tissue compartments or cleared more rapidly by the kidenys than is phenolphthalein. On balance, it would appear that there are several factors which could have given rise to the absence of a secondary peak in the systemic blood levels of morphine, LSD and diphenylacetic acid in intact rats, these being the relative rates and extent of:-

i) Absorption from the gut following EHC

ii) Hepatic extraction

iii) Distribution of the drug between the systemic blood and tissue compartments.

iv) Renal clearance.

Other drugs known to undergo EHC and give rise to a secondary plasma peak in the systemic blood of intact animals or a significantly higher systemic level of the drug in intact relative to bile duct cannulated animals, include oxazepam (Bertagni et al., 1975), rifamycin AMP (Curci and Loscalzo, 1966), BRL 1341 (Steward, 1961), benzylthio-5-trifluoro-methyl benzoic acid (Taylor, 1973) and digoxin (Abshagen et al., 1972). On the basis of the evidence available for their metabolism and excretion, there did not appear to be any single factor common to these particular drugs which could account for the occurence of a secondary plasma peak arising from EHC.

An additional factor which may have contributed to the absence of a secondary peak with morphine and LSD is the low dose level at which these compounds were administered (5mg/Kg and 1mg/Kg respectively) relative to phenolphthalein and diphenylacetic acid (25 mg/Kg).

Summary

1) The relationship between the physicochemical properties of xenobiotics and their ability to undergo EHC.

i) The physicochemical requirements of EHC

Constraints, in the form of specific physicochemical requirements, are known to exist for both the biliary excretion and intestinal absorption of xenobiotics. In order that a compound can be extensively excreted in the bile, it must possess a minimum molecular weight, which varies with species (325 ± 50, 400 ± 50 and 475 ± 50 for the rat, guinea pig and rabbit, respectively), and a polarionisable group, or be provided with such by conjugation with an agent such as glucuronic acid (Millburn et al, 1967; Hirom et al., 1972). The absorption of most xenobiotics from the gut is known to depend primarily on the degree of lipophilicity exhibited by the compound (Schanker, 1960), such that compounds of low lipid solubility are less readily absorbed from the gut than those of higher lipid solubility.

Compounds participating in an EHC undergo both biliary excretion and intestinal absorption and thus must meet the specific physicochemical requirements specific for each process. As the polarity requirement for biliary excretion is to some extent incompatible with that necessary for intestinal absorption, it follows that compounds would require a certain degree of ampiphilicity in order to satisfy the physicochemical requirements of EHC. In practice, it is found that very few compounds possess the necessary ampiphilicity to undergo EHC as the unchanged compound, an exception being rifamycin which is a zwitterion (Maffi et al., 1961). The majority of xenobiotics undergoing EHC are relatively lipid soluble compounds and are readily absorbed from the gut and acquire the polarity necessary for biliary excretion by forming a conjugate with an agent such as glucuronic acid. In order that the polar biliary conjugate may then undergo absorption from the gut, it must be hydrolysed to liberate the lipid soluble free compound or metabolite. Compounds excreted in the bile, but not undergoing EHC are generally highly polar and are unable to meet the lipid solubility requirement for the intestinal absorption phase of EHC, examples being chromoglycate and oxyphenonium

ii) The importance of gut microfloral hydrolysis to EHC

Our studies were carried out exclusively with compounds excreted in the bile as glucuronic acid conjugates. The microfloral hydrolysis of the biliary metabolites of the compounds studied was shown to be a prerequisite to their extensive absorption from the gut and the rate limiting step in the intestinal phase of EHC. This requirement for hydrolysis was shown to result from the poor absorption of biliary conjugates from the gut which could be related to their low lipophilicity relative to their deconjugation products.

Taking the differences between the biliary recovery in recipients (i/d) with the biliary metabolites of the compounds studied and that following infusion of their deconjugation products as a rough estimate of the hydrolysis rate, suggested that the biliary conjugates of phenolphthalein, LSD and diphenylacetic acid were hydrolysed at similarly rapid rates while morphine was apparently hydrolysed at a slower rate. On the limited evidence we have collected, it is tentatively suggested that ester glucuronic acid conjugates (those of diphenylacetic acid) are hydrolysed in the gut in a manner similar to that of ether glucuronic acid conjugates (those of phenolphthalein, morphine and LSD).

iii) The relationship between the lipid solubilities of deconjugation products of hydrolysis and their ability to undergo absorption from the gut.

The rate and extent to which the deconjugation products of the gut microfloral hydrolysis of their corresponding biliary conjugates were absorbed from the gut was shown to correspond with their relative lipid solubilities. Phenolphthalein and diphenylacetic acid, being considerably more lipid soluble than morphine and 13-hydroxy-LSD, were found to be more readily absorbed from the gut than either morphine or 13-hydroxy-LSD. With morphine, in addition to lipid solubility considerations, the slower hydrolysis of its biliary conjugates as compared with other compounds, is likely to have been a factor contributing to its rate and extent of absorption from the gut.

iv) Compounds excreted in the bile both in free and conjugated form

It has been suggested that biliary glucuronic acid conjugates are poorly absorbed from the gut relative to their deconjugation products and consequently hydrolysis would be the rate limiting step in their intestinal phase of EHC.

On this basis, the (i/d) infusion of the biliary metabolites of a compound excreted in the bile, both in the free and conjugated form, would be expected to be recovered in recipient bile more rapidly than those of a compound excreted in the bile exclusively as its glucuronic acid conjugate; providing that the hydrolysis rate for the biliary conjugates and the intestinal absorption rate for the deconjugation products was similar for both compounds. Diphenylacetic acid (13% free and 87% conjugated in the bile) and phenolphthalein (100% conjugated in bile) provided examples of two such compounds. However, no significant increase in the rate of biliary recovery of recipients infused (i/d) with the biliary metabolites of diphenylacetic acid was observed as compared with that in recipients infused with the biliary metabolites of phenolphthalein. It could be calculated that the fraction (13%) of the biliary metabolites of diphenylacetic acid present in the bile as the free acid would give an increased initial recovery in recipient bile of 4 - 5% which would not be a significant increase.

It has previously been shown that following the intragastric (i/g) injection of pregnanolone biliary metabolites (predominantly the glucuronic acid conjugate and 4% as the free steroid) in bile duct cannulated recipients, the recovery pattern in recipient bile gave a biphasic pattern. A minor peak was observed at 1 hour after dosing, thought to represent the rapid intestinal absorption of the free steroid present in the dose and a major peak at 3 - 6 hours after dosing corresponding to the hydrolysis of the conjugates and their consequently slower absorption (Long and Soyka, 1975). On the basis of these findings, we may have expected diphenylacetic acid to behave in a similar manner, however, the (i/g) administration of pregnanolone biliary metabolites differed from the (i/d) infusion used in our studies for diphenylacetic acid. It is considered that the (i/g) injection represents a totally un-physiological delivery of biliary metabolites to the gut, giving rise to, in the case of pregnanolone, a biphasic recovery in recipient bile which, on the basis of our studies, would be an event unlikely to occur in the intact animal.

2) The systemic availability of xenobiotics undergoing EHC

A comparison of the urinary excretion of morphine, LSD, diphenylacetic acid and phenolphthalein between intact and bile duct cannulated rats would

suggest that all are systemically available from EHC. Only in the case of phenolphthalein was systemic availability from EHC accompanied by an apparent increase in systemic blood levels of the drug in intact rats as against levels in bile duct cannulated rats in which an EHC was absent.

3) The intestinal mucosal conjugation of compounds undergoing EHC

Intestinal perfusion studies with phenolphthalein and morphine suggested the possibility that following the hydrolysis of their biliary conjugates in the gut, absorption from the intestine was accompanied by a reconjugation of the free compounds with glucuronic acid. As a result of the design of the perfusion studies, it was not possible to differentiate with certainty between the relative contributions of intestinal mucosal and hepatic conjugations. In the following Chapter (5), the influence of the intestinal mucosal conjugation to the overall metabolism of these compounds is considered.

CHAPTER FIVE

The intestinal mucosal conjugation of morphine, phenolphthalein, diphenylacetic acid, phenol and 1-naphthol.

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Introduction

Earlier studies had suggested that during the intestinal phase of EHC, morphine and to a lesser extent phenolphthalein, may undergo conjugation with glucuronic acid in the intestinal mucosa. Although these studies were indicative of intestinal mucosal conjugation with morphine and phenolphthalein, they were not designed to differentiate between hepatic and intestinal conjugation and thus there remained some doubt as to the exact site of conjugation of these two drugs. In the past, methods used to investigate intestinal mucosal metabolism have included *in vitro* techniques, involving the use of supernatants from homogenates of intestinal mucosa (Tredger and Chhabra, 1976), everted gut sacs (Fischer and Millburn, 1970, Powell *et al.*, 1974), various *in situ* intestinal loop preparations (Winne, 1966, Ochsenfahrt, 1969 and George *et al.*, 1974), intestinal perfusion studies (Powell *et al.*, 1974) and indirect estimations from serially sampling portal or mesenteric blood (Iwamoto and Klaassen, 1977, Collins *et al.*, 1976). Our investigations demanded an ability to determine intestinal absorption and intestinal mucosal conjugation simultaneously under the most physiological conditions possible and with these requirements in mind, the everted gut sac and *in situ* intestinal sac preparations were employed.

