ABSTRACT

BILE COMPOSITION IN NORMAL FEMALE AND PREGNANT RABBITS

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Summary

In order to examine the hypothesis that the higher incidence of gallstones in multiparous women is caused by changes in bile composition during pregnancy, a study has been made of the bile acid, cholesterol and lipid-phosphorous content of hepatic and gallbladder bile of non-pregnant (control) and pregnant rabbits.

The relative and absolute concentrations of bile acids in hepatic bile were the same for the two groups of animals. GDC was the major bile acid, while GCDC was the second most abundant. The relative concentration of glycine conjugates was higher in the gallbladder bile than in the hepatic bile, while concentrations of lithocholate, unconjugated dihydroxy acids and 2 minor unidentified acids were lower. No significant differences were seen in the cholesterol content, lipid-phosphorous nor in the bile flow rate of both groups, but the bile volume of gallbladder bile was significantly higher in the pregnant group.

No evidence has been obtained from this study that pregnancy causes changes in the composition of hepatic and gallbladder bile that could account for the higher incidence of gallstones in multiparous women.

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ABSTRACT

In order to examine the hypothesis that the higher incidence of gallstones in multiparous women is caused by changes in bile composition during pregnancy, a study has been made of the bile acid, cholesterol and lipid-phosphorous content of hepatic and gallbladder bile of non-pregnant (control) and pregnant white New Zealand rabbits.

Bile samples were collected from the gallbladder by aspiration and the volume was measured. Hepatic bile was collected by cannulation of the common bile duct over a period of one hour and the bile flow rate estimated. Cholesterol and lipid-phosphorous were extracted from the bile with petroleum ether and estimated colorimetrically; bile acids were extracted with absolute ethanol, separated by thin-layer chromatography and estimated by an enzymatic procedure. Losses during the entire procedure were monitored by introducing labelled 14-C bile acids to bile samples and estimating their recovery.

The relative and absolute concentrations of bile acids in hepatic bile were the same for the two groups of animals. GDC was the major bile acid, while GCDC was the second most abundant. The relative concentration of glycine conjugates was higher in the gallbladder bile than in the hepatic bile, while concentrations of lithocholate, unconjugated dihydroxy acids and 2 minor unidentified acids were lower. These differences are most readily explained by a greater selective reabsorption of unconjugated mono-and dihydroxy acids through the gallbladder wall.

No significant differences were seen in the cholesterol, lipid-phosphorous content nor in the bile flow rate of both groups, but the volume of gallbladder bile was significantly higher in the pregnant group.

No evidence has been obtained from this study that pregnancy causes changes in the composition of hepatic and gallbladder bile that could account for the higher incidence of gallstones in multiparous women.

LIST OF ABBREVIATIONS

TC	-	Taurocholic Acid
GDC	-	Glycodeoxycholic Acid
UDC	-	Ursodeoxycholic Acid
CDC	-	Chemodeoxycholic Acid
DC	-	Deoxyocholic Acid
GC	-	Glycocholic Acid
GCDC	-	Glycochenodeoxycholic Acid
C	-	Cholic Acid
GLiC	-	Glycolithocholic Acid
LiC	-	Lithocholic Acid
NaTDC	-	Sodium Taurodeoxycholic Acid
ATP	-	Adenosine Triphosphate
CoA	-	Coenzyme A
Mg	-	Magnesium
NAD	-	Nicotinamide Adenosine Diphosphate
NADH	-	Reduced NAD

CHAPTER I

REVIEW OF BILE METABOLISM AND GALLSTONES

(i) GENERAL INTRODUCTION

(a) Descriptive Outline

An important function of the liver is the continuous secretion of bile. Bile is largely a digestive secretion which not only aids in the intestinal absorption of fats, but also serves as an important excretory and regulatory metabolic fluid.

Although bile contains no digestive enzymes, it has a two-fold digestive role. Its alkalinity helps neutralize acid food entering the stomach and creates a pH favourable for the action of pancreatic and intestinal enzymes. The bile salts present in bile, function in emulsifying fats, breaking them up into smaller globules, thereby providing more surfaces on which fat-splitting enzymes can act. They are essential for the absorption of fats (1), fat-soluble vitamins (A,D and K) as well as the water-soluble vitamin C and members of the B complex (2). They also represent the major excretory products of cholesterol. They are not eliminated from the body but are reabsorbed along with the fats and are carried back to the liver via the blood of the portal system to be used again. This enterohepatic circulation of bile salts exerts a regulatory influence on cholesterol metabolism. The colour of bile (green, yellow, red according to the species) is due to the presence of bile pigments, excretory products derived from the breakdown of haemoglobin in the liver.

Bile is thus an important digestive, excretory and metabolic fluid. Without bile, the digestion and absorption of fats and lipid are greatly impaired and vitamin deficiency may develop. (20).

Bile serves many other auxilliary functions. Indirectly it aids in the regulation of salt and water balance and of the acid-base balance through the biliary excretaion of alkaline substances.(2). It also aids in the absorption of Ca, Fe and Cu. The elimination of many organic poisons e.g. strychnine and quinine are excreted in the bile in combination with the bile acids. Certain drugs, e.g. penicillin, streptomycin, salicylates are excreted in the bile. It thus aids in the detoxifying processes of the body.

Prolonged absence of bile, caused for example by the creation of external fistulas, results in dehydration, weight loss with intestinal disturbances, macrocytic anaemia and other complications and eventually death.

(b) Historical Background

The existence of bile has been known for centuries. The earliest recorded notice of gallstones, a pathological condition of bile, was made by a Greek Physician, Alexander Trallianus who lived about the 5th century A.D. (3). Trallianus' work however, was not published until 1548. Little evidence of research on bile seems to have been done up to this time, and it is not until the 18th and 19th centuries that one must look for the first satisfactory attempts on the analysis of bile. In the 18th and 19th centuries much research on bile itself

and its pathology was done, and several works on these subjects were published (4,5,6,7).

Many of the important constituents of bile, such as the bile salts and cholesterol were only discovered in the 18th and 19th centuries. In 1757, Robert Ramsay, first noted "the resin in bile which was afterwards to be known as the bile acids" (7). The identification of these bile acids is attributed to Tiedmann and Gmelin in 1826-27, who reported a large number of analyses of bile in their work on the digestion of animals (7,8). The discovery of cholesterol, was made by de la Salle in 1769 and named "cholestearin" by Chevreul (8).

While the actual composition of bile was not worked out until fairly recently in the 20th century, its peculiar characteristics have been known for hundreds of years. The detergent effect of bile was mentioned by Coe in his treatise (4). He noted that people used aqueous solutions of bile to clean grease and stains from cloth and to mix colours in paints. Coe also noted that bile aided in uniting the "oily and watery" components of the contents of the intestinal tract prior to digestion. The colloidal state of cholesterol in bile, its solubility in bile salt solutions and the dissolving power of the bile salts were recognized in the late 19th century by several researchers (9,10,11).

In the early 20th century, lecithin, another component of bile was recognized as also having a solvating effect (12,13).

Bile from various sources has since been studied and analysed (14,15,16,17,18,19). Although its composition varies considerably, not

only from species to species, but also among individuals of single species under various circumstances, its general composition is now fairly well established.

(c) Bile Composition

97-98% of bile is water while the remaining 2-3% is made up of a variety of solids. In addition to the bile salts, cholesterol, pigment and lecithin, there are phosphates, soluble and insoluble sulphates, fats, fatty acids, thyronines, pressor amines, conjugates of the body hormones, alkaline phosphatase, reducing sugars, mucin, albumin and other plasma proteins, amino acids, uric acid, and creatinine, urea, electrolytes, the major ones being Na, K and Cl ions. Bollman (20), classified substances in hepatic bile as belonging to the following categories:-(1) those in similar concentration to plasma filtrate, such as Na, K and Cl ions; (2), those less concentrated than the same substances in plasma. Included here are alkaline phosphatase, cholesterol, phospholipids, sucrose; (3), those found in bile in concentration up to 1000 times those found in blood, such as the bile salts, pigment and steroid conjugates. A great range of concentration is to be encountered with most of these constituents. Most notably in this respect are cholesterol, bile salts and the pigments. In those animals possessing a gallbladder, where the hepatic bile is stored and exposed to the concentrating activity of that organ, the concentrations of these substances in gallbladder bile differ considerably from hepatic. During its stay in the gallbladder, bile is reduced in volume some 90%, through the reabsorption

of water and certain other solutes, but the bile pigments, bile acids and cholesterol undergo practically no reabsorption and therefore increase up to 10-fold in concentration (21). Despite this, however, hepatic bile and gallbladder bile, at all stages of reabsorption and concentration remain isotonic with plasma (21,22).

(ii) BILE METABOLISM

(a) Formation and Secretion of Bile

Because of the inaccessibility of the biliary tract to direct investigation, the exact mode of formation and secretion of bile remains for the most part unsatisfactorily explained.

It is known that bile is secreted continuously by the hepatic cells or hepatocytes. Each hepatic cell is in intimate contact with a hepatic sinusoid (23). Microvilli of the hepatic cells project into the sinusoidal space making intimate contact with the extremely thin endothelial lining of the biliary ducts or canaliculi. The latter are surrounded by a network of arterioles and branches of the portal vein as well as by a network of lymphatics. With this arrangement of hepatic cell and biliary ductules, one would expect a simple filtration process for water and other substances which are in concentration in bile similar to that of blood, would suffice to explain the secretion of these substances into bile, but several physiological observations refute this view. Bile is secreted from the low pressure region of the hepatic sinusoid and the presence or absence of arterial pressure does not alter bile volume (24). The substances which appear

in bile in a much higher concentration than in the blood must necessarily be actively secreted into the bile. The energy required for this is probably derived from the metabolism of glucose (20). At present it is surmised that a primary secretion originates in the canaliculi, and that the principal driving force for fluid transfer at this site is an osmotic gradient generated by active solute secretion (25). Active secretion of bile acids by the hepatocytes has been demonstrated by Erlinger et al (26). Subsequent to active secretion, osmotic diffusion of water and electrolytes takes place, as well as secretion of an electrolyte solution by the biliary epithelium (26). Chenderovitch (27), in a study designed to investigate the secretory and absorptive functions of the bile channels, showed that in the distal segment of the bile ducts (the bile ductules) an electrolyte solution isosmotic with primary bile is secreted. Into this an excess of K is added probably by goblet cell secretion, while in the proximal segment (the bile canaliculi) water is caused to move across the hepatic cells by modifications of hydrostatic and/or osmotic pressure gradients. Forker (25), in his study on bile secretion, noted that liver cells were capable of achieving high concentrations of solutes such as mannitol which are not thought to be susceptible to any sort of specific transport and which do not readily penetrate cell membranes. This would indicate that solutes the size of mannitol or smaller enter bile principally by osmotic filtration rather than by diffusion. Larger solutes probably enter bile by simple diffusion (25). Bilirubin has been shown to be secreted in bile canaliculi, while biliary alkaline phosphatase is secreted by the hepatic cells of bile canaliculi and biliary cells of the distal segment of the bile ducts.

An active process seems also to be involved in the transfer of water, K and Cl ions from the blood to the bile (20). Concentrations of these substances in the bile parallel their concentrations in the blood. Even when the plasma osmolarity is changed, the equality of electrolyte and osmotic pressures between the hepatic cell and bile ductule is still maintained. Under these circumstances, the rate of bile flow is reduced, indicating that cellular secretion governs the rate of bile flow (20).

(b) Regulation of Bile Metabolism

The enterohepatic circulation of the bile salts themselves, nervous stimuli, hormonal mechanisms, and diet, influence the formation and secretion of bile, the rate of bile flow and the composition of bile.

The enterohepatic cycling of bile salts exerts a homeostatic regulation on the entire secretory process. Subsequent to the absorption of lipids, the bile salts are absorbed in the ileum where an active transport system is located (28). The absorbed bile salts are returned to the liver mainly by the hepatic portal system, where they are resecreted into the bile thereby reinitiating the cycle. A small loss of bile salts occurs in each cycle but this is replaced in the liver by biosynthesis from cholesterol. The rate of this biosynthesis is regulated by a feedback mechanism which keeps the pool of circulating acids constant. When the concentration of bile salts in the portal vein is reduced, so that the amount of bile salts returning to the liver is reduced, the synthesis of bile acids may increase several fold. Thus Bergstrom and Danielsson(29),

showed that when the enterohepatic circulation of bile is interrupted by fistula, a marked increase in synthesis of bile ensues, indicating a homeostatic regulation of bile acid formation. The natural bile salts themselves and their synthetic derivatives, are known to be potent choleretic agents (22).

Observations on the effects of nervous stimulation of the biliary tree have been inconsistent. Both augmentation and inhibition of bile flow have been observed after direct stimulation of the vagus nerve (22).

The hormone, secretin, secreted by the mucosal lining of the small intestine, increases bile flow. Consequently, all factors causing secretin liberation, such as entry of gastric contents into the duodenum, will correspondingly cause an increase in bile flow. Exogenous administration of secretin has also been observed to stimulate the output of bile (22).