The everted gut sac preparation, first introduced by Wilson and Wiseman (1954), has been widely used in the study of the intestinal absorption of xenobiotics (Dolvisio *et al*, 1969 and Houston *et al.*, 1974) and the metabolic capacity of the intestinal mucosa (Hertz *et al.*, 1961; Arias *et al.*, 1963; Kreek *et al.*, 1963; Fischer and Millburn, 1970). Although this preparation is generally considered capable of yielding realistic absorptional and metabolic functions, it has been criticised on the grounds that its isolation from the intact animal has been shown to cause a loss in the structural integrity of the mucosal cells (Levine, *et al.*, 1970a). However, other workers have found no histological changes in the mucosal tissue even after 90 minute incubations (Fischer and Millburn, 1970).

The major difference between the everted gut sac and *in situ* intestinal sacs is that while the former is an isolated *in vitro* preparation, the latter retains an intact blood supply as a result of which this preparation has been shown to maintain a high functional integrity over long periods of time (Winne, 1966 and Ochsenfahrt, 1969).

In addition to morphine, diphenylacetic acid and phenolphthalein, the intestinal absorption and mucosal conjugation of phenol and 1-naphthol were studied as they have previously been reported to undergo intestinal conjugation *in vitro* with glucuronic acid and sulphate (Bock, 1974; Powell *et al.*, 1974), but in the case of phenol, this had not been clearly demonstrated *in vivo*.

Methods

1) Everted gut sac incubations

Everted gut sacs, prepared as described in Chapter 2, were incubated for 90 minutes in mucosal buffer containing [^{14}C]-1-Naphthol and [^{14}C]-Phenol. Following incubation, aliquots of the serosal and mucosal buffers and homogenate of the gut sac were counted and analysed chromatographically as described in Chapter 2.

2) In situ intestinal sac preparations

[^{14}C]-Morphine, [^{14}C]-Diphenylacetic acid, [^{14}C]-1-Naphthol and [^{14}C]-Phenol were introduced by injection into the lumen of in situ intestinal sacs, prepared as described in Chapter 2. Blood was collected continuously for 30 minutes after which the rat was killed by a blow to the head and the intestinal sac removed. Aliquots of blood, sac contents and the metabolites in each sample were analysed by t.l.c.

3) Intestinal perfusion

Rats, prepared for intestinal perfusion as described in Chapter 2, were perfused for 90 minutes with buffer containing [^{14}C]-1-Naphthol and [^{14}C]-Phenol. Aliquots of the perfusate, bile, blood and tissue collected during or at the end of perfusion, were counted and metabolites identified by t.l.c. as described in Chapter 2.

Amounts of compounds used in methods (1) - (3)

In previous intestinal perfusion studies, 5 mg of the compounds were added to the perfusate, which for 200 g rats, would represent an oral dose of approximately 25 mg/kg. The amount of each compound added to everted gut sac incubations and in situ intestinal sacs was 0.5 mg, based on their wet weight of 0.7 - 0.9 g in proportion to that of perfused intestinal sections (7 - 9 g).

Results and Discussion

1) Studies in everted gut sacs

a) 1-Naphthol

After a 90 minute incubation, 30.0 - 40.7 % of the [^{14}C] present at the start of the incubation, was lost from the mucosal buffers (%absorbed) and recovered in the serosal buffers (3.9 - 6.2%) and tissue (25.3 - 33.8%) of proximal, medial and distal everted gut sacs (see Table 5.1). Loss of [^{14}C] from the mucosal buffer was found to be significantly greater in medial than in proximal or distal sacs, but this increased loss was found to be associated with an increased accumulation of [^{14}C] in the tissue rather than a mucosal-serosal transfer. Fischer and Millburn (1970) have reported that the loss of stilboestrol from the mucosal buffer of everted gut sacs was lower in sacs taken from the proximal region and increased progressively on moving distally along the intestine. A similar finding has been reported for testosterone in everted gut sacs (Kreek et al., 1963).

Identification of the metabolites of 1-naphthol in the mucosal buffer, serosal buffer and tissue of the sac (see Table 5.2), revealed that a considerable fraction of the [^{14}C] present in the serosal buffer was present as 1-naphthyl glucuronide (58.6 - 63.3%) and only trace amounts as the sulphate (0.0 - 0.1%). The mucosal buffer and tissue of the gut sacs contained predominantly unchanged 1-naphthol, but 9.1 - 24.8% and 7.8 - 9.4% respectively were present as the glucuronic acid conjugate. Taking the [^{14}C] present in the mucosal buffer, serosal buffer and tissue collectively, 1-naphthyl glucuronide accounted for 22.2% of the total [^{14}C], 1-naphthyl sulphate 0.2% and the remainder as unchanged 1-naphthol in proximal everted gut sacs. In distal gut sacs, the % of the total [^{14}C] present as conjugates was found to be only half that amount found in proximal sacs, (see Table 5.2). Similarly, stilboestrol has been shown to be more extensively conjugated with glucuronic acid in proximal than in medial or distal gut sacs (Fischer and Millburn, 1970).

From the total [^{14}C]-1-Naphthol conjugated (ng's) over the 90 minute incubation in proximal and distal sacs, the overall conjugation rates were calculated to be 2.5 and 1.0 ng/min/mg tissue respectively with glucuronic acid and 0.02 and 0.03 ng/min/mg tissue respectively with sulphate.

Table 5.1 The distribution of [^{14}C] in everted gut sac preparations after a 90 min incubation in mucosal buffer containing [^{14}C]-1-Naphthol.

Section	% [^{14}C] present in the mucosal buffer	% [^{14}C] present in the serosal buffer	% [^{14}C] present in the sac tissue
Proximal	70.0 (1.4)	3.9 (0.5)	25.3 (1.9)
Medial	59.3 (2.2)	4.1 (0.3)	33.8 (1.2)
Distal	60.1 (2.1)	6.2 (0.4)	27.1 (1.2)

% [^{14}C] = % of the pre-incubation dose of [^{14}C]-1-Naphthol (0.5mg) - (100% present in mucosal buffer at start of incubation). Values are the means of three animals and the figures in brackets, the standard errors of the means. Proximal, medial and distal refers to the section of the small intestine from which the gut sac was prepared, (see Chapter 2).

Table 5.2 Identification of 1-naphthol and its metabolites in the mucosal buffer, serosal buffer and tissue of everted gut sacs incubated with [^{14}C]-1-Naphthol.

Section		% as free 1-naphthol		% as 1-naphthyl sulphate		% as 1-naphthyl glucuronide	
Proximal	Mucosal	72.6		ND		24.8	
	Serosal	31.5	(74.4)	ND	(0.2)	63.3	(22.2)
	Tissue	88.8		0.8		9.4	
Distal	Mucosal	90.9		ND		9.1	
	Serosal	41.4	(81.9)	0.1	(0.3)	58.6	(11.2)
	Tissue	91.2		1.0		7.8	

Everted gut sacs, prepared from proximal and distal sections of rat intestine, were incubated for 90 min in mucosal buffer containing [^{14}C]-1-Naphthol (0.5mg). Following incubation, samples of mucosal buffer, serosal buffer and tissue from each sac were counted (see Table 5.) and analysed by t.l.c. using solvent H (see Chapter 2). Values represent the % of [^{14}C] occuring in each individual sample present as 1-naphthol and its metabolites. Values in brackets, represent the % of the [^{14}C] occuring collectively in the mucosal buffer, serosal buffer and tissue as 1-naphthol and its metabolites.

Table 5.3 The distribution of [^{14}C in everted gut sac preparations after a 90 min incubation in mucosal buffer containing [^{14}C]-Phenol.

Section	% [^{14}C] present in the mucosal buffer	% [^{14}C] present in the serosal buffer	% [^{14}C] present in the sac tissue
Proximal	74.4 (4.1)	6.3 (0.6)	14.9 (0.4)
Medial	75.7 (3.2)	7.8 (0.7)	16.9 (0.2)
Distal	78.1 (2.7)	9.1 (0.8)	10.3 (2.8)

% [^{14}C] = % of the pre-incubation dose of [^{14}C]-Phenol (0.5 mg) ; (100% present in mucosal buffer at start of incubation). Values are the means of three animals and the figures in brackets, the standard errorsof the means. Proximal, medial and distal refers to the section of the small intestine from which the gut sac was prepared (see Chapter 2).

Table 5.4 Identification of phenol and its metabolites in the mucosal and serosal buffers and tissue of everted gut sacs incubated with [^{14}C]-Phenol.

Section of intestine	Sample	% of total as phenol		% of total as phenyl sulphate		% of total as phenyl glucuronide	
Proximal	Mucosal buffer	52.2		1.3		46.2	
	Serosal buffer	4.2	(48.2)	1.9	(1.6)	93.9	(50.3)
	Tissue	42.4		4.9		52.7	
Medial	Mucosal buffer	42.8		2.9		54.3	
	Serosal buffer	10.8	(40.1)	2.4	(3.0)	86.9	(56.9)
	Tissue	43.9		5.0		51.0	
Distal	Mucosal buffer	56.1		5.5		42.9	
	Serosal buffer	3.8	(46.5)	1.9	(5.1)	94.4	(48.5)
	Tissue	45.3		5.1		49.6	

Everted gut sacs, prepared from proximal, medial and distal sections of rat intestine, were incubated for 90 min in mucosal buffer containing [^{14}C]-Phenol (0.5mg). Following incubation, samples of mucosal and serosal buffer and tissue from each sac were counted (see Table 5.3) and aliquots analysed by tlc using solvents A and G, (see Chapter 2). Values represent the % of [^{14}C] occuring in each individual sample (mucosal buffer, serosal buffer and tissue) present as phenol and its metabolites. Values in brackets represent the % of the [^{14}C] occuring in the samples collectively, present as phenol and its metabolites.

b) Phenol

After a 90 minute incubation, 21.9 - 25.6% of the [^{14}C] present at the start of incubation was lost from the mucosal buffers (% absorbed) and recovered in the serosal buffers (6.3 - 9.1%) and tissue of proximal, medial and distal gut sacs (see Table 5.3). No significant difference in the % [^{14}C] absorbed was observed between proximal, medial or distal sacs.

Identification of the metabolites present in the mucosal buffer, serosal buffer and tissue of the sacs (see Table 5.4) showed that most of the [^{14}C] present in the serosal buffer was present as phenyl glucuronide (86.9 - 93.9%), the remainder as phenyl sulphate (1.9 - 2.4%) and unchanged phenol (3.8 - 10.8%). The tissue and mucosal buffer were found to contain unchanged phenol and phenyl glucuronide in roughly equal proportions and a small amount of phenyl sulphate (see Table 5.4). Of the total [^{14}C] present in the incubation i.e. that in the mucosal buffer, serosal buffer and tissue, 48.5 - 56.9% was present as the glucuronide, 1.6 - 5.1% as the sulphate and the remainder as unchanged phenol. The extent to which phenol was conjugated with glucuronic acid was found to be greater in medial sacs than in proximal and distal sacs, increasing progressively towards the distal end of the gut.

The overall conjugation rates for phenol, calculated as described previously for 1-Naphthol, were 4.3, 3.8 and 3.7 ng/min/mg tissue for proximal, medial and distal sacs respectively with glucuronic acid and the responding rates with sulphate 0.1, 0.2 and 0.4 ng/min/mg tissue. Powell et al., (1974) have shown in isolated gut sac preparations perfused with buffer containing [^{14}C]-Phenol, that 50-80% of the [^{14}C] present in the perfusate was absorbed over 2 hours. At the end of perfusion, 95% of the total [^{14}C] was present as phenyl-glucuronide and 5% as phenyl sulphate.

Comparing the results obtained in everted gut sacs for 1-naphthol with those for phenol, it appears that although 1-naphthol was more extensively absorbed than was phenol, it was less readily conjugated with glucuronic acid than was phenol. However, for both compounds, their ability to undergo conjugation with glucuronic acid was considerably greater than with sulphate.

2) Studies using the in situ intestinal sac preparation

a) The appearance rate of [^{14}C] in the venous blood

Following the administration of [^{14}C] labelled compounds studied to the lumen of in situ intestinal sacs, [^{14}C] was detectable in the venous blood draining from the sac within 5 minutes. During the 30 minutes over which the experiments were conducted, the % [^{14}C] instilled into the lumen appearing in the venous blood was found to be similar for the majority of the compounds studied, (31 - 47%), but significantly lower in the case of morphine (2.1%) (see Table 5.5).

The slower appearance rate of morphine in the blood is likely to be the result of a lower affinity for absorption with this compound. Morphine has been shown in previous studies to be absorbed slowly from the intestine relative to the other compounds we have studied, which was suggested may be related to its lower lipid solubility relative to these other compounds, (see Table 4.3).

The appearance rate in the venous blood of all the compounds studied, was found to be maximal over the first 10 to 15 minutes after their administration, declining progressively after this time. The time at which the maximal appearance rate occured was found to coincide with a marginally faster blood flow rate over this period. Blood flow rate through the in situ sacs was found to be relatively constant over the 30 minute period of the experiment at 0.30 - 0.45 ml/min per g. of tissue. Bock and Winne (1975) have reported blood flow rates of up to 1 ml/min/g. tissue from the mesenteric vein draining in situ jejunal loops.

b) The appearance of conjugates in the blood

Identification of the metabolites present in the venous blood samples collected from in situ sacs using t.l.c. revealed that with the exception of diphenylacetic acid, compounds appeared in the venous blood both as the unchanged compounds and their corresponding conjugates. The % of the total [^{14}C] appearing in the venous blood present as glucuronic acid conjugates was found to be similar (30 - 38%) for morphine, 1-naphthol and phenol, but significantly lower for phenolphthalein (1.2%). The appearance of sulphoconjugates in the venous blood was only detected for phenol and 1-naphthol and the amount appearing was small by comparison with that of glucuronic acid conjugates.

The appearance rate of both the free compounds and their conjugates in the blood was found to be maximal over the first 15 minutes following their instillation. After this time, the rate declined for the free compounds while that of the

glucuronic acid conjugates remained constant for phenolphthalein and morphine glucuronides (see Figs. 5.1 and 5.2) or declined, either at a slower rate than that found with the free compounds as with phenyl glucuronide (see Fig. 5.3) or was followed by a marginal increase in rate as with 1-naphthyl glucuronide (see Fig 5.4). At the end of the experiment, the venous blood draining from sacs instilled with phenol and 1-naphthol contained predominantly glucuronic acid conjugates (80 and 54% respectively), while blood from the sacs instilled with morphine and phenolphthalein contained mainly the unchanged compounds (65 and 99% respectively). The variations in the relative rates of appearance of the free compounds and conjugates in the venous blood between the compounds studied, is thought to be related to differences in their relative capacities for intestinal absorption and conjugation.

The appearance rates of sulphoconjugates in the venous blood of sacs instilled with phenol and 1-naphthol was found to be at a maximum over the first 10 - 15 minutes, declining after this time (see Figs 5.3 and 5.4). In terms of the relative proportions of free to conjugated compound in each serial sample of blood, the amounts of both glucuronic acid and sulphate conjugates of phenol and 1-naphthol were found to increase as the experiments progressed, while that of the free compounds decreased proportionately.

c) The nature of the metabolites in the sac tissue and luminal fluid

At 30 minutes after the start of the experiments, the residual [^{14}C] not appearing in the blood was recovered in the sac tissue (14 - 23%) and luminal fluid (19 - 76%), (see Table 5.6). With the exception of diphenylacetic acid, the sac tissue and luminal fluid were found to contain both the unchanged compounds and their conjugates (see Table 5.10).

The presence of conjugates in the luminal fluid may explain the variation in the appearance rates of compounds and their conjugates in the blood. The initial maximum appearance rate of [^{14}C] in the venous blood was shown to result largely from the appearance of the readily absorbed unchanged compounds, while the subsequent decline in the appearance rate of the latter may correspond with the increasing proportion of conjugated to free compound appearing in the luminal fluid as a result of mucosal conjugation. As the conjugates would be absorbed slowly relative to the free compounds, the overall appearance rate of [^{14}C] in the venous blood would decline. This would agree with the observed increases in

Table 5.5 Distribution of [^{14}C] in the venous blood, sac tissue and luminal fluid at 30 min after administration of certain [^{14}C] labelled compounds to in situ intestinal sac preparations.

Compound	% of [^{14}C] in blood	% of [^{14}C] in sac tissue	% of [^{14}C] in luminal fluid
1-Naphthol	46.6 (1.3)	23.2 (4.9)	19.1 (1.7)
Phenol	31.3 (2.3)	22.5 (2.6)	40.6 (3.4)
Morphine	2.1 (0.5)	15.5 (3.2)	75.6 (6.7)
Phenolphthalein	35.8 (3.4)	18.4 (6.3)	32.4 (1.8)
Diphenylacetic acid	38.3 (4.6)	13.6 (6.9)	37.5 (1.3)

Each value represents the mean of three animals and the figures in brackets, the standard error of the means. Compounds were administered at a dose of 0.5 mg/sac. % of [^{14}C]=% of the [^{14}C] labelled dose delivered to the lumen of the intestinal sac at the start of the experiment.

Table 5. 6 The nature of metabolites present in venous blood, sac tissue and luminal fluid at 30 min after administration of certain [^{14}C] labelled compounds to in situ intestinal sac preparations.

Compound	Total metab. in blood			Total metab. in sac tissue			Total metab. in luminal fluid		
	% U	% G	% S	% U	% G	%S	% U	% G	% S
1-Naphthol	66.6	29.6	3.8	89.1	9.0	0.1	83.6	12.3	0.1
Phenol	59.2	37.6	3.2	87.9	9.8	2.3	72.4	26.0	1.6
Morphine	64.3	32.6	ND	98.3	1.2	ND	61.8	32.7	ND
Phenolphthalein	96.7	1.2	ND	95.5	3.2	ND	100	ND	ND
Diphenylacetic acid	100	ND	ND	100	ND	ND	100	ND	ND

% U, G and S = the % of the total metabolites present in each tissue or fluid as unchanged compounds, glucuronides and sulphates.