The influence of thyroid activity on bile metabolism has been studied extensively. It is well known that in man serum cholesterol levels tend to be high in hypothyroidism and low in hyperthyroidism. This tendency has also been observed in rats (30). Bile fistula rats excrete more cholesterol in the bile when treated with thyroxine. Consequently, it is not surprising that thyroid activity exerts a significant effect on bile metabolism, since bile acid synthesis is dependent on the rate of cholesterol oxidation. In bile fistula rats, it has been observed that the secretion of bile acids is lower in the hypothyroid state than in the euthyroid, while the total secretion of bile acids in the

hypertyroid rats is about the same as in the euthyroid rats (31). It has also been shown that in man, hypothyroid patients have a lower daily fecal output of bile acids than euthyroid control subjects. Treatment of these patients with thyroid hormone resulted in a return of the fecal value of bile acid output to normal (32). Hellstrom and Sjovall (33), found that the pattern of conjugation was influenced by the state of the thyroid. In the hyperthyroid rat an enhanced rate of secretion of CDC occurs concomitant with a decrease in C output. On the other hand, in the hypothyroid rat, the secretion of both CDC and C decreases, with CDC output showing marked dimunition. These findings have led to the speculation that the thyroid hormone may inhibit the 12 α-hydroxylating system while promoting the oxidation of the cholesterol side chain.

The pituitary is reported to affect bile synthesis. Hypophysectomy has been observed to cause a significant depression of bile acid synthesis in the rat (34). Thyroid treatment of hypophysectomised rats tended to increase the rate of bile acid production. In the intact rat however, no significant changes were observed during thyroid administration. These authors have offered the possible explanation that the thyroid and the pituitary work antagonistically regarding bile acid metabolism.

The influence of diet on bile metabolism has been investigated. In general no consistent differences have been observed by the administration of a high fat diet, whether it is made up of saturated or unsaturated fat. Under these circumstances, there is no change in bile acid secretion or in the excretion of total neutral fecal steroid (35,36).

A change from a regular diet to a semisynthetic one, containing casein, starch, sucrose, glucose, fat, salts, vitamins and cellulose, leads to an increase in the half-life of bile acids and to a consequent decrease in bile acid production (31). Danielsson and Tchen(31), also point out the important fact that some of the changes in the bile acid production induced by different diets may be attributable to changes in the intestinal flora brought about by these diets as well as by changes in the rate of passage of contents through the intestinal tract. Experimental evidence suggests that addition of cholesterol to the diet results in an absolute and relative increase in bile acid secretion (37), and that the magnitude of bile acid excretion is linked to the amount of cholesterol absorbed.

(c) Gallbladder Function

In animals possessing a gallbladder, the flow of bile to the intestine is not continuous. A considerable portion is side-tracked into the gallbladder for storage. The gallbladder is a thin-walled sac-like organ, lined internally by a thin mucosa. It is joined to the common bile duct which carries bile from the liver to the intestine, by the cystic duct. The entry and exit of bile from the gallbladder is governed by a sphincter, the sphincter of Oddi. Between periods of digestion, bile is delivered via the cystic duct into the gallbladder where it is stored and concentrated. Some time after feeding, the gallbladder contracts. Relaxation of the sphincter and contraction of the gallbladder are controlled by the vagi nerves. In addition, the hormone, cholecystokinin, elaborated by the cells of the gastric mucosa, stimulates contraction.

While in the gallbladder, bile undergoes physical and chemical changes. The gallbladder mucosa absorbs water, solutes and electrolytes (Na,K ions), but not bile salts, pigment or cholesterol, which are correspondingly concentrated several times (21). The exact mode of selective absorption is not known, but it has been demonstrated in studies with fish gallbladder that active secretion is the process involved during reabsorption (38). Studies with canine gallbladder revealed the reabsorption of a virtually isotonic solution of NaCl and $NaHCO_3$ from hepatic bile. Similarly, the gallbladder of rabbit exhibits in vitro, a capacity to absorb a nearly isotonic solution whose principal solute constituents are also NaCl and $NaHCO_3$(39). When both surfaces of rabbit gallbladder were bathed in Kreb's bicarbonate buffer, a net movement of solutes and water from the mucosa to serosa of the gallbladder was observed. Na^+ and Cl^- movement accounted for most of the net solute flux, however there was also an appreciable net bicarbonate flux in the same direction. The net flux of K was negligible. Na^+ and Cl^- ions appeared to be actively transported as a neutral ion pair. The net solute flux was directly proportional to the Na flux.

The mechanism of water reabsorption however remains obscure. Movement of water against an osmotic gradient has been observed in fish and rabbit gallbladder under circumstances in which simple osmosis cannot account for its movement (39). Net water reabsorption has been observed to take place against significant osmotic gradients, as when hypertonic solutions consisting of up to 220 milliequivalents/litre NaCl are placed within the lumen of the gallbladder. The capacity to move water against an adverse osmotic gradient can only occur in a structure

where solvent movement is coupled with active solute transport. This theory of water reabsorption, provides a valid explanation for the observed movement of water but the exact physical nature of the coupling process has not yet been determined.

While the transport of solute and water from the lumen of the gallbladder can proceed effectively when Cl^- is replaced by another ion, the transport process cannot take place in the absence of Na^+ and specifically Cl^- transport only occurs when Na ion is available.

Pinocytosis has been suggested to explain the active transport, but this process appears doubtful as it would not explain the selectivity of the reabsorptive process, for example of Na as opposed to K. The possibility has been suggested that the transported solution is actually formed within or between the mucosal cells in secretory channels or in vacuoles which are discharged at the serosal surface, and that the coupling between active solute transport and water would depend upon local osmotic gradients developed at the sites of solute transport (40). This possibility however, is difficult to reconcile with Diamond's observation that the coupling between solute and solvent movement is the same in both directions.

(d) Bile Acid Synthesis

By far the most important solid constituents in bile are the bile salts (conjugated bile acids). It is to these compounds that the detergent, solvating and emulsifying properties of bile must be attributed.

Bile acids are C24-C27 carboxylic acids, having a saturated cyclopentanophenanthrene (steroidal) nucleus, containing hydroxylic substituents and a branched side chain of 5-8 C atoms. The A/B ring is cis-in the bile acids of most higher vertebrates although A/B trans-bile acids and alcohols occur in lower vertebrates (52), or may be induced in higher vertebrates by feeding cholestanol, the saturated analog of cholesterol (41).

The most common bile acids occurring naturally, are cholic, chenodesoxycholic and deoxycholic acids. These are the 5 α-cholanic acids (C24) and have α-hydroxy groups in the C-3, C-7 and/or C-12 positions. Other C-24 bile acids having hydroxyl groups in some or all of these positions and in the C-6, C-16 and C-23 positions have been found in different species. Bile acids occur in the bile as conjugates of taurine or glycine. Conjugation with glycine occurs only in mammals (31).

The stage in evolution at which bile salts appear has not been clearly defined. They do not occur in invertebrates, but have been detected in all vertebrate species. Of the mammalian species, the pig and seal have bile acids unique to each, viz. hyocholic and phocaecholic respectively, while the mouse and rat have hydroxylated acids α- and β-muricholic acids and these also seem to be species-specific. Otherwise the bile acid pattern in mammals is relatively uniform (31). From an evolutionary point of view, the C-24 bile acids are typical of modern animals (bony fishes, snakes, birds and mammals).

Bile acids are formed from cholesterol. This was first deomonstrated by Bloch, Berg and Rittenberg in 1943 (42). Cholic and chenodeoxycholic acids are referred to as the primary bile acids since these are manufactured directly as end products of cholesterol by the liver. The mechanisms of conversion of cholesterol to bile salts have been studied in several lower species and in mammals, mainly the rat and mouse (31). During its conversion to bile acids, the steroid nucleus of cholesterol is hydroxylated at the 7α-and 12α-positions while the 3 β-hydroxyl group undergoes inversion. Subsequent to this, the side chain isopropyl group is removed with oxidation at the C-24 position to a carboxyl group. In this sequence of reactions cholic acid is formed. In another sequence, only the 7α-position is hydroxylated, followed by the inversion of the 3β-hydroxyl group forming chenodeoxycholic acid (43). Conjugation of bile acids with glycine or taurine is carried out by the microsomes of liver parenchymal cells. The reaction requires ATP, CoA and Mg ions (44). The pattern of conjugation is infuenced by diet, hormones and different diseases.(43) Since the pH of bile is in the neutral range, the bile acids are usually secreted in bile in the form of their sodium salts (45). Upon reaching the lower part of the small intestine, the bile acids become subjected to the action of intestinal microorganisms, and continue through the large intestine. One of the main reactions deals with the removal of the 7α-hydroxyl group from cholic and chenodeoxycholic acids, yielding deoxcholic and lithocholic acids respectively (46).

Bile acids are absorbed mainly in the lower part of the ileum (47). The main compounds reabsorbed from the intestine are deoxycholic and the primary bile acids cholic and chenodeoxycholic acids, partly in the form of conjugates. Smaller amounts of lithocholic acid are also absorbed (31). Upon reaching the liver via the portal circulation, the free bile acids are conjugated and re-excreted in the bile.

In many mammalian species, deoxycholic acid is not further metabolized (except for conjugation), and is a major bile acid under physiological conditions (48). In some species e.g. the rat lithocholic acid is further metabolized while in others e.g. man, it does not give rise to other metabolites but becomes conjugated not only with taurine and glycine, but also with unknown compounds giving rise to conjugates more stable to hydrolysis than glycine and taurine conjugates (49).

Bile acids appear in the feces only in the free form (50). Under normal conditions, only a negligible amount of bile acids is found in the general circulation. These are referred to as the serum bile acids. The type of bile acids appearing in the feces is influenced by several factors such as changes in the intestinal flora induced by diet or antibiotics (51).

(iii) PHYSICOCHEMICAL CHARACTERISTICS AND PROPERTIES OF BILE

The importance of bile in the digestion and absorption of fats is largely attributable to the bile salts. In addition, they are responsible for solubilizing cholesterol (which is water-insoluble and whose concentration reaches great magnitudes in the gallbladder).

Because of their characteristic structure, the bile salts have the property of lowering the surface tension of aqueous solutions, thereby allowing the formation of stable solutions or emulsions of fatty materials.

In the physiological situation of the biliary system, the bile salts are not found alone, but in association with other substances, especially lipids. Interaction with these lipids to form macromolecular complexes, results in solubilization of cholesterol and emulsification of intestinal contents during fat digestion.

The lipids encountered in the biliary system are of the polar type. Many of them are amphipaths, having both dissymetric polar and non-polar regions, the former being hydrophilic, orienting towards the aqueous phase, while the latter are hydrophobic. Hoffman and Small (52), classify the lipids according to the following scheme:-

(a) Insoluble amphipaths which orient at air-water and oil-water interface. Such lipids include triglycerides and cholesterol.

(b) Swelling amphipaths (amphiphiles). Molecules of this type hydrate in water but remain associated. The molecules have appropriately arranged polar and non-polar regions such that they hydrate in water which surrounds their polar regions while the latter remain closely associated by short range forces. Swelling amphipaths are of physiological importance, and in bile include such substances as lecithin, cephalin and monoglyceride. Lecithin is the predominant phospholipid in bile.

(c) Soluble amphipaths. These have finite solubility in water. The bile salts belong to this category. The structure of the bile salts is not

unlike the soaps and detergents and is reflected by their surface active properties. Like the soaps and detergents, the bile salt molecule has a rigid polycyclic structure. On one side of the molecule is the strongly polar terminal ionic group of either a carboxyl or sulfonic group depending on whether the molecule is conjugated with glycine or taurine. This side chain confers on the molecule its hydrophilic properties. The peptide linkage being strongly polar, lowers the pKa of the bile salt and increases its solubility in acid solutions, a fact of obvious physiological significance if the bile is to function in the acid environment of the duodenum. The other side of the molecule contains 2 or 3 hydroxyl groups thus giving the molecule its hydrophobic properties.

Bile is a mixed micellar solution. Micelles are molecular aggregates, which exist in aqueous media. They are spherical in shape and have a liquid interior of hydrocarbons. The polar ends of the molecules forming the aggregate, being hydrophilic, orient towards the aqueous phase, while the hydrophobic hydrocarbon ends orient toward the center of the micelle. Micelles form in solutions when the amphipathic concentration exceeds a specific value known as the critical micellar concentration and they are completely stable. Their formation is influenced by temperature. Above a critical temperature, the Krafft point, micelle formation takes place and the solubility of oils and fats in the solution is therefore greatly increased. The Krafft point for bile salts is well below room temperature (45), a fact which enhances their detergent action in the body.

Swelling amphipaths cannot by themselves form micelles presumably because of their low solubility, while the soluble amphipaths are capable of forming micelles by themselves. Micelles formed by bile salts are formed partly by intermolecular forces such as hydrogen bonding (45).

The bile salts do not all have the same micellar characteristics. The number of hydroxyl groups occurring on the bile salt molecule, and their location on the molecule, influence their polarity and determine whether the micelles formed by them are predominantly polar or non-polar. Micellar complexes formed by the dihydroxy bile salts are reportedly more non-polar than those of the trihdroxy cholate salts because of the smaller number of hydroxyl groups on the former (45). The dihydroxy bile salts form micelles at much lower concentrations than trihydroxy salts and have a higher molecular weight, since there are more molecules in each micelle. Thus dihydroxy salts have a greater solvating capacity for non-polar compounds than trihydroxy. Complexes made up of deoxycholic acid are slightly more non-polar than chenodeoxycholic acid, though they are both dihydroxy salts. The C-12 hydroxyl group of deoxycholate is close to the non-polar methyl groups while the C-7 hydroxyl group of chenodeoxycholic acid is not; therefore steric hindrance of the C-12 hydroxyl group of deoxycholic makes deoxycholic acid more non-polar than chenodeoxycholic (45).