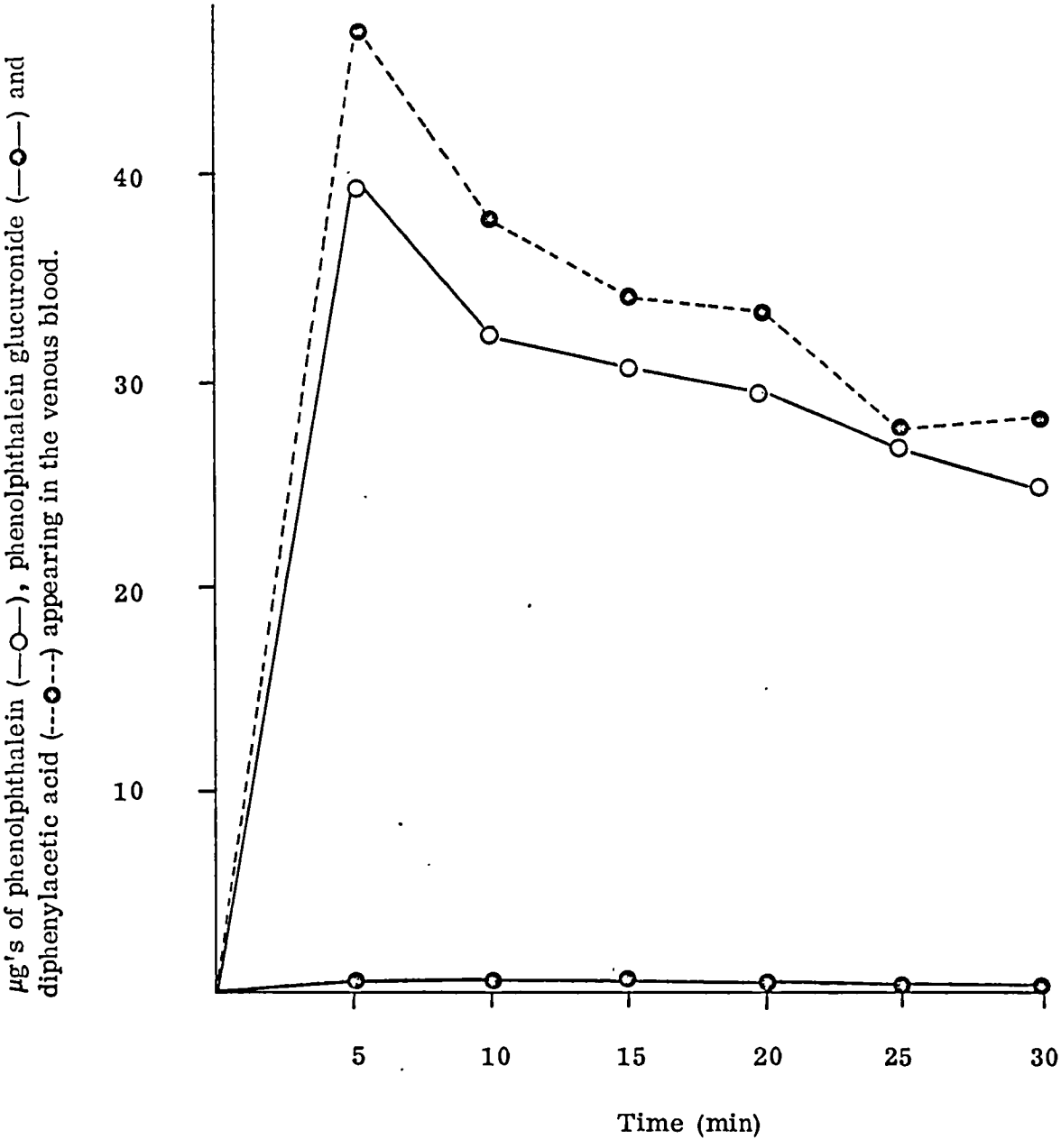

Fig 5.1 The appearance rates of phenolphthalein, phenolphthalein glucuronide and diphenylacetic acid in the venous blood draining from in situ intestinal sacs.

Venous blood draining from in situ sacs instilled with [^{14}C]-Phenolphthalein (0.5mg) or [^{14}C]-Diphenylacetic acid (0.5 mg) was collected at 5 min intervals, an aliquot of which was counted and the remainder of each sample pooled with the corresponding samples from two similarly treated sacs. Each pooled sample was prepared for chromatography (see Chapter 2) and analysed by t.l.c. using solvents A and B or H and I. The [^{14}C] present in each sample as phenolphthalein, and phenolphthalein glucuronide or diphenylacetic acid is expressed here in μg's.

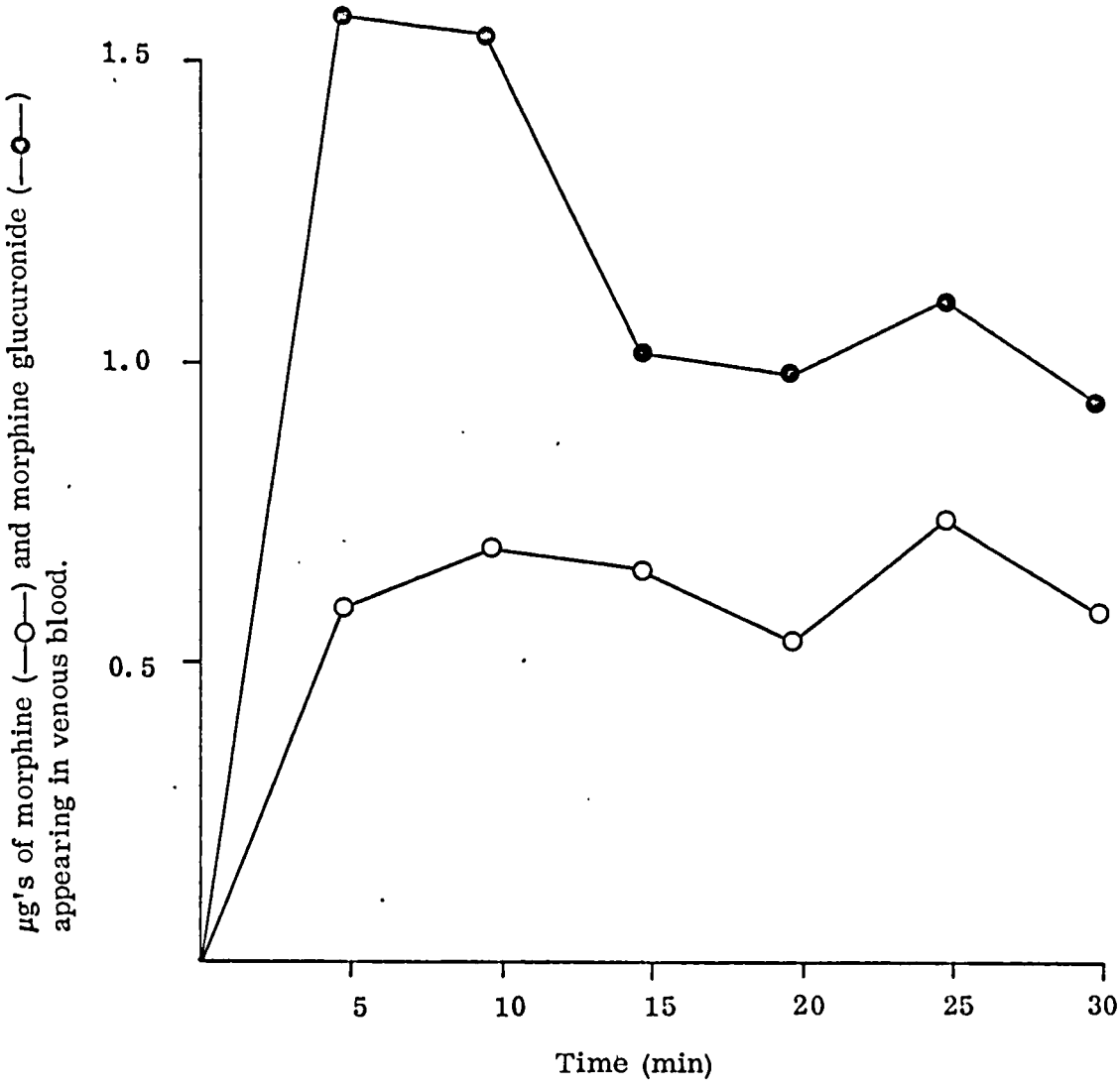

Fig 5.2 The appearance rates of morphine and morphine-3-glucuronide in the venous blood draining from in situ intestinal sacs instilled with [^{14}C]-Morphine].

Venous blood, collected from in situ sacs containing [^{14}C]-Morphine (0.5 mg) was collected at 5 min intervals, an aliquot counted and the remainder of each sample pooled with the corresponding samples collected from two similarly treated sacs. Each pooled sample was prepared for chromatography (see Chapter 2) and analysed on t.l.c. using solvents E and F. The [^{14}C] present in each sample as morphine and its glucuronide is expressed here in μg's.

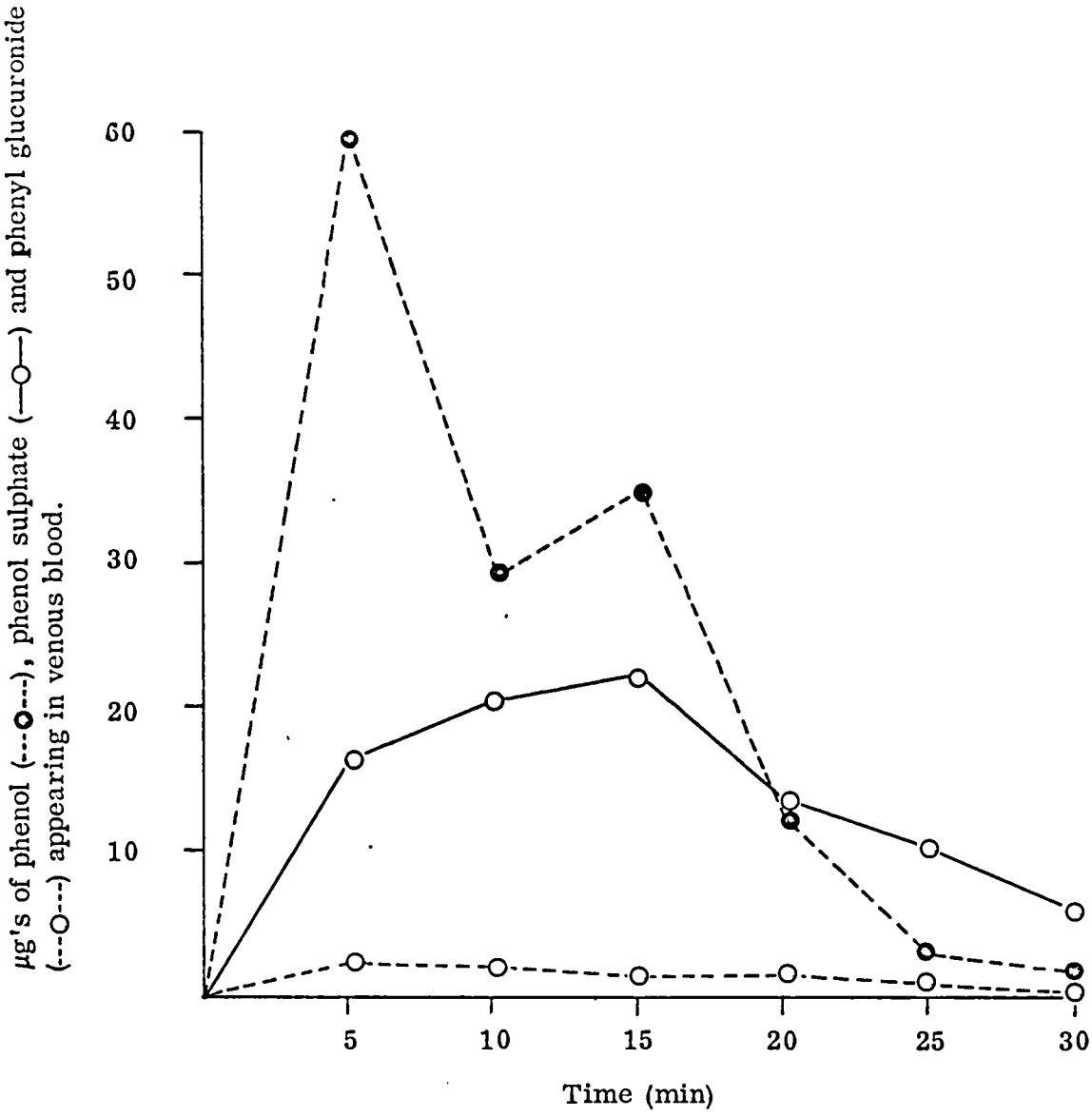

Fig 5.3 The appearance rate of phenol and its conjugates in the venous blood draining from in situ intestinal sacs instilled with [^{14}C]-Phenol.

Blood draining from sacs instilled with [^{14}C]-Phenol (0.5mg) was collected at 5 min intervals, an aliquot counted and the remainder of each sample pooled with the corresponding samples collected from two similarly treated sacs. Each pooled sample was prepared for chromatography (see Chapter 2) and analysed using t.l.c. with solvents A and G. The [^{14}C] present in each sample as phenol, phenyl glucuronide and phenyl sulphate is expressed here in µg's.

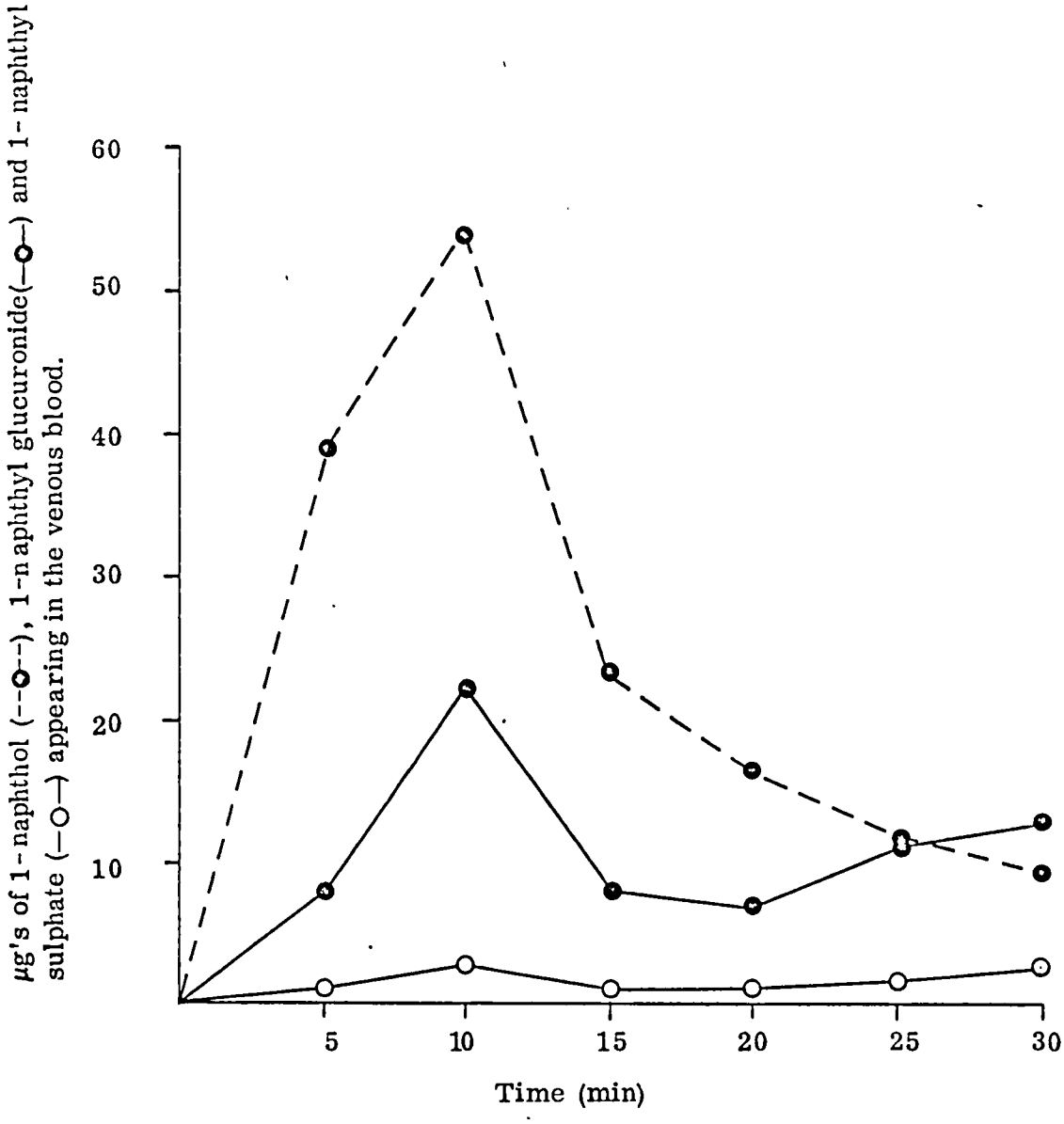

Fig 5.4 The appearance rates of 1-naphthol, 1-naphthyl glucuronide and 1-naphthyl sulphate in the venous blood draining from in situ intestinal sacs instilled with [^{14}C]-1-Naphthol.

Venous blood collected from in situ sacs containing [^{14}C]-1-Naphthol (0.5mg) was collected at 5 min intervals, an aliquot counted and the remainder of each sample pooled with the corresponding samples collected from two similarly treated sacs. Each pooled sample was prepared for chromatography (see Chapter 2) and analysed using solvent H. The [^{14}C] present in each pooled sample as 1-naphthol, 1-naphthyl glucuronide and 1-naphthyl sulphate is expressed here in μg's.

the proportions of conjugated to free compound appearing in the venous blood with time. Bock and Winne (1975) have reported similar findings following the instillation of 1-naphthol in jejunal loops, but were able to attain steady state conditions by preventing an accumulation of conjugated products in the luminal fluid using a single pass perfusion of the loop. The possibility that the decline in the appearance rate of [^{14}C] in the blood results from conjugation, may explain why that phenolphthalein and diphenylacetic acid, which appear to be poorly conjugated in the gut, showed a less marked reduction in their appearance rates in the venous blood than was observed for the other compounds, (see Fig. 5.1).