In bile, lecithin takes part in micellar formation by aggregating with the detergent bile salt molecules to form mixed micelles. This high micellar solubility of lecithin in bile salt solutions, results in mixed or expanded particles which can solubilize more cholesterol

than the bile salt solution alone(45). High solubility of cholesterol in bile is possible only because of this cooperative effect of lecithin with bile salt. It has been shown in vitro that in a conjugated bile salt-water cholesterol system, less than one molecule of cholesterol was dissolved per 40 molecules bile salt, while in a bile salt-lecithin system a maximum amount of 4% by weight of cholesterol could be solubilized in the mixed micellar phase (52).

Hoffman and Small (52), also propose a scheme describing the mechanism whereby the bile salts succeed in emulsifying the contents of the intestines. Pancreatic lipase adsorbs to the glyceride/water interface and hydrolyses the 1-ester linkages to produce 2-monoglyceride and fatty acids. Lipase is then displaced by bile salts and adsorbs to a new interface. The bile salt/fatty acid/monoglyceride complex then equilibrates with smaller aggregates of micellar size in the aqueous phase. By this means there is continuous adsorption of lipase to fresh triglyceride/water interfaces and dispersion of the reaction products (53).

Hoffman and Borgstrom (54), observed the intestinal contents during fat absorption to consist of 2 phases, an oil phase composed of di-and triglycerides and un-ionized fatty acids and a micellar phase of bile salt, monoglyceride and partially ionized fatty acid, while the extremely non-polar substances such as cholesterol and fat-soluble vitamins have partition coefficients in favour of the oil phase. The cholesterol and fat-soluble vitamins do achieve finite concentrations in the micellar phase and are thus absorbed (55).

(iv) GALLSTONES

(a) Description

One of the most commonly occurring diseases of the biliary system, and the one with which this thesis is primarily concerned is gallstones. This disease is marked by the occurrence of calculi or concretions (gallstones) in the gallbladder or biliary passages. It is a common disease of man, ranking fifth among the causes of hospitalisation in the Unites States (56). A painful disease, it may result in considerable disability and (57),and by predisposing to other serious diseases such as obstructive jaundice, biliary cirrhosis, cholecystitis, and carcinoma of the gallbladder, may ultimately result in death (57). The incidence of gallstones is not restricted to man only. Gallstones are sometimes found in ox, horse, pig, beaver, goose, sheep, goat, monkey and dog (58). They are rarely found in the rabbit in its natural state. Gallstone disease has been recognised for centuries having been first described by Trallianus (3).

(b) Composition and Classification of Gallstones

Gallstones consist of cholesterol, pigment, carbonate, protein and calcium in varying degrees. Several classifications of gallstones have been made on the basis of their characteristic shape, size, occurrence and composition. A broad classification, based on what appears to be the predominant constituent in gallstones is as follows:-

(1) Pure Stones.

(i) Cholesterol Stones.

This type is rare and usually occurs singly, although two, three or even four in a gallbladder have been reported (8). Yellowish or grey in colour, this type of stone has a glittering appearance (due to the presence of crystallised cholesterol) and its surface is often granular or crystalline. A cross-section of the cholesterol stone shows radiating lines of crystals (cholesterol) pointing towards the centre.

(ii) Pigment Stones.

These are more common than the cholesterol stone. They are usually small and multiple, but sometimes occur singly or in two's and three's, when they are larger. In composition they contain chiefly bilirubin.

(iii) Carbonate Stones.

These stones are very rare. They are hard and may be incorporated as small spheres in a larger stone.

(2) Mixed Stones.

(i) Cholesterol-Pigment-Calcium Stones.

This is the most common type. They may occur singly or in multiples, and may be large, small, rounded of faceted. They can have either rough or smooth surfaces. In cross-section this type of stone shows radiating crystals of cholesterol mixed with concentric layers

of pigment. Sometimes they have a laminated appearance with alternating layers of cholesterol, bilirubin, calcium carbonate and mixtures of all three components.

(ii) Pigment-Calcium Stones.

These are also very common. They are small, multiple and hard with glistening irridescent or lustrous appearances. Commonly called 'mulberry' stones, they vary in shape and appear to be made up of small spheres which have coalesced giving an overall appearance of a mulberry. In composition they contain chiefly billirubin with an admixture of calcium.

(c) Etiology of Gallstone Disease and Stone Formation

The etiology of gallstone disease is largely unknown, but several theories have been proposed and discussed. It is evident from their constitution that gallstones result from the precipitation of material normally dissolved in bile in the gallbladder or extrahepatic ducts. Bile is a supersaturated solution with respect to pigment and cholesterol. The latter is kept in micellar solution with bile salts, fatty acids and phospholipids as previously described (59,60). Cholesterol solubility appears to be the most important single factor in gallstone formation. It has been observed that human bile is always saturated with cholesterol and most human gallstones have cholesterol as the chief component(58). Human gallstones can be dissolved in vitro by lecithin-sodium cholate complex and when placed in the gallbladder of a living dog, will disappear. Dog bile is low in cholesterol content and when saturated with cholesterol it no longer dissolves gallstones (61). Therefore, variation

in the balance of these cholesterol dissolving components and cholesterol towards a preponderance of the latter, can be said to be of major importance in gallstone formation (61,45).

Bile itself is a labile solution, and slight changes in its composition can affect the solubilizing complexes necessary for holding cholesterol in solution. This may lead to precipitation of either cholesterol or pigment plus an admixture of calcium salts or proteins, thus predisposing to stone formation. A lowering of the bile acid to cholesterol ratio or qualitative changes in the bile acids themselves, rendering them less effective in solubilizing biliary components, might promote gallstone formation (45).

It is therefore important to consider the circumstances in which the cholesterol-holding capacity of bile changes so that cholesterol is precipitated. The most commonly discussed factors causing changes in bile composition are infection, inflammation, stasis and metabolic disturbances. Such disturbances may be due to exogenous factors such as diet, and to endogenous factors such as certain diseases (diabetes, hypercholesterolaemia, hypothyroidism) or the physiological state of the animal as in pregnancy. These factors will be discussed separately.

(d) Causal Factors Related to Gallstone Disease

(1) Infection and Inflammation

Infection and/or inflammation as causal factors in gallstone formation based on the evidence that certain pathogenic bacteria are excreted in bile. Aerobic and anaerobic bacteria reach the liver through

the intestinal tract from the portal vein or from the general circulation via the hepatic artery and are excreted in bile. Many common strains of bacteria have been identified in human bile. Found most frequently are Escherichia coli, Bacillus spp., and Aerobacter aerogenes. The presence of pathogenic microorganisms of the coli-typhoid group in the centre of gallstones has been demonstrated repeatedly (8). The overall evidence obtained from research concerning infection as a cause of stone formation, is that clumps of dead bacteria and desquamated cells arise in the bile through the action of these pathogens (8). The clumps of dead bacteria and cells initiate stone formation either by providing a nucleus onto which cholesterol and other biliary components aggregate or adhere, or by causing inflammation of the gallbladder or biliary passages by their toxic products. Recently it has been shown using guinea pig gallbladder, that the absorption of conjugated bile acids increases when the gallbladder mucosa is injured (62). For example, the absorption of cholate was increased 2 to 3-fold in the presence of spontaneous cholelithiasis. It is possible therefore, that mucosal injury may promote gallstone formation by reducing bile salt concentrations, thus diminishing cholesterol solubility in gallbladder bile to the extent that cholesterol is precipitated.

Infection may also play a part by creating metabolic disturbances in the liver thus leading to the formation of abnormal bile acid conjugates. Infection of guinea pigs with the organism Klebsiella pneumoniae and its toxic products, by injection or parenteral administration, has been found to lead to the excretion in bile of ornithocholanic acid

(an abnormal bile acid conjugate in this animal) and the rapid formation of cholesterol and pigment precipitates in the gallbladder (63). Ornithicholanic acid and small soft gallstones have also been observed in bile of patients from which K. pneumoniae was isolated (64). The exact mechanisms through which K. pneumoniae and perhaps other microorganisms lead to the precipitation of bile constituents have not been established. Peric-Golia and Jones (63) in explaining these findings, postulate that a possible mechanism would be a metabolic derangement in the liver, perhaps in the ornithine-arginine-urea cycle, resulting in the conjugation of L-ornithine to the free bile acids. The SO_3H radical of taurine has been thought to be important in micelle formation (65). The ornithine conjugates do not possess this SO_3H group. Thus precipitation of cholesterol and pigment could follow changes in the micellar suspension of bile acids due to the presence of the abnormal ornithocholanic acids especially during concentration of bile in the gallbladder. The precipitate could serve as a nucleus for the further accumulation of pigment and cholesterol. Irritation of the gallbladder by concentrated bile may produce sufficient damage to result in increased permeability of the wall. Ostrow (62), recently observed that exposure of the gallbladder of guinea pigs to high concentrations of deoxycholic acid (10 mm) for short periods of time (10-15 minutes) or to dilute bile salt- saline-solutions for longer periods (4-6 hours) resulted in mucosal injury with subsequent increase in absorption of taurocholic acid.

Gallstones form without biliary tract infection or inflammation, so that these two factors are not necessarily a prerequisite for stone formation.

(2) Stasis

Bile stasis as a cause of gallstone formation is one of the oldest theories (8). As a contributory factor, stasis is based on the evidence that within the gallbladder, bile tends to stratify in layers (66). The gallbladder is a one duct reservoir organ in which bile undergoes an increase in viscosity which impedes emptying. Normally the gallbladder never completely empties its contents even under maximum stimulation with cholecystokinin, and situations exist where fresh hepatic bile comes into contact with old concentrated bile (66). Gallbladder bile is therefore non-homogeneous and interfaces do exist between layers of bile of different specific gravities and viscosities. In vitro work has shown that spheroliths can form at the interface of two collodial solutions or two proteinaceous solutions of different specific gravities (67). In vitro, precipitation of cholesterol has been observed to occur at the interface of fresh hepatic bile and old concentrated gallbladder bile, a situation simulating the condition of gallbladder bile (68). Such a process of precipitation of cholesterol can probably take place in gallbladder, thereby favouring the formation of spheroliths and thus providing a nucleus for stone formation.

Functional disturbances in the extra-hepatic biliary system can cause stasis of a more pronounced nature, such that there is temporary cessation of bile flow to the intestine (61). This can cause a change in the composition of bile, in the gallbladder, for when bile does not reach the intestine, bile salts cannot be absorbed by the intestine and resecreted by the liver. This results in pronounced dimunition of bile salts and other cholesterol dissolving components. This consequently

leads to decreased solubility of cholesterol and also other substances which depend on bile salts for their solubility. Conditions are thus made favourable for the precipitation of cholesterol as well as other substances and hence stone formation.

While the above evidence suggests the mechanism by which stasis may play a role in gallstone formation, other experiments have cast doubt on the importance of stasis as a causative factor. Lindelof and Van der Linden (69), studied the effect of impairing the evacuation of the gallbladder of rabbits by inducing periductal fibrosis, a condition under which the formation of gallstones has been shown to develop (105,106). While the authors did not prove that they had produced stasis, they found no difference in the tendency to stone formation between animals with and without fibrosis. Both their experimental and control animals developed gallstones at approximately the same frequency. However, as Rains points out (67), without stasis small stone "embryos" or bile grit would pass down the bile ducts and not remain in the gallbladder, so that aggregation and addition of layers around these stone "embryos" would not be possible. Stasis may indeed be a crucial factor in gallstone formation.

(3) Metabolic Disturbances-Exogenous Factors

Diet

Extensive experimental studies in animals, involving the manipulation of the dietary regime, have been carried out in an effort to identify factors responsible for gallstones formation.

Dietary induction of gallstones has been reported in hamsters (70), rabbits (71), guinea pigs (72,73),mice (74), dogs (75), and rats (76).

These studies indicate that dietary manipulation alters the chemical and physical environment in the gallbladder and bile ducts, presumably resulting in one or more of the following situations:-

(1) altered permeability of the gallbladder mucosa through the irritation and inflammation of the gallbladder wall with a consequent selective absorption of bile acids relative to cholesterol (62).

(2) decrease in bile acid production by the liver (93).

(3) alteration in the composition of various bile salts or production of abnormal bile acid conjugations less effective in solubilizing cholesterol (63).

All these situations relate to a change in the solubility relationships in bile which permit or favour the precipitation of cholesterol. In Schoenfield's and Sjovall's study on guinea pigs (73), a high cholesterol concentration in association with a lowered bile acid concentration was only found in the stone-forming groups. Recent evidence has shown human lithogenic gallbladder bile to contain a significantly smaller amount of lecithin than normal (77).

The part diet plays in gallstone formation depends in part on the species undergoing investigation. Christensen and his associates (78), were unable to induce formation in mice fed the same diet that had been used successfully in the hamster.

It has recently been reported that gallstones may be produced in dogs by a diet consisting of normal foodstuffs designed to alter the bile salt-phospholipid-cholesterol-pigment relationship (75). It was low in protein and high in sugar; it had a low content of total and unsaturated fat (to alter phospholipids and bile salts in bile), and contained added cholesterol, to saturate the cholesterol-holding capacity of dog bile. On analysis, the gallstones produced were qualitatively similar to human gallstones. This finding illustrates that gallstones can be caused not only by metabolic disease or by any pathological conditions in the hepatobiliary system, but merely by diet alone. The diet resembles the kind that might normally be encountered in the daily food intake of some individuals. Differences in gallstone incidence and race have been recognised and attributed mainly to differences in the dietary habits between the races investgated (8).

Endogenous Factors

Hypercholesterolaemia, Hypothyroidism, Obesity, Diabetes

These conditions are commonly associated with the occurrence of cholesterol or mixed stones. In all of these conditions, serum cholesterol levels tend to be high. The composition of bile does not necessarily reflect that of plasma, (in some animals, stones develop on low cholesterol diets and have no relation to blood cholesterol (107)), but studies have shown that these conditions influence the type of bile acid synthesised as well as the total production of bile acids. In hypothyroid rats, for example, the excretion of cholic and chenodeoxycholic

acids in diminished (107). The control of cholesterol metabolism is exerted by the liver and is regulated by a homeostatic mechanism. As previously discussed, a high level of circulating cholesterol suppresses cholesterol oxidation in the liver so that the excretion of bile acids is reduced. Such a change could account for the occurrence of gallstones which so often accompanies metabolic states in which hypercholesterolaemia is manifest.

(4) Age, Sex, Endocrine Function

In his studies on gallstones, Rains (8,67), points that (a) gallstone disease is essentially a disease of later life, the frequency increasing with age ,(b) in the middle years they are about twice as common in women as in men ,(c) the increase in incidence with age affects women in the child-bearing age but after the menopause, the rate of increase falls off and the incidence in males increases to such an extent that the sex difference may disappear. These facts point to differences between males and females in hormone secretion, particularly estrogens as causative factors in gallstone formation. There are several pieces of experimental evidence that bear out such a concept. Imamoglu et al (79), was able to induce gallstones in rabbits by the administration of the placental hormones, estradiol and progesterone. Tepperman's study on mice (80), showed that estradiol increased the rates of bile flow in males while adnrogens decreased the rate in females. Females showed a significantly higher rate of bile production than did the males. Not only did females tend to develop stones earlier than males, but stone formation occurred under circumstances in which large volumes

of bile were being secreted and concentrated by the gallbaldder. In another study, Pedreira and Tepperman (81) showed that the rate of stone formation when induced by a cholesterol-cholic diet was found to be significantly higher in females than in males. Further treatment of females with testosterone and of males with estrone caused a reversal of sex difference. These authors suggest that a contributory mechanism of the serum cholesterol-lowering effect of estrogens may be an increase in the rate of cholesterol secretion into the biliary tract. This hypothesis partially explains the relationship of age, sex, and hormonal status to the incidence of gallstones.

(5) Pregnancy

The increased frequency of gallstones in women who have had children suggests that pregnancy heightens the risk of stone formation. Here again hormone influence is indicated. Changes in the pattern of secretion of sex hormones during pregnancy and the possible effects of these changes are prime factors for consideration in this problem.

During the reproductive years in the non-pregnant females, the estrogen production follows a cyclic pattern in accordance with the rhythmic fluctuations of gonadotrophins. After conception, the relatively high levels of estrogen persist and increase very slowly subsequently. Not only is there hyperestrogenism, but there is also a marked elevation of ester and total cholesterol of the plasma. These two changes seem at variance with each other, for estrogens are known to exert a cholesterol-lowering effect in the plasma. No reasonable explanation has been given for this. Oliver (82), postulates that the

lipid-depressant action of estrogens is antagonised or lost during pregnancy. Estrogen administration has been observed to increase bile flow and augment the tendency of stone formation (80). The hyperestrogenism of pregnancy may play a similar role. The hypercholesterolaemia evident in pregnancy may contribute to stone formation by decreasing cholesterol oxidation and hence bile acid excretion, as previously discussed. On the other hand there may be an increase in the rate of secretion of cholesterol into the biliary tract. Cholesterol stones are alleged to be more common in multiparous women, suggesting an abnormality in the biliary excretion of lipids, particularly cholesterol (57).

An equally important factor with respect to gallstones and pregnancy is stasis. During pregnancy, there is altered smooth muscle function in the gatrointestinal and genitourinary tracts (8). Incomplete emptying of the gallbladder due to impaired muscle function could be a contributory factor in stone formation during pregnancy. A delay in evacuation of gallbladder bile in women during the last weeks of pregnancy has been observed (83). Other interesting observations regarding this problem have been noted. Potter (84), observed that the gallbladder was distended in 75% of pregnant women undergoing Caesarean section. The gallbladder wall in these cases appeared normal but the bile resembled hepatic bile in its high chloride content and low bile acid content. This observation, although opposing the concept of stasis, provides a situation which could promote gallstone formation (due to the low bile salt concentration and thus diminished cholesterol solubility). In addition, the absorptive function of the gallbladder practically

ceases during parturition (21). The hormone oxytocin, released during childbirth to contract the uterus, fully inhibits the absorptive function of the gallbladder in vitro (21). This would account for the cessation of absorption by the gallbladder during parturition.

The relationship between pregnancy and gallstones is probably related to all these processes and mechanisms operative during pregnancy and childbirth.

One aspect of gallstone formation, however, which deserves consideration but which has received little attention in the past, is the possible change in bile composition (bile salt content, cholesterol and phospholipid concentrations) which could conceivably take place during pregnancy. It is to this aspect that the present work is mainly directed. Infection inflammation, hormone imbalance and the metabolic diseases already referred to, are factors which are not necessarily related to pregnancy. While these factors may contribute to gallstone formation in general, they do not explain the direct relationship between the increased frequency of gallstones and pregnancy.

Variation in the proportion of bile acids may alter the composition of the micelles and consequently either increase or decrease their solvating capacities. As discussed previously, differencesexist among the bile acids in their ability to solubilize cholesterol. Like colloidal particles, the surface changes on the micelles are responsible for their size and stability. The latter derive mainly from their non-polar character. A bile salt micelle made up largely of trihydroxy conjugates is more stable and resistant to aggregate formation since it has a larger number of polar

groups, while one made up chiefly of dihydroxy conjugates, being less polar is larger. The trihydroxy-cholate complexes solubilize cholesterol efficiently, while the dihydroxy complexes being larger, are less efficient solubilizers of cholesterol. Thus the latter have a higher saturation ratio for cholesterol than do the trihydroxy complexes. An increase in dihydroxy conjugates or a decrease in the trihydroxy, could conceivably bring about emulsion changes which would favour the precipitation of cholesterol thus promoting gallstone formation. Studies by Sjovall (86) reveal a significantly increased concentration of dihydroxy bile acids in gallbladder bile of patients with cholelithiasis, suggesting that an emulsion change such as described above could be related to gallstone formation.

(e) Mechanism of Stone Formation

As discussed previously, the bile salts, like colloidal electrolytes, with sufficient increase in concentration, will form colloidal aggregates or micelles. At the critical micellar concentration the total amount of cholesterol dissolved is increased. A measurement of the capacity of bile salts to dissolve cholesterol is called the critical ratio between bile salt and cholesterol. If the ratio falls below the critical value by an increase in cholesterol or a decrease in bile salt concentration, cholesterol will come out of solution. Several values have been given for this critical ratio depending on the source of reference (8). Reported values range from 1: 10-12 to 1: 18.

The precise mechanism by which stones form and then grow is poorly understood. The pioneer work done on this aspect was performed by Rainey (85). Similar investigations have since been carried out (87, 88,89). Rains (8), proposes the following theory of stone formation. First, there is the appearance in bile of microspheroliths. These are defined as paracrystalline structures, liquid crystals or mesomorphs. They represent a half-way stage toward pure crystallisation of cholesterol, pigment and calcium carbonate in a colloid medium. These microspheroliths coalesce and thus increase in size. As more spheroliths are added they change into permanent radiating crystals which extend into, and absorb successive adhering spheroids. The crystalline spheroliths form a stone by further coalescence of smaller spheroliths which in turn crystallise. The radiation of crystals gradually enlarges the stone.

The appearance of spheroliths is probably due to physico - chemical changes at an interface of different surface tensions. As a result there is differential diffusion of solids out of the colloid system which carries them in solution. In bile different interfaces may exist. Some of the possible interfaces which could result in spherolith formation and coalescence are a bile/bile interface (biles of different specific gravities adjacent to each other as a result of stasis), protein/bile interface and a cholesterol/bile interface.

(vi) AIM OF RESEARCH

This research project was undertaken to investigate the composition of bile of pregnant and non-pregnant rabbits with respect to the bile acid, cholesterol and phospholipid content of both the hepatic and gallbladder bile in these animals.

Large et al (90), investigated the bile of pregnant women, and found no evidence to suggest that the composition of liver bile or gallbladder bile was altered in any way which might induce gallstones, while others have reported striking changes, such as increased cholesterol concentration and decreased bile salt concentration, in bile of pregnant women (91,92).

The composition of fistula bile of the normal rabbit has been investigated (19),but as yet no comparison of the bile acid composition of the hepatic and gallbladder biles in the normal non-pregnant rabbit and the pregnant animal, nor has a comparison been made of bile acids of hepatic and gallbladder biles in the same animal.

The rabbit does not form gallstones frequently in the natural state (19). In view of this and of the conflicting reports concerning the composition of bile from pregnant women, it was thought worthwhile to analyze and compare the composition of gallbladder and hepatic bile of pregnant and non-pregnant rabbits. Information concerning the pathogenesis of gallstone formation with respect to bile composition might thus be obtained.

CHAPTER II
METHODOLOGY

(i) INTRODUCTION

Briefly, the experimental procedure consisted of collecting the hepatic and gallbladder bile from healthy pregnant and non-pregnant rabbits. From the samples of bile, aliquots were taken for the quantitative estimation of cholesterol and lipid-phosphorous. To the remaining portions of bile, internal standards of radioactive bile acids were added. The resulting radioactive bile was chromatographed by thin-layer chromatography; the individual bile acids were subsequently eluted and either quantitatively estimated directly, or in the case of some of the conjugated bile acids, hydrolysed, rechromatographed, eluted and then estimated.

(ii) MATERIALS

^{14}C-Cholic acid (^{14}C-C), used as an internal standard in the estimation of the recovery of bile acids and in the preparation of ^{14}C-taurocholic (^{14}C-TC) and ^{14}C-glycocholic (^{14}C-GC) acids, was obtained fron New England Nuclear Corp.. It was purified before use by preparative thin-layer chromatography as described later. ^{14}C-Glycine used in the preparation of the other internal standard, ^{14}C-glycodeoxycholic acid (^{14}C-GDC), was also obtained fron New England Corp. and was used without further purification. Reagents used in the synthesis of the radioactive bile acids included dioxane (Fisher Scientific Co.) which was used directly, and tri-n-buytlamine(Eastman Kodak Ltd.) which was redistilled over BaO. Ethylchlorocarbonate (Eastman Kodak Ltd.) was redistilled. Silica gel G,

used throughout for thin-layer chromatography, was obtained from Fisher Scientific Co. Isooctane, Isopropyl ether, isopropyl alcohol, n-butanol, solvents used in the preparation of developing solvent systems for thin-layer chromatography, were all obtained from Fisher Scientific Co. and were redistilled before use. Certified glacial acetic acid (Fisher Scientific Co.) was used directly. Ehtanol and methanol, solvents for the bile acids, were also obtained from Fisher Scientific Co. and were redistilled. Other inorganic solvents, chloroform-methanol 2:1 and petroleum-ether b.p 60-80C (Fisher), were used directly.

For the quantitative determination of bile acids, sodium pyrophosphate buffer pH 9.5 was obtained from Anachaemia Ltd., nicotinamide adenine diphosphate (NAD) and its reduced form (NADH) were purchased from Sigma Chemical Co., hydrazine hydrate from British Drug Houses and β-steroid dehydrogenase from Worthington Biochemical Co.,

(iii) METHODS

(a) Thin-layer Chromatography

For thin-layer chromatography, the chromatoplates were coated with silica gel. A slurry of silica gel was prepared by mixing 30 gm. silica gel with 10% ethanol (94), in a Waring blendor for 20 seconds. Before use, the silica gel was washed twice with non-distilled methanol and finally with redistilled methanol. After the final washing, the excess methanol was drained off and the silica gel was dried for 12 hours at 100C. The slurry was spread on glass plates measuring 200 x 20 cm. 0.2.mm. thick with the aid of a commercial apparatus (95). Just before use, the plates were wiped with ethanol to facilitate even coating. The

prepared plates were then activated by drying them in an oven at 100C for 2 hours. They were then cooled and used immediately or stored over dessicant unitl ready for use.

Two solvent systems were used for the separation of the bile acids. The system, n-butanol:glacial acetic acid: water (2:1:1) (96), was used in the preparative chromatography of the radioactive bile acids. The other system consisted of isooctane:isopropyl ether:isopropanol: glacial acetic acid 2:1:1:1 (95), and was used to separate biological samples of bile. Figures 1 and 2 show the separation of some typical bile acids using these solvents. As can be seen, the n-butanol system provides good separation for the radioactive acids TC,C, GC and GDC, while the isooctane system gives good separation for the free and most of the conjugated bile acids to be encountered in the bile.

All chromatography was carried out at room temperature (23-25C) in sealed glass tanks 28.5 x 60 cm. When the isooctance system was used, the tank was lined, after pouring in the appropriate amount of solvent, with Whatman 3MM filter paper to effect equilibration, and the glass lid sealed with masking tape. In the case of the n-butanol system equilibration was not found to be necessary. Samples were applied to plates in streaks using a Hamilton microlitre syringe with the aid of a Camag Chromatocharger (Research Specialities Co.), or in spots, in which case a microlitre pipette was employed.