3) Intestinal perfusion studies

a) 1-Naphthol

After 90 minutes of perfusion, 44.8 ± 2.6% of the dose present at the start of perfusion was lost from the perfusate (% absorbed) and was recovered in the bile (17.9 ± 2.2%), urine (10.3 ± 2.7%), the tissue of the perfused section (8.8 ± 3.4%) and the liver (5.6 ± 1.1%), (see Table 5.7). Identification of the metabolites present in the various samples collected at the end of perfusion (see Table 5.8) revealed that while the bile contained exclusively 1-naphthyl glucuronide, the urine contained both the glucuronide (59.4%) and 1-naphthyl sulphate (40.6%). The absence of 1-naphthyl sulphate in the bile may be a reflection of its lower molecular weight (224) relative to 1-naphthyl glucuronide (320), the former being below the minimum molecular weight threshold for extensive ($<$ 10% of the dose) biliary excretion shown to be 325 ± 50 in the rat (Hirom et al., 1972b). 1-naphthyl glucuronide and 1-naphthyl sulphate, in roughly equal proportions, have previously been identified in the urine of rats receiving 1-naphthol intra peritoneally, (Capel et al., 1974).

Systemic blood was shown to contain exclusively conjugates of 1-naphthol while the portal blood contained free 1-naphthol in addition to its conjugates. The unchanged 1-naphthol present in the portal blood was presumably derived from the perfusate and on reaching the liver is though to be extracted from the portal blood to undergo hepatic conjugation and partially excreted in the bile which would explain the absence of the free compound in systemic blood and the significantly higher [^{14}C] levels in the portal blood relative to systemic blood (see Table 5.7).

Table 5.7 The intestinal absorption, biliary excretion and blood levels of [^{14}C] in rats perfused intestinally with [^{14}C]-1-Naphthol

Time after start of perfusion (min)	C % of the dose absorbed	C % of the absorbed dose in the bile	portal blood μg/ml	Systemic blood μg/ml	P<
10	22.1 (6.0)	3.2 (2.8)	48.9 (7.3)	17.3 (2.2)	0.01
20	27.6 (1.4)	5.9 (1.2)	26.6 (4.1)	18.3 (0.5)	0.01
30	33.3 (2.2)	11.4 (2.4)	28.9 (7.0)	10.0 (2.3)	0.01
45	39.3 (0.8)	15.3 (2.2)	23.5 (5.0)	16.2 (1.4)	NS
60	42.3 (0.1)	16.7 (2.2)	24.8 (4.5)	15.2 (0.6)	0.05
90	44.8 (2.6)	17.9 (2.2)	25.6 (4.4)	15.0 (1.1)	0.05

Rats were perfused intestinally for 90 min with buffer containing [^{14}C]-1-Naphthol. Samples of perfusate, bile and blood were collected at the times indicated. C % of the dose = cumulative % of the [^{14}C]-1-Naphthol (5.0 mg). present in the perfusate at the start of perfusion. Each value represents the mean of three animals and the figures in brackets, the standard error of the means. The [^{14}C] levels measured in the blood are expressed here as μg [^{14}C]-1-Naphthol / ml blood. The degrees of significant difference between systemic and portal blood levels of 1-naphthol are expressed as P values. (NS = no significant difference, i.e. $P > 0.05$).

Table 5.8 Identification of the metabolites present in tissues of rats perfused intestinally with [^{14}C]-1-Naphthol.

Tissue	% as free 1- naphthol	% as 1-naphthyl sulphate	% as 1-naphthyl glucuronide
Perfusate	29.2	36.2	34.8
Bile	ND	ND	100
Urine	ND	40.6	59.4
Portal blood	10.5	64.1	25.4
Systemic blood	ND	69.7	30.3
Homogenate of the perfused intestinal section	95.2	3.0	1.8

Rats were perfused intestinally for 90 min with perfusate containing 5 mg [^{14}C]-1-Naphthol. At the end of perfusion, the tissues were removed and metabolites extracted as described in Chapter 2. 1-naphthol and its metabolites were identified by t.l.c. using solvent H (see Chapter 2). Each value represents the % of the total [^{14}C] occuring in each tissue, present as 1-naphthol, 1-naphthyl sulphate and 1-naphthyl glucuronide (ND = not detected).

At the end of perfusion, the perfusate itself was shown to contain both 1-naphthyl glucuronide (34.8%) and 1-naphthyl sulphate (36.2%) in addition to 1-naphthol (29.2%). Previous studies with 1-naphthol in everted gut sacs and in situ intestinal sacs have shown that the intestinal mucosa is capable of conjugating this compound extensively with glucuronic acid, but has only a limited capacity for sulphoconjugation. On the basis of these earlier studies, it has been estimated that the conjugation capacity of the intestinal mucosa would be capable of accounting for the 1-naphthyl glucuronide present in the perfusate but could provide less than 1% of the 1-naphthyl sulphate present, suggesting that the bulk of the sulphate is of hepatic origin.

b) Phenol

At the end of perfusion, 63.2 ± 5.7% of the dose present at the start of perfusion was lost from the perfusate (the% absorbed) and was recovered in the bile (4.6 ± 1.0%), urine (35.0 ± 2.4%), tissue of the perfused section (8.0 ± 1.2%) and liver (6.4 ± 0.3%), (see Table 5.9).

Identification of the metabolites present in the various samples collected at the end of perfusion (see Table 5.10), revealed that the urine contained both phenyl glucuronide (45.2%) and sulphate (54.8%), while the bile contained exclusively phenyl glucuronide. The absence of phenyl sulphate in the bile may be due to its low molecular weight (174) as compared with the glucuronide (270). It has previously been reported that the urine of rats dosed intra-peritoneally with phenol contained both phenyl sulphate (55%) and phenyl glucuronide (44%), (Capel et al., 1972).

Both the portal and systemic blood were found to contain exclusively conjugates of phenol, suggesting that at this stage of the perfusion, relatively little of the [^{14}C] present in the perfusate was undergoing absorption as phenol itself. The absence of a significant difference between systemic and portal [^{14}C] levels for the most part of the perfusion suggests that the hepatic uptake of phenol from portal blood may be relatively low, as indicated by the poor biliary excretion of this compound. The perfusate was shown to contain predominantly conjugates of phenol which may explain why unchanged phenol was not detected in the portal blood sampled at the end of perfusion. Previous studies in everted gut sacs and in situ intestinal sacs have shown phenol to be extensively conjugated by the

Table 5.9 The intestinal absorption, biliary excretion and blood levels of [^{14}C] in rats perfused intestinally with [^{14}C]-Phenol

Time after start of perfusion (min)	C % of the dose absorbed	C % of the absorbed dose in the bile	portal blood μg/ml	systemic blood μg/ml	P<
10	8.3 (4.9)	0.1 (0.1)	17.5 (2.2)	11.5 (1.5)	0.02
20	23.7 (2.0)	0.6 (0.4)	19.8 (2.0)	15.7 (1.5)	NS
30	31.8 (1.7)	0.8 (0.5)	24.0 (3.1)	18.4 (1.8)	NS
45	47.0 (3.3)	2.2 (0.6)	25.1 (2.9)	26.9 (5.1)	0.05
60	56.1 (5.4)	2.7 (0.6)	26.4 (5.1)	26.6 (6.5)	NS
90	63.2 (5.7)	4.6 (1.0)	32.1 (4.7)	33.5 (4.7)	NS

Rats were perfused intestinally for 90 min with buffer containing [^{14}C]-Phenol (5 mg). Samples of perfusate, bile and blood were collected at the times indicated above. C % = the cumulative % of the [^{14}C]-Phenol present in the perfusate at the start of the perfusion. The levels of [^{14}C] measured in portal and systemic blood are expressed here as μg phenol/ml blood. Each value represents the mean of three animals and the figures in brackets, the standard errors of the means. The degrees of significant difference between portal and systemic levels are expressed as P values (NS = no significant difference i.e. $P > 0.05$).

Table 5.10 Identification of the metabolites present in various tissues of rats perfused intestinally with [^{14}C]-Phenol.

Tissue	% as free phenol	% as phenyl glucuronide	% as phenyl sulphate
Perfusate	26.6	56.3	17.1
Bile	ND	98.2	ND
Urine	ND	45.2	54.8
Portal blood	ND	38.5	61.5
Systemic blood	ND	38.1	61.9
Gut	40.3	35.4	24.3

Rats were perfused intestinally for 90 min with buffer containing 5 mg [^{14}C]-Phenol. At the end of perfusion, the tissues were removed and metabolites extracted as described in Chapter 2. Phenol and its metabolites were identified by t. l. c. using solvents A and G (see Chapter 2). Each value represents the % of the total [^{14}C] in each tissue, present as phenol phenyl glucuronide and phenyl sulphate.

intestinal mucosa with glucuronic acid, but poorly with sulphate. From these earlier studies it could be estimated that intestinal mucosal conjugation could account for the phenyl glucuronide present in the perfusate, but for only 1 - 2% of the phenyl sulphate present, suggesting that most of the phenyl sulphate must have been derived from hepatic conjugation. These findings with phenol are similar to those reported by Powell et al., (1974) who showed that after 2 hours of intestinal perfusion, phenol could not be detected in the perfusate as the unchanged compound, present only as its conjugates.

The intestinal perfusion studies with phenol and 1-naphthol have indicated the limitations of this technique in the study of intestinal mucosal metabolism as we have shown that conjugates appearing in the perfusate are not necessarily of intestinal mucosal origin and could equally have been formed in the liver.