Plates were removed from the tanks when the moving front was 1-2 cm. from the top. The time required for this was 1-2 hours in the case of the isooctane system, and 3-4 hours when the n-butanol system was used.

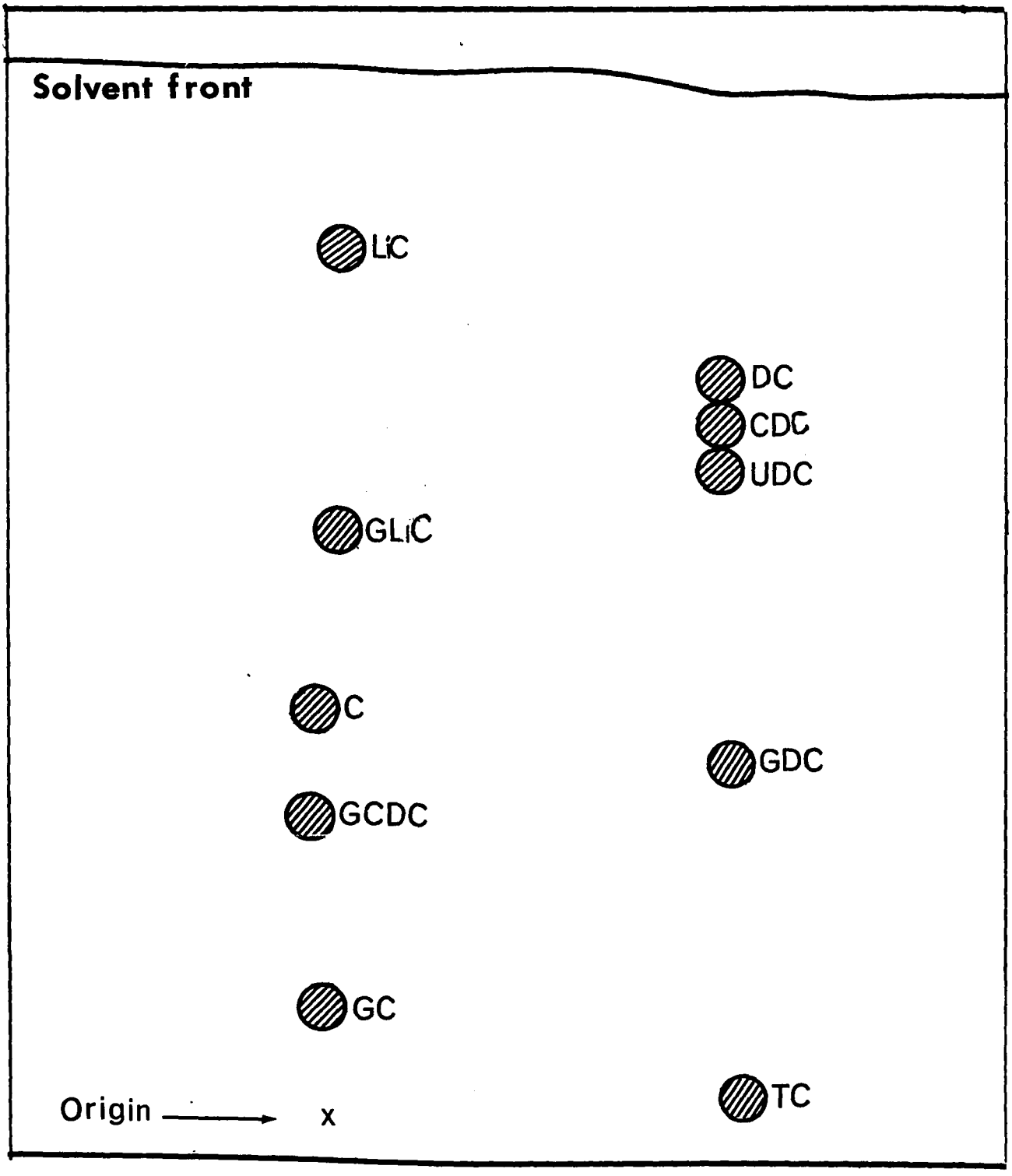

Figure 1. Thin-layer separation of bile acids using the isooctane: isopropanol: isopropyl ether: glacial acetic acid system. (For abbreviations see p. iii)

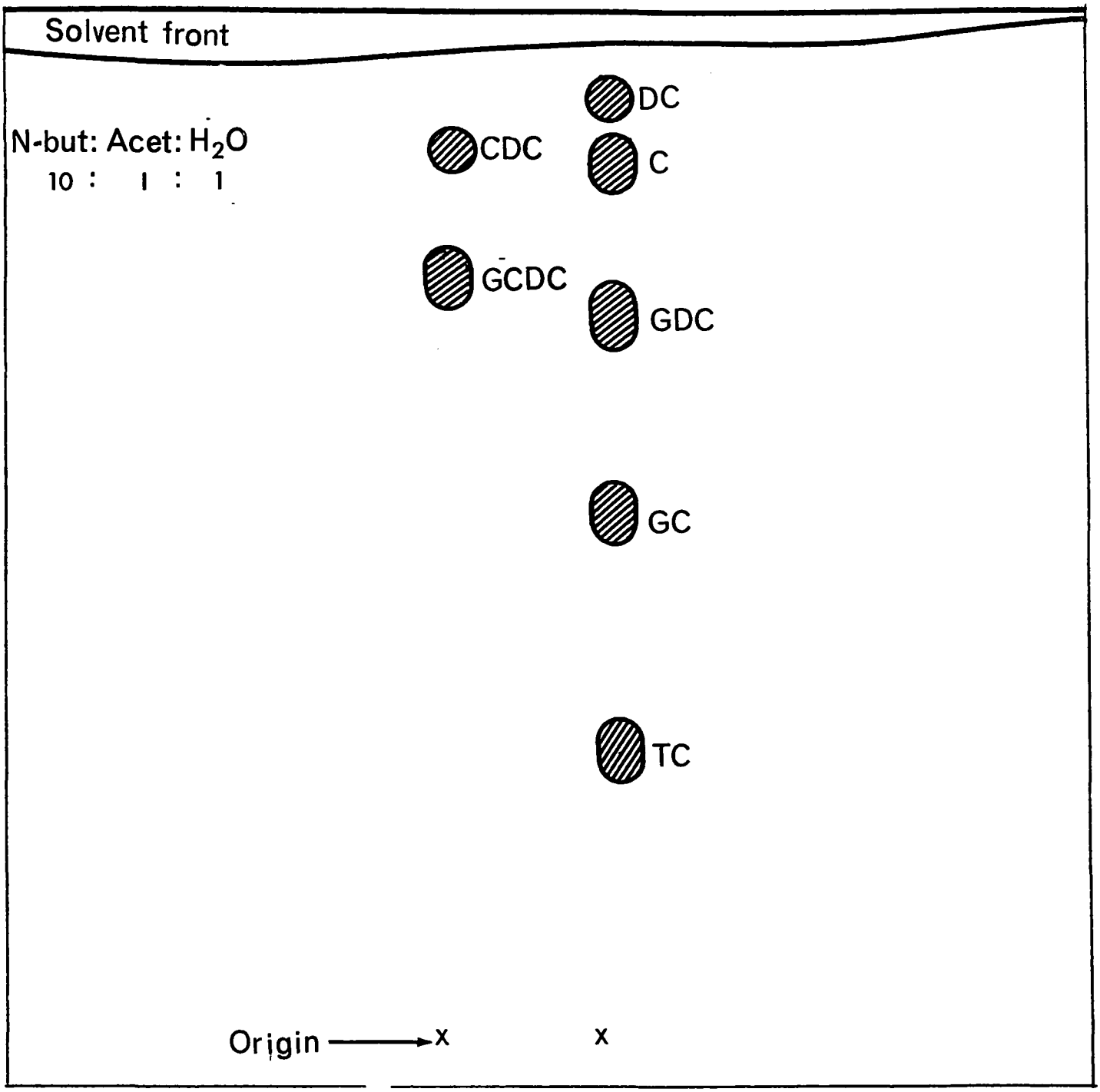

Figure 2. Thin-layer separation of bile acids in the n-butanol: glacial acetic acid: water system. (For abbreviations see p.iii)

After development and drying, the bile acids were detected by spraying with 10% phosphomolybdic acid in absolute ethanol and heating for 5-10 minutes at 100 C, when the characteristic blue spots representing the bile acids appear .

(b) Preparation of Radioactive Bile Acids

Synthesis of radioactive bile acids were carried out by the procedures of Norman (96). ^{14}C-C was tested for purity by thin-layer chromatography using the n-buanol system. The commercial ^{14}C-C revealed only one spot and therefore was used directly in the preparation of ^{14}C-TC and ^{14}C-GC acids.

Preparation of ^{14}C-GC

204.3 mg. ^{14}C-C(0.5 mmoles were added 0.12 ml (0.5 mmoles) of tri-n-butylamine and 2 ml dioxane. The mixture was cooled to 8C and 0.048 ml (0.5 mmoles) of ethylchlorocarbonate was added. After 15 minutes at this temperature, 0.55 moles of glycine in 0.55 ml N NaOH was added. The solution was then evaporated to dryness, dissolved in about 20 ml distilled water, acidified to pH 1 with concentrated HCl and extracted with ether. The ether extracts were evaported in vacuo to dryness and the residue was dissolved in a small volume of ethanol. The radioactive ethanolic bile acid solution obtained, was then purified by preparative thin-layer chromatography. This was done by applying samples of the ethanolic solution to the chromatoplates in 10 cm.-long streaks. Standards of chromatographically pure GC were spotted on either side of the plate. These were sprayed after development, and served as guides to locate the area where the ^{14}C-GC was resolved. In addition, after development, the plates were scanned by

means of a Geiger counting device to assist in locating the radioactive sample. The area giving the highest number of counts corresponded to the standard GC spots at the sides of the plate, and thus represented the exact location of the ^{14}C-GC. This area was removed from the plate by scraping the silica gel off with a sharp stainless steel blade and placing the powder into a large stoppered test tube. Several plates were similarly prepared. The silica gel powder was pooled and the ^{14}C-GC extracted from it by shaking vigorously 4 times with 25 ml of ethanol. The washings were pooled and evaporated in vacuo to a small volume. This was again tested for purity and was found to contain only ^{14}C-GC. It was then ready to be used in subsequent experiments.

Preparation of ^{14}C-TC

This acid was prepared and purified in a similar manner to ^{14}C-GC. The same proportion of reagents was used except that taurine was used instead of glycine.

Preparation of ^{14}C-GDC

In the preparation of this acid, ^{14}C-glycine was used to conjugate the deoxycholic acid (DC) since ^{14}C-DC was not readily available. 98.4 mg (0.25 mmoles) of DC and 0.06 ml (0.25 mmoles) tri-n-butylamine in 2 ml dioxane was cooled to 8C and 0.024 ml (0.25 mmoles) ethylchlorocarbonate added. After 15 minutes at this temperature, 0.275 mmoles ^{14}C-glycine in 0.275 ml N NaOH was added. After 15 minutes, the reaction mixture was evaporated to dryness in vacuo, and the residue dissolved in about 5 ml distilled water. This was acidified to pH 1 with concentrated HCl, and

the resulting precipitate was washed with water until free of HCl. The crude product was crystallised from a few ml of hot ethanol by adding water to incipient cloudiness. The cloudy mixture was stored overnight at 0C. The excess solution was poured off and the crystals were dried in a dessicator. The dried crystals were then dissolved in a small volume of ethanol. The resulting ethanolic solution was then purified as described previously.

Counting of radioactive samples

The amount of radioactivity of each sample was measured by liquid scintillation counting. The sample to be tested was placed in a glass vial of 30 ml capacity. To this was added 15 ml of a counting solution consisting of 200 gm naphthalene, 20 gm. 2,5-diphenyloxazole (PPO) and 0.5 gm. 1,4 bis (2,5)-phenyloxazolyl (POPOP) benzene in 2 litres toluene. Counting was carried out in a Nuclear Chicago liquid Scintillator Counter. Quench correction was carried out by the channels ratio method, and results were expressed in dpm.

Known volumes (100 µ l) of each of the radioactive samples were taken for counting and the following values were obtained:-

Name of bile acid	dpm/100 µl
C	2460
TC	14120
GC	7980
GDC	6980

The concentration of each acid was obtained by measuring the amount of bile acid contained in 100 µl by the enzyme method of

quantitative estimation (see p.8). In each case the O.D. obtained was less than 0.005. The least determinable amount of bile acid by this method is 5 µg, (99), an amount which gives an O.D. of 0.025, 100µl therefore of each radioactive acid contained less than 1 µg.

(c) Quantitative Estimation of Bile Acids

Various methods for the quantitative determination of bile acids have been developed. Some of these methods are based on the Pettenkofer reaction which reflects the concentration of C only (108). Conjugated acids have been estimated by the difference between the amino nitrogen before and after hydrolysis of bile. Sulfur determinations have also been carried out to determine the amount of taurine-conjugated bile acids. These methods however, have been limited to the determination of only one bile acid, the total sum of bile acids or of fractions composed of different amounts of various bile acids. Attempts have been made to determine individual bile acids in mixtures containing 2 or more bile acids. Glycine and taurine conjugates have been estimated by combining some or all of these methods. Other methods involving the determination of free bile acids in 65% sulfuric acid have been used (97).

For the present study, these methods were found to be tedious and time-consuming for the routine analysis required for the type of experiments involved. A rapid, accurate and reproducible method of quantitation was therefore sought. The method found most suitable was the enzymatic determination of bile acids (99). The principle underlying this method is the oxidation of the 3 α-or 3 β-hydroxyl group of the bile acid by the enzyme β-steroid dehydrogenase. In the reaction, the coenzyme NAD

acts as hydrogen acceptor and is reduced. The NADH formed is measured spectrophotometrically at 340 mμ . The reaction may be schematically represented thus:-

NAD + Enzyme + Bile acid → NADH + Enzyme + oxidised bile acid. Using this procedure, only bile acid and NAD react to give NADH, and therefore the measure of NADH formed is a quantitative estimate of the amount of bile acid present. Iwata and Yamasaki (99), have shown that in this system, only the 3α- or 3β-hydroxyl group is oxidised with any kind of di-or tri-OH acid or conjugated acid which has a 3α- or 3β-hydroxyl group in the molecule.

In this study the reaction mixture was modified to contain 2.0 ml of 2.5 μ moles/ml NAD in 0.1M sodium pyrophosphate buffer pH 9.5, 0.15 ml of 0.02M hydrazine hydrate, 0.5 ml of 1% hydroxy-steroid dehydrogenase (prepared by homogenising 100 mg steroid dehydrogenase with 10 ml ice-cold water with a glass homogenizer for 5 minutes and centrifuging the homogenate at 20,000g for 20 minutes), and 0.5 ml of methanolic bile acid solution. The final mixture was incubated at 40C for 30 minutes in a water bath. Readings were taken at 340 mμ with a Bausch and Lomb or Beckman Spectrophotometer in a cuvette of 1 cm light path. Samples were read against a blank containing the same proportion of ingredients but substituting methanol for the bile acid (100,101). In this proportion of reactants and under these conditions of incubation 90-100% oxidation of bile acids was achieved. The % oxidation is derived by the following simple equation:-

$$\% \text{ oxidation} = \frac{\text{NADH formed } \mu\text{moles}}{\text{Bile acid added } \mu \text{ moles}} \times 100$$

Since bile acid and NAD react in equimolar proportions,(Figure 3) the amount of NADH formed in μ moles is equivalent to the amount of bile acid present in μ moles. The latter is derived by converting the amount of NADH in mg. to μ moles by multiplying by a factor of 1.28, the molar equivalent of 1 mg. $NADH.4H_2O$. From a standard curve of $NADH.4H_2O$ versus O.D. 340 mμ, (Figure 4) the amount of NADH (mg) can be extrapolated from the O.D. reading of the sample

The technique as published by Iwata and Yamasaki (99) failed to give a spectrum when the system was assayed. Reference to previous work done with this enzyme (101) revealed that the incubation system should contain 3μ moles of hydrazine hydrate instead of 3 mmoles. Even so, under these conditions maximum oxidation was not achieved (see Table I).

In order to obtain maximum oxidation of bile acids, the concentration of NAD, incubation time and temperature was varied. Table I shows the effect of different concentrations of NAD on the % oxidation of some bile acids, while Table II shows the effect of temperature and incubation time. The % oxidation achieved with some representative bile acids in the modified incubation mixture and conditions stated above, is shown in Table III.

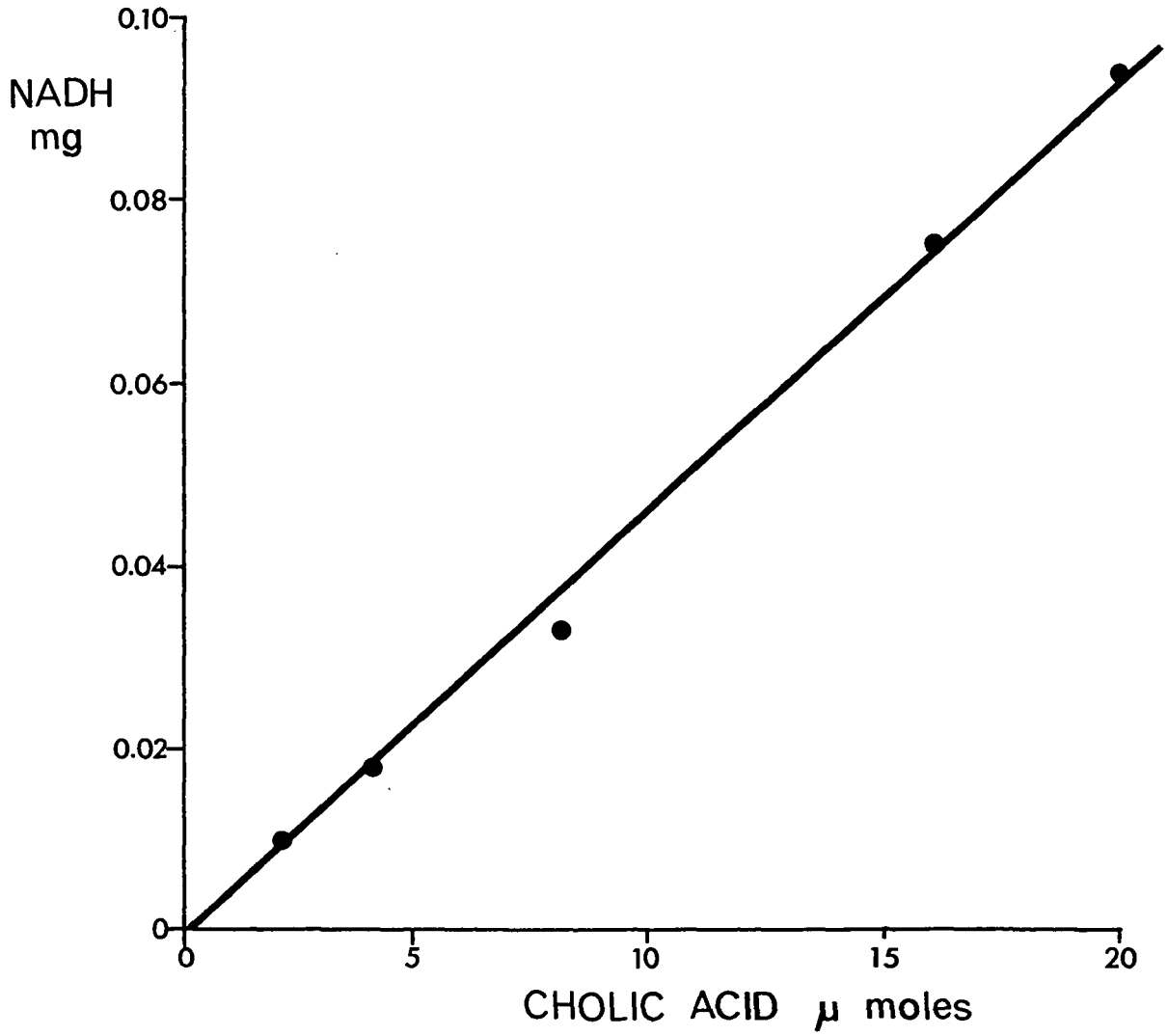

Figure 3. Linear relationship between the amount of bile acid (cholic acid) present and the amount of NADH formed, showing that bile acid and NAD react in equimolar proportions. 94-98% oxidation achieved at each concentration of bile acid.

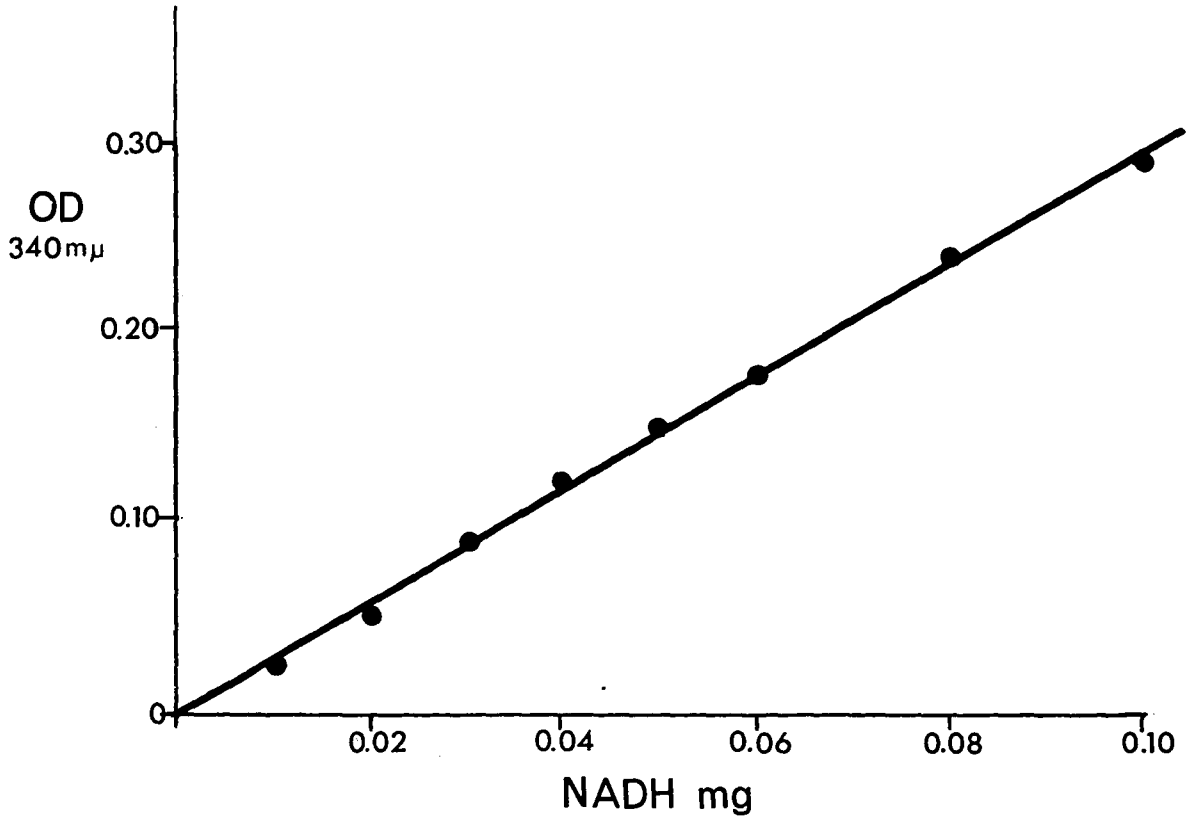

Figure 4. Typical NADH standard curve.

TABLE I

Effect of concentration of NAD on the % oxidation of Bile acid (Cholic acid)

NAD present in Incubation Mixture*	NADH (μ moles)	% oxidation
0.68 μ moles	0.0973	81.1
	0.0998	83.2
1.36 μ moles	0.1088	90.7
	0.1114	92.8
5.0 μ moles	0.1216	101.3
	0.1216	101.3

* The incubation system contained 0.5 ml of 0.24 μ moles/ml of cholic acid in methanol, 0.5 ml of 1% steroid dehydrogenase, 0.15 ml of 0.02 M hydrazine hydrate, and varying amounts of the NAD in 0.1 M sodium pyrophosphate buffer pH 9.5 as shown in the left column. Incubation time 30 min. temperature 40°C.

TABLE II

Effect of temperature and time on % oxidation of Bile Acids

Temperature (C^{o})	Time (min)	Bile Acid	% Oxidation
30	30	C	100
		GC	56.5
		GDC	52.3
35	30	C	100
		GC	73.6
		GDC	88.5
37	60	C	100
		GC	77.8
		GDC	100
40	15	C	99.2
		GC	71.5
		GDC	100
40	30	C	100
		GC	91.0
		GDC	100

Incubation system as in Table I (For abbreviations see p.iii)

TABLE III

Oxidation Quotients of Various Bile Acids

Bile Acid	O.D 340 mμ	NADH formed (μ moles)	% oxidation
C	0.230	0.119	99.2 (97.1-99.2)+
DC	0.220	0.115	95.9 (90.8-97.0)
CDC	0.210	0.109	90.8
UDC	0.205	0.108	90.0
LiC	0.205	0.108	90.0
GC	0.220	0.115	95.9
GDC	0.230	0.119	99.2 (94.9-100)
GLiC	0.210	0.109	90.8 (85.6-99.2)
TC	0.205	0.108	90.0 (85.6-95.9)
NaTC	0.210	0.109	90.8

The incubation system contained 0.12 moles of methanolic bile acid solution 5.0 moles NAD, 3.0 moles hydrazine hydrate, 5.0 mg steroid dehydrogenase. Incubation time 30 minutes, temperature 40C. (For abbreviations see p.111)

* Mean

\+ Range

(d) Examination of the validity of the enzyme method with thin-layer chromatography.

In the experimental procedure, bile acids were estimated quantitatively after they were chromatographically separated by thin-layer chromatography. Experiments were therefore carried out to ascertain that the enzyme reaction proceeded optimally after the application, development and elution of bile acids to the thin-layer plates.

Effect of Silica gel on the enzyme system.

Washing the silica gel as described previously, removes anything which might interfere with the enzyme reaction. This was proven by carrying out the following experiment.