The differences between the compounds studied as to their relative capacities for intestinal mucosal conjugation (see Table 5.11) suggests that they possess widely differing enzyme specificities for the UDP-glucuronyl transferases and phenol sulphotransferases present in the intestinal mucosa.

Table 5.11 Extent and rate of conjugation for various compounds with glucuronic acid and sulphate in the in situ intestinal sac.

Compound	Total overall conjugated		Overall rate of conjugation	
	% glucuronide	% sulphate	% glucuronide	% sulphate
1-Naphthol	18.0	1.8	3.9	0.4
Phenol	26.1	2.3	5.4	0.5
Morphine	25.6	ND	5.5	ND
Phenolphthalein	2.4	ND	0.5	ND
Diphenylacetic acid	ND	ND	-	-

Total overall conjugated = % of the total [^{14}C] in the venous blood, sac tissue and luminal fluid collectively, present at the end of the experiment as glucuronide and sulphate conjugates. Overall rate of conjugation = the overall rates of conjugation of compounds with glucuronic acid and sulphate (ng/min/mg tissue) calculated from the % of the total [^{14}C] in the venous blood, sac tissue and luminal fluid collectively, present as their respective glucuronic acid conjugates and sulphoconjugates.

Summary

1) The relative capacity of the intestinal mucosa for conjugation with glucuronic acid and sulphate.

Our studies have shown that conjugation with glucuronic acid in the intestinal mucosa was extensive for morphine, phenol and 1-naphthol, but relatively poor with phenolphthalein and not detectable with diphenylacetic acid. Sulphoconjugation in the intestinal mucosa was found to occur only with phenol and 1-naphthol. The sulphation rates for these compounds was estimated to be approximately 10 times lower than their corresponding rates of conjugation with glucuronic acid, (see Table 5.11), which is in agreement with recent in vitro studies in this laboratory for these compounds using the 600g supernatant of rat intestinal mucosal homogenates (Mehta et al., 1977).

2) A comparison of the methods used to investigate intestinal mucosal conjugation

It was proposed earlier that the appearance of conjugates in the perfusate following intestinal perfusion may either have arisen as a result of intestinal mucosal conjugation or be of hepatic origin. Further investigations in in situ and everted gut sacs have suggested that although intestinal mucosal conjugation could account for most of the glucuronic acid conjugates present in the perfusate, the relatively slow sulphoconjugation observed in the intestinal mucosa could not account for the large amounts of phenyl and 1-naphthyl sulphates present. It is thus proposed that the bulk of the sulphates of phenol and 1-naphthol shown to occur in the perfusate, are derived from the liver and not from the intestinal mucosa as has been inferred by Powell et al., (1974) for phenol.

The absorption rates for the compounds studied were found to be comparable for those measured in intestinal perfusion preparations and in situ intestinal sacs, but significantly lower when estimated in everted gut sacs which may be due to the absence of an intact blood supply in the latter, or a loss in the structural integrity of the cells of everted gut sacs as has been reported by Levine et al., (1970a). Glucuronic acid conjugation and sulphation rates in everted gut sacs (see above) were generally found to be lower than those estimated using in situ intestinal sacs which may have resulted from a depletion of UDP-glucuronic acid and PAPS in everted gut sacs, a situation thought unlikely to exist for UDP-glucuronic acid in situ (Bock and Winne, 1975).

CHAPTER SIX

Concluding Remarks.

Concluding Remarks

a) Phenolphthalein - a model compound for sudying the EHC of xenobiotics?

One of the aims of the present work was to evaluate the compounds we have investigated in terms of potential model compounds for the study of the EHC of xenobiotics. The basic requirementsof a model compound are first and foremost the need to be representative of the majority of compounds on which it is modelled and secondly to closely resemble the theoretical model it is attempting to describe. Most xenobiotics known to undergo EHC are excreted in the bile as glucuronic acid conjugates of either the unchanged compound or its Phase I metabolite (s), thus phenolphthalein would be representative of the former group and LSD of the latter. However, as we have shown the EHC of LSD to be in essence similar to that of phenolphthalein, the latter must prove a better model for EHC by virtue of its straight forward metabolism and the efficiency with which it undergoes EHC.

b) An evaluation of the physicochemical requirements for EHC

It was suggested earlier that xenobiotics undergoing EHC required a degree of ampiphilicity in order to satisfy the physicochemical requirements for both biliary excretion and intestinal absorption. In order to define this degree of ampiphilicity in terms of a measurable parameter such as log P, the individual polarity requirements for both biliary excretion and intestinal absorption must themselves be described in these terms. Hirom et al., (1974) have suggested that compounds excreted extensively (<10% of the dose) in the bile require a certain balance between their lipid and water solubilities (ampiphilicity) showing that compounds of log P>-2 (i.e. ratio of lipid / water solubility is low) tend to be excreted preferentially in the urine (i.e. > 10% of the dose in the bile) while compounds of log P <−2, reflecting a certain balance between their lipid and water solubilities, tend to be preferentially excreted in the bile (<10% of the dose). It has also been suggested that when the ratio of lipid and water solubilities becomes high, biliary excretion tends to decline. This was shown by the progressive reduction in the extent to which a series of penicillins were excreted in the bile when their log P values increased progressively from 1.1 to 3.2, (Ryrefeldt, 1971). Although the relationship appears to hold for the majority of compounds, exceptions such as phenol (log P +1.3; 5% excreted in

the bile - Abou-El-Makarem, 1967) and morphine (log P + 0.09; >1% of the dose excreted in the bile as free morphine) suggest that the polarity requirement for biliary excretion cannot rigorously be defined on the basis of ampiphilicity alone. In comparison, the relationship between lipid solubility and intestinal absorption rate is more clearly defined (Bates and Gibaldi, 1970). Our studies and those of other workers suggest that polar compounds of log P values approximately > -1 to -2 tend to be poorly absorbed from the gut (Hogben et al., 1959). It has been shown for a homologous series of carbamates that for analogues (C_1 - C_4), with lipid solubilities increasing from log P -0.7 to +0.9, their absorption rates from the intestine increased linearly with the rise in lipid solubility. Further extentions of chain length (C_5 - C_8), accompaned by increasing lipid solubility from log P +1.3 to +2.9, was shown to result in a decline in their absorption rates. This decline in absorption rate with further increases in lipid solubility was suggested to result from the existence of a hydrophilic barrier in addition to the lipophilic barrier (Houston et al., 1974).

From the data we have considered, it appears that there may be some overlap between the lipid solubility requirements of biliary excretion and those of intestinal absorption which would explain why certain compounds (see Table 1.12) are able to undergo EHC as the unchanged compound.

c) Systemic availability from EHC

Our studies and those of other workers suggest that most compounds undergoing EHC are systemically available to some extent from the EHC (see Table 1.12). The extent of systemic availability will depend upon the degree to which a compound is extracted from the portal blood by the liver and thus we have shown that morphine, which is poorly excreted in the bile, relative to other compounds we have studied, was found to be more systemically available than the other compounds investigated. It has been reported for many compounds undergoing EHC as glucuronic acid or other conjugates and is confirmed by our studies, that the systemic availability from EHC is generally restricted to the conjugates rather than the parent drug itself. As conjugates are generally accepted to be pharmacologically inert relative to their parent drugs, it follows that any increased pharmacological response arising directly from EHC will be restricted to those compounds under-

going EHC as the free unchanged drug or its active unconjugated metabolites. As most compounds known to undergo EHC do so as conjugates, it is thus not surprising that there are so few examples of increased pharmacological responses arising from the EHC of drugs. Drugs such as diazepam and digitoxin, known to be excreted in the bile as their active metabolites or the unchanged drug, have been shown to give rise to enhanced pharmacological responses that could be attributed to their EHC (Baird and Hailey, 1972; Caldwell and Greenberger, 1971 a,b). The EHC of compounds such as phenprocoumon (Meinertz et al., 1977), phenobarbital and progesterone (Marselos et al., 1975) have been inferred to significantly enhance or prolong their pharmacological responses despite their being excreted in the bile as the inactive glucuronic acid conjugates, suggesting that these drugs must in part be systemically available from EHC as the active unconjugated drugs, or their active unconjugated metabolites.

d) The contribution of the intestinal mucosa to the overall conjugation of xenobiotics in the body.

Several workers have questioned the importance of the liver in the overall metabolism of certain compounds administered by an oral route (Harrison and Riegelman, 1969; Powell et al., 1974; Bennett et al., 1974; Gugler et al., 1975). Powell et al., (1974) have suggested that when phenol is administered intravenously, so as to by-pass the intestine, the compound is metabolised primarily by the liver but when given orally, they conclude from investigations with in vitro gut preparations and intestinal perfusion studies that phenol is metabolised mainly in the intestinal mucosa. Our studies with phenol would dispute these conclusions to some extent in that we have shown sulphation to be a major conjugation pathway for phenol from intestinal perfusion studies while finding a limited capacity for phenol sulphation in the intestinal mucosa as has been confirmed by in vitro microsomal studies (Metha et al., 1977). Although it was not possible to accurately estimate the contribution of the intestinal mucosa to the overall metabolism of orally ingested phenol from our studies in the absence of steady state conditions, it is tentatively suggested that no more than 50 - 60% of the dose is conjugated in the intestine, as we have shown that approximately 50% of the phenol absorbed during intestinal perfusion is present as its sulphate.