A quantity of unwashed silica gel, representing an area of about 1 inch square of powder scraped from a plate, was placed into a test tube. The same was done with washed silica gel powder. To the powder was added 20 μ moles of C dissolved in 10 μl of methanol. The mixture was washed 3 times with about 5 ml of ethanol; the washings were pooled, centrifuged at about 13,000 rpm for 1/2 hr. The resulting supernatant was filtered through a Millipore filter (22 microns pore size) to remove the last traces of powder. The filtrate was finally evaporated to dryness under nitrogen. The residue was dissolved in 1.0 ml methanol from which 0.5 ml was taken for the enzyme determination. Samples were prepared in triplicate. Blanks for each set of samples contained 10 μl pure methanol instead of bile acid solution.

and tap water ad libitum. Prior to experimentation, they were fasted overnight and anaesthetised the next day with pentobarbital (1 ml/kg body weight) by injection through the ear vein. The abdomen was opened through a midline incision. The cystic duct was clamped and the gall-bladder bile was collected by aspiration using a syringe and a 25 G needle. The cystic duct clamp was left in place and the common bile duct was cannulated with polyethylene tubing for collection of hepatic bile over an interval of one hour. The gallbladder was then removed and in the case of the pregnant animals one or two of the foetuses were taken to confirm the stage of pregnancy. Samples of bile were either used immediately or kept frozen until ready for use.

(v) EXTRACTION PROCEDURE AND CHROMATOGRAPHY OF BILE ACIDS FROM BILE

Known volumes of bile were taken from both the gallbladder and hepatic specimens for extraction of bile acids. In order to estimate losses throughout the entire procedure internal standards of radio-active bile acids were added to the measured volumes. Measured aliquots of ^{14}C-C, ^{14}C-TC, ^{14}C-GC and ^{14}C-GDC were added to two large stoppered tubes and evaporated to dryness. To one tube was added the gallbladder bile and to the other, the hepatic bile of the same animal. At the same time, 5 µl of each of the radioactive bile acids were taken for counting. These served as controls to determine the amount of radio-activity added to the bile samples.

The samples of bile were then mixed thoroughly with the radio-active residues. The resulting radioactive bile was then freed of lipids and cholesterol by extraction with large volumes of petroleum

ether (b.p. 60-80C). The bile was then mixed with 10 volumes absolute ethanol and allowed to stand overnight. The precipitated proteins were removed by filtration and washed three times with 10 ml ethanol. The combined washings and filtrate were evaporated on a rotary vaccum and the residue taken up in a small volume of ethanol (usually 1.0-3.0 ml). From this alcoholic bile acid extract, aliquots (15-45 µl) were taken for chromatography by the method of Gregg (95) previously described. On each chromatoplate were placed two applications of a sample together with standard bile acid mixtures. After chromatographic development, the standards as well as one applied sample were stained for guidance in eluting the unstained sample. A typical chromatogram showing the separation of bile acids in a bile sample is shown in Figure 5. In this system, the bile pigment was sharply resolved between the taurine conjugates and GC, while good separation was obtained of the unconjugated bile acids as well as of GLiC and GC acids, all of which were eluted individually. Two other eluates, one containing GDC and GCDC, and the other containing unresolved taurine conjugates, were eluted and hydrolysed in order to estimate the proportion of individual acids in these eluates.

After development, appropriate areas of the powder were scraped from the plate with a stainless steel blade and the powder placed in teflon-stoppered tubes. An area of powder between C and GLiC acids was removed and treated as a blank. The taurine conjugates were eluted with absolute ethanol while the other acids were eluted with chloroform–methanol (2:1). Elution was carried out by constant shaking on an automatic

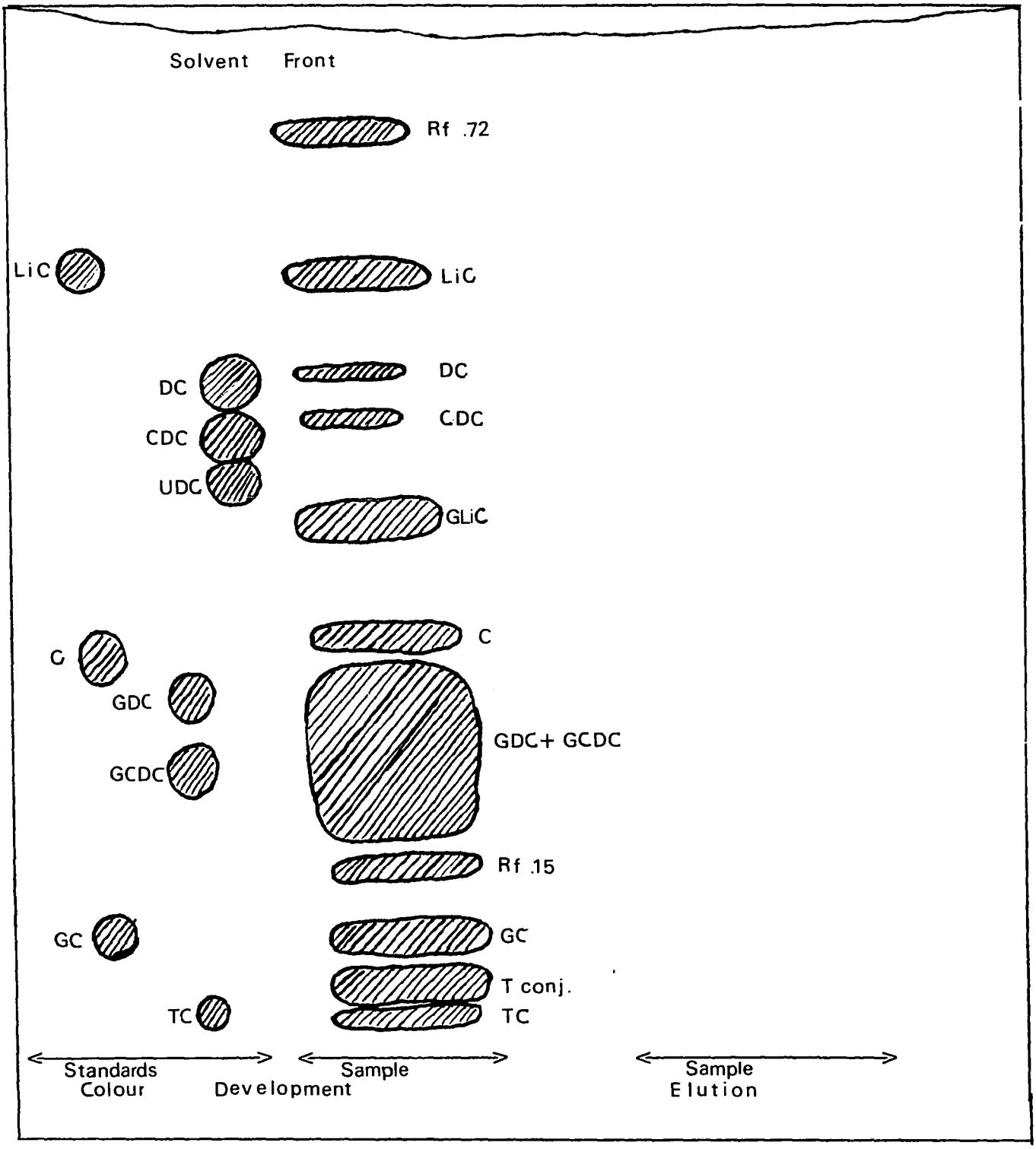

Figure 5. Typical chromatogram showing separation of a bile sample into bile acids. Separation into individual bile acids of standard bile acid mixtures is also shown. (For abbreviations see p. iii)

shaker for 16 hours at 60C. The eluates were filtered under vacuum and evaporated to dryness under N. The residues were then dissolved in appropriate volumes of methanol. From these methanolic bile acid solutions (except the GDC + GCDC and the taurine conjugates) aliquots were taken for the enzymatic determination of the bile acids. In addition, measured aliquots were taken from the C,GC, GDC + GCDC and the taurine conjugate samples for assay of radioactivity in a liquid scintillator. A mean of the dmp recovered was used to calculate the loss of bile acids in the gallbladder and hepatic bile acid samples. Table X (Chapter III) shows the mean and standard error of the % recoveries of these 4 eluates.

(a) Hydrolysis of conjugated bile acids

Known volumes from the eluates containing the taurine conjugates and the GDC + GCDC conjugates were evaporated to dryness and hydrolysed for 3 hours in 2 ml of 5N NaOH in sealed ampoules, in an autoclave at 21 lbs pressure and 255F. Each hydrolysate was diluted with an equal volume of water and acidified to pH 1 with 10 N HCl. The free bile acids were extracted with chloroform-methanol 2:1, and evaporated to dryness; the residues were dissolved in ethanol and from this, known aliquots chromatographed as before.

By this method, recovery was 83-93% after hydrolysis and the recovery after hydrolysis followed by chromatography and elution of the hydrolysates was 71-78% of the starting material,as determined by the enzyme method. This was established by performing hydrolysis experiments

with known amounts of standard conjugated bile acids as follows.500 µl of ethanolic solutions of NaTDC and GLiC acids of concentration 1µg/ml were placed in ampoules, evaporated to dryness and hydrolysed as described. After hydrolysis, samples were extracted, evaporated to dryness and 0.5 ml methanol added to the residues. Two sets of 100 µl aliquots were taken from each sample. One set of aliquots was evaporated to dryness and 0.5 ml of methanol added for the enzyme reaction, in order to determine the % hydrolysis, while the other set was applied to chromatoplates, chromatographed and eluted in the usual manner. The eluates were evaporated to dryness, 0.5 ml methanol added and the total taken for the enzyme reaction. From this result, the % recovery for the entire hydrolysis procedure can be obtained. 500 µl ethanol, taken through exactly the same procedure as the samples served as a blank. For controls, 100 µl of the same solutions of NaTDC and GLiC were placed in test tubes and evaporated to dryness under N. 0.5 ml of methanol was added to each residue and used for the enzyme reaction in order to determine the equivalent amount of NADH formed by the standard solutions used for hydrolysis. Controls were done in duplicate while samples were done in triplicate. The results are shown in Table VIII.

TABLE VIII

% Hydrolysis and % recovery of conjugated bile acids

Bile Acid	Control	Recovery after hydrolysis		Recovery after chromatography	
	µ moles	µ moles	% recovery	µ moles	% recovery
NaTDC	0.164* (0.159-0.169)+	0.133	83 (80-86)	0.125	78 (74 - 82)
GLiC	0.225 (0.223-0.228)	0.210	93 (78-108)	0.161	72 (71 - 73)

* Mean
\+ Range

(vi) MEASUREMENT OF CHOLESTEROL AND LIPID PHOSPHOROUS

Aliquots of bile were extracted on a steam bath with 40 volumes of chloroform-methanol (2:1). The mixture was then filtered and the filtrate evaporated to dryness in vacuo. The residue was dissolved in 2-3 ml of water and extracted with petroleum ether (b.p. 60-80C). The extract was made up to 50 ml from which 3 aliquots of 5.0 ml each were taken for cholesterol determination (109) and 3 aliquots of 10.0 ml each were taken for lipid-phosphorous determination (110).

Outline followed throughout the entire procedure and sample calculation of typical hepatic and gallgladder bile samples.

Female rabbit No. 14.

Volume of gallbladder bile -0.82 ml

Volume of hepatic bile -17.5 ml/hr.

Volume of gallbladder bile used for bile acid extraction -0.4 ml

Volume hepatic bile used for bile acid extraction -9.5 ml

Volume taken for cholesterol and lipid-phosphorous determinations from gallbladder and hepatic biles -0.3 and 8.0 ml. respectively

Internal ^{14}C acids added to bile aliquots:-

50 µl TC

100 µl GC

100 µl GDC

250 µl C

Amount gallbladder and hepatic extracts applied to chromatoplates -30 µl each

No. of plates chromatographed and eluted -7

Total volume gallbladder and hepatic bile acid extract eluted .210 µl each

volume methanol added to dried residues from gallbladder and hepatic eluates:-

Name of eluate	Volume methanol (ml)
Blank	2.0
Taurine conj.	1.5
GC	1.5
Rf 0.15	1.0
GDC+GCDC	10.0
C	1.5
GLiC	1.0
UDC	1.0
CDC	1.0
DC	1.0
Rf 0.72	1.0
LiC	1.0

Volume taken from methanolic bile acid extracts for counting:-

Name of extract	Volume
Blank	1.0
T conj.	0.5
GC	0.5
GDC	5.0
C	0.5

Results of counting of standard ^{14}C bile acids:-

Name and volume of bile acid	dpm
TC 50 µl	7600
GC 100 µl	7420
GDC 100 µl	5340
C 250 µl	6150

Results of counting of methanolic bile acid extracts and % recoveries:-

	Gallbladder		Hepatic	
Name of bile acid	dpm	% rec.	dpm	% rec.
T. conj.	4427	58.3	5226	68.8
GC	3370	45.4	5055	68.1
GDC	4493	84.1	4893	91.6
C	5911	96.1	4912	79.9
Mean		70.8		77.1

Results of cholesterol and lipid-phosphorous determinations

Mean O.D. of gallbladder Cholesterol sample = 0.012 ≡ 1.5 µg cholesterol

0.3 ml gallbladder bile contains 60 µg

Concentration of cholesterol in gallbladder bile = 200 µg/ml bile, or

20 mg/100 ml bile

Mean O.D. of hepatic cholesterol samples = 0.017 ≡ 2.2 µg cholesterol

8.0 ml hepatic bile contains 8.8 µg

Concentration of cholesterol in hepatic bile 88 µg

Concentration of cholesterol in hepatic bile = 11 µg/ml bile or

1.1 mg/100 ml bile.