The potential pharmacological consequences of intestinal mucosal conjugation would be that the conjugation would reduce the amount of drug absorbed as the free pharmacologically active compound. This would infer the necessity to administer larger oral doses of the drug than would be required to attain the same order of pharmacological response arising from an intravenous dose. The intestinal mucosa has been shown to conjugate isoprenaline (Conolly et al., 1972; George et al., 1974), salicylamide (Levy et al., 1967; Gugler et al., 1975) and acetylsalicylic acid (Harris and Riegelman, 1969) to form their respective sulphoconjugates. Bennett et al., (1975) have shown that both the systemic blood levels and pharmacological response to isoprenaline in dogs was increased when given orally in combination with salicylamide, suggesting that these two drugs compete for sulphoconjugation in the gut wall, such that more of the active unchanged isoprenaline is absorbed.

A reduced pharmacological response has been noted with morphine following its oral administration (Houde et al., 1965) despite an apparently complete absorption from the gut (Cochin et al., 1954) which has been explained by its 'first pass' metabolism (Iwamoto and Klaassen, 1977). In vitro studies have shown that morphine can be conjugated by microsomal preparations of intestinal mucosa (Del Villar et al., 1974). Iwamoto and Klaassen (1977) have estimated by pharmacokinetic means that approximately 45% of an oral dose of morphine (5mg/kg) is conjugated with glucuronic acid by the intestinal mucosa in the rat, which compares favourably with the value of approximately 35% we have determined from our studies with this compound.

Our studies and those of other workers have indicated that the intestinal mucosa is capable of making a considerable contribution to the overall metabolism of several compounds following their oral administration but our studies in particular suggest a need to distinguish carefully between the relative contributions of hepatic and intestinal mucosal conjugation as we have shown that the inferences drawn from certain in vivo techniques were misleading in this respect.

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The enterohepatic circulation of ^{3}H-phenolphthalein in the rat

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Many drugs are excreted in bile and, consequently, may undergo an enterohepatic circulation (EHC) (see Smith & Millburn, 1975). In the rat, phenolphthalein is conjugated with glucuronic acid and then extensively excreted in bile (Millburn, Smith & Williams, 1967). This paper reports on the EHC of phenolphthalein.

In bile-duct-cannulated rats injected with [^{3}H]-phenolphthalein (25 mg/kg i.p.), 89 ± 1.6% (n = 3) of the ^{3}H was excreted in bile in 3 h, whereas in intact rats four days are required for the elimination of 86 ± 3.9% (n = 3) in faeces. This delayed faecal excretion appears to be due to EHC.

Following intraduodenal infusion of [^{3}H]-phenolphthalein into bile-duct-cannulated rats, there is a rapid biliary excretion of radioactivity (Figure 1a). By contrast, on infusion of bile containing [^{3}H]-phenolphthalein glucuronide obtained from rats injected with [^{3}H]-phenolphthalein, there is a lag period of some 4 h before a comparable rate of excretion occurs (Figure 1a). This suggests that the glucuronide may require hydrolysis to the aglycone before significant absorption occurs, as is the case for stiboestrol glucuronide (Fischer, Millburn, Smith & Williams, 1966).

Rats were treated daily for 3 days with antibiotics to suppress the intestinal microflora. Figure 1b shows that this treatment did not inhibit the absorption of free phenolphthalein. However, on the intraduodenal infusion of bile containing [^{3}H]-phenolphthalein glucuronide the biliary excretion of ^{3}H (Figure 1b) was much lower than in untreated animals (Figure 1a). This indicates that hydrolysis of phenolphthalein glucuronide by bacterial β-glucuronidase is an important step in the EHC of phenolphthalein.

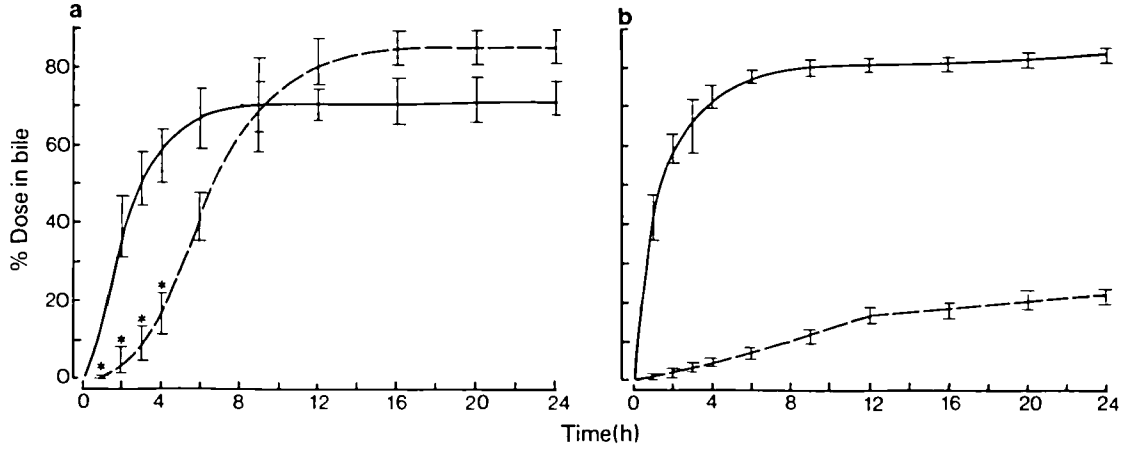

Figure 1 Biliary excretion of radioactivity after intraduodenal infusion of ^{3}H-phenolphthalein (——) or ^{3}H-phenolphthalein glucuronide (— — —) into bile-duct-cannulated female Wistar albino rats (200-250 g body weight). The dose was 79 μmol/kg. The vertical bars represent s.e. mean (n = 3).
a: without antibiotic treatment. *These values are significantly ($P < 0.05$) lower than the corresponding values for phenolphthalein.
b: after 3 days treatment with neomycin (100 mg kg^{-1} day^{-1}), tetracycline (50 mg kg^{-1} day^{-1}) and bacitracin (50 mg kg^{-1} day^{-1}).

Carotid arterial blood level measurements suggest that phenolphthalein may be systemically bioavailable from the EHC. Thus, in intact but not in bile-duct-cannulated rats there is a secondary plasma peak of radioactivity 5-6 h after the intravenous administration of [^{3}H]-phenolphthalein. This peak coincides with the absorption of the aglycone from the intestine following the bacterial hydrolysis of phenolphthalein glucuronide (see Figure 1a).

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The effects of cyproheptadine pretreatment on insulin release from isolated pancreatic islets

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Cyproheptadine, an antiserotonin-antihistaminic agent with a chemical structure similar to the tricyclic antidepressants (Stone, Wenger, Ludden, Stavorski & Ross, 1961) inhibits glucose-mediated insulin release by an immediate and direct effect on the rat pancreatic islet of Langerhans (Richardson, McDaniel & Lacy, 1975).

The present studies describe the effects of different secretogogues on insulin release from cyproheptadine-pretreated rat islets. Approximately 200 islets were isolated from two 200-300 g ten week old male albino rats (OFA Sandoz SPF strain) by the collagenase technique (Lacy & Kostianovsky, 1967). An equal number of islets were placed in each of two perfusion chambers and perifused at 37° C and pH 7.40 with Krebs Ringer bicarbonate containing 5.6 mM D-glucose at a rate of 1 ml/min as described previously (Lacy, Walker & Fink, 1972). After 45 min, the test islets were exposed to 100 μM cyproheptadine hydrochloride monohydrate for five minutes. Subsequently both chambers were stimulated with an insulin secretogogue for a further 60 min as indicated in the Table. The perfusate was collected at 1- or 5-min intervals throughout the study. The insulin content was determined by radioimmunoassay (Wright, Makulu, Vichick & Sussman, 1971) and expressed as (μU/islet)/minute. All data was subjected to complete statistical analysis.

Cyproheptadine pretreatment completely abolished tolbutamide- or glucose-evoked insulin release. Conversely the responsiveness of islets to

Table 1 The effects of cyproheptadine pretreatment on insulin release from perifused islets

Mean rate of secretion with 5.6 mM D-glucose (μU/islet)/min ± s.e.		*Insulin secretogogue added*	*n*	*Mean rate of secretion after addition of secretogogue μu/islet per min ± s.e. mean*		**P*	*% Inhibition*
Control	*Test*			*Control*	*Test*		
0.59 ± 0.04	0.54 ± 0.09	1.1 mM tolbutamide	3	1.37 ± 0.08	0.48 ± 0.09	< 0.001	100.0
0.47 ± 0.21	0.78 ± 0.29	11.1 mM D-glucose	3	2.67 ± 0.65	0.72 ± 0.22	< 0.01	100.0
0.73 ± 0.15	0.67 ± 0.08	11.1 mM D-glucose + 6.0 mEq/1. Ca^{++}	3	3.17 ± 0.37	0.96 ± 0.15	< 0.001	88.5
0.53 ± 0.35	0.49 ± 0.24	5.0 mM theophylline	4	1.19 ± 0.44	1.17 ± 0.33	NS	0.0

* Control versus test values after addition of secretogogue
NS = not significant