Mean O.D. of gallbladder lipid-phosphorous sample = 0.001 ≡ 0.0025 µg P

Mean O.D. of hepatic lipid-phosphorous sample 0.019 = 0.04 µg

8.0 ml hepatic bile contains 0.2 µg phosphorous

Concentration of lipid-phosphorous in hepatic bile = 0.025 µg/ml bile.

TABLE IX

Results of Enzyme determination and corrected values of bile acids:

Gallbladder

Name of Bile acid	O.D.	NADH mg.	NADH µmoles	Total in sample µmoles	Total in extract µmoles	Corrected* Amt. µmoles	Conc. µmoles/ml
T conj.	0.070	0.026	0.033	0.099	0.942	1.62	4.05
GC	0.120	0.044	0.056	0.168	1.60	3.52	8.80
Rf 0.15	0.120	0.044	0.056	0.112	1.066	1.50	3.75
GDC+GCDC	0.390	0.157	0.188	3.760	35.795	42.56	106.40
C	0.090	0.033	0.042	0.126	1.20	1.25	3.13
GLiC	0.120	0.044	0.056	0.112	1.066	1.50	3.75
UDC	0.070	0.026	0.033	0.066	0.628	0.88	2.20
CDC	0.070	0.026	0.033	0.066	0.628	0.88	2.20
DC	0.085	0.032	0.041	0.082	0.781	1.10	2.75
Rf 0.72	0.140	0.052	0.067	0.134	1.276	1.80	4.50
LiC	0.120	0.044	0.056	0.112	1.066	1.50	3.75
Hepatic							
T conj.	0.085	0.032	0.041	0.123	1.171	1.70	0.18
GC	0.100	0.037	0.047	0.141	1.342	1.97	0.21
Rf. 0.15	0.090	0.033	0.042	0.084	0.80	1.04	0.11
GDC+GCDC	0.440	0.166	0.212	4.240	40.364	44.07	4.64
C	0.060	0.022	0.028	0.084	0.80	1.00	0.11
GLiC	0.160	0.060	0.077	0.154	1.466	1.90	0.20
UDC	0.090	0.033	0.042	0.084	0.80	1.04	0.11
CDC	0.100	0.037	0.047	0.094	0.895	1.15	0.12
DC	0.120	0.044	0.056	0.112	1.066	1.38	0.14
Rf. 0.72	0.130	0.048	0.061	0.122	1.161	1.50	0.16
LiC	0.160	0.060	0.077	0.154	1.466	1.90	0.20

CHAPTER III

RESULTS

The results presented are those obtained with 6 non-pregnant (controls) and 6 pregnant animals. The absolute concentration of the individual bile acids in the gallbladder and hepatic bile of each group of animals is shown in Table XI. These values are calculated from a mean of the radioactivity recovered from the ^{14}C bile acids in each sample . Table X shows the mean of the recoveries of the ^{14}C bile acids in the gallbladder and hepatic bile samples of each animal.

There were 4 cases, 2 samples of the controls and 2 of the pregnant in which it was not possible to obtain the proportion of the individual bile acids in the GDC-GCDC eluates. In no case was it possible to derive the proportion of the individual acids in the taurine conjugate samples, the total concentration of taurine conjugates being too small to permit this measurement. However, as a result of hydrolysis of these samples TLiC, TDC and TCDC were identified (Figure 6). Two acids, identified only by their Rf. values appeared consistently as minor components.

In order to determine whether the differences observed in the absolute concentrations of the bile acids were significant, the mean concentration of each acid was correlated in pairs between the pregnant and non-pregnant groups and an analysis of variance at 10 degrees of freedom was applied to these values. An F test was first conducted

Table X. % Recovery of Radioactivity in Bile

	Non-pregnant			Pregnant	
Animal	Gallbladder	Hepatic	Animal	Gallbladder	Hepatic
1	70.1±9.6*	51.1±3.8	1	89.6±5.8	98.4±11.3
2	67.4±8.5	79.2±6.1	2	68.7±8.8	66.3±10.0
3	70.9±11.6	77.1±5.6	3	67.2±11.5	81.3±6.9
4	74.8±7.6	79.9±5.3	4	61.9±15.5	61.3±14.1
5	78.3±14.8	97.2±13.8	5	87.3±23.5	70.9±7.5
6	80.1±7.9	70.4±11.6	6	60.2±11.2	59.7±10.5

* Mean and S.E. of ^{14}C bile acids in each bile sample.

TABLE XI Absolute concentrations (μmoles/ml) of individual bile acids in non-pregnant and pregnant animals

Non-pregnant Rabbits

Bile acid	1		2		3		4		5		6		Mean ± S.E	
	gb	hep	gb	hep	gb	hep	gb	hep	gb	hep	gb	hep	gb	hep
GC	11.97	0.94	9.80	0.45	8.80	0.21	12.38	0.44	5.40	0.70	13.31	0.34	10.27 ± 1.1	0.51 ± 0.1
GDC+GCDC	85.33	4.86	65.44	4.99	106.39	4.64	135.43	6.80	272.40	3.20	138.64	6.77	133.94 ± 2.7	5.17 ± 0.5
GLiC	8.73	0.56	7.56	0.48	3.75	0.20	2.98	0.28	12.66	0.50	10.16	0.57	7.54 ± 1.3	0.43 ± 0.04
T.Conjs.	2.53	0.28	5.92	0.09	4.05	0.18	0.05	5.80	5.80	0.19	9.36	0.16	5.31 ± 0.9	0.16 ± 0.04
C	5.70	0.48	3.44	0.19	3.18	0.11	4.15	0.09	4.68	0.24	5.56	0.33	4.44 ± 0.4	0.24 ± 0.04
UDC	1.97	0.39	3.52	0.15	2.20	0.11	3.88	0.14	5.98	0.43	2.20	0.19	3.29 ± 0.6	0.24 ± 0.04
CDC	2.10	0.27	3.72	0.09	2.20	0.12	3.40	0.03	5.64	0.22	4.33	0.25	3.56 ± 0.5	0.16 ± 0.04
DC	4.93	0.67	4.08	0.19	2.75	0.14	3.88	0.52	9.76	0.40	5.91	0.49	5.22 ± 0.9	0.40 ± 0.1
LiC	4.70	0.39	4.08	0.22	3.75	0.20	2.98	0.28	8.94	0.49	6.24	0.38	5.03 ± 0.7	0.33 ± 0.04
Rf. 0.15	22.77	0.63	2.28	0.17	3.75	0.11	1.93	0.11	8.18	0.32	4.67	0.22	3.93 ± 0.9	0.26 ± 0.1
Rf. 0.72	4.00	0.41	4.08	0.22	4.05	0.16	6.08	0.28	9.74	0.49	4.67	0.28	5.51 ± 0.8	0.31 ± 0.04
Total	134.73	9.88	113.39	7.04	145.27	6.18	181.27	9.08	348.58	7.18	205.05	9.98	188.05 ± 31.8	8.21 ± 0.6

Pregnant Rabbits

GC	9.31	0.78	23.06	0.40	18.67	1.86	17.75	0.76	8.87	0.71	14.02	0.23	15.28 ± 2.1	0.79 ± 0.2
GDC+GCDC	194.58	5.69	152.80	4.35	85.42	3.93	49.63	2.65	270.09	11.29	133.28	5.84	147.63 ± 29.3	5.63 ± 1.1
GLiC	1.62	0.67	6.60	0.28	2.76	0.61	6.30	0.65	4.82	0.71	6.70	0.35	4.80 ± 0.8	0.55 ± 0.1
T. Conjs.	6.29	0.29	9.90	0.40	9.63	0.45	8.60	0.63	4.16	0.37	13.86	0.19	8.74 ± 1.2*	0.39 ± 0.04***
C	2.29	0.20	7.14	0.04	1.41	0.28	11.02	0.37	1.61	0.31	3.58	0.14	4.51 ± 1.14	0.22 ± 0.04
UDC	2.47	0.26	6.60	0.37	0.19	0.41	2.55	0.74	2.20	0.32	3.82	0.24	2.97 ± 0.8	0.39 ± 0.1
CDC	1.36	0.58	2.96	0.56	0.14	0.14	-	0.54	1.10	0.32	2.50	0.29	1.61 ± 0.4	0.41 ± 0.7**
DC	3.53	0.58	6.34	0.35	-	0.31	0.33	0.54	2.02	0.32	3.82	0.43	3.81 ± 0.6	0.47 ± 0.04
LiC	-	0.58	14.76	0.96	2.19	0.49	4.63	0.98	2.20	0.56	9.30	0.56	6.62 ± 2.0	0.69 ± 0.1***
Rf 0.15	4.64	0.36	5.95	0.29	1.81	0.18	1.35	0.28	3.17	0.66	5.98	0.46	38.2 ± 0.7	0.37 ± 0.1
Rf 0.72	4.34	0.54	-	0.96	2.19	-	3.33	1.26	2.75	0.43	9.30	0.24	3.94 ± 0.7	0.69 ± 0.2*
Total	230.43	10.53	236.10	8.96	124.41	8.66	109.49	9.40	302.99	16.00	204.16	8.97	201.18 ± 27.3	10.42 ± 1.1

* significance of difference between values in paired samples of gallbladder and hepatic bile. gb.- gallbladder; hep. - hepatic

* $p < 0.05$

** $p < 0.02$

*** $p < 0.01$

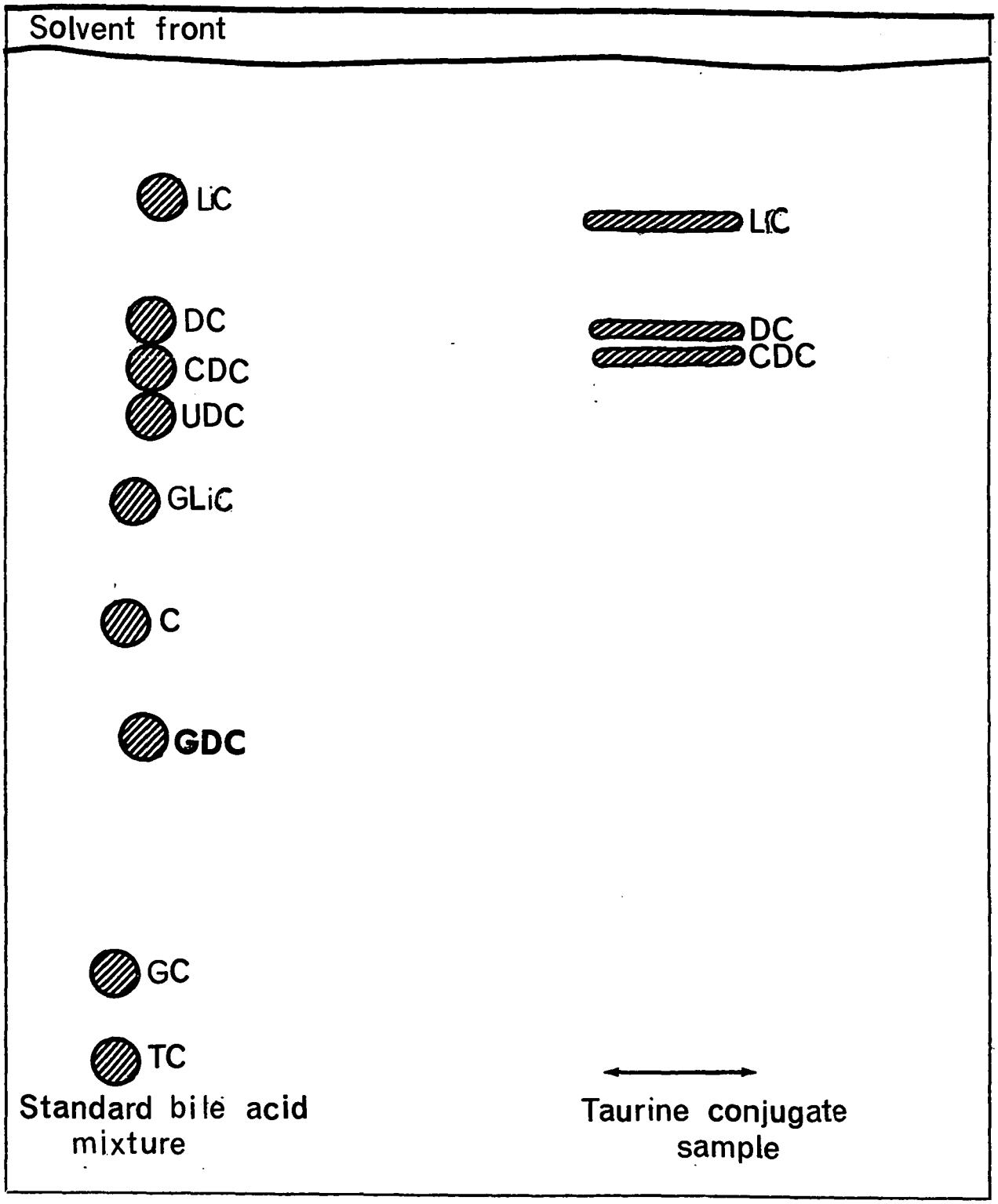

Figure 6. Chromatogram of a Hydrolysed Taurine Conjugate Sample

(For appreviation see p. iii)

on each pair of values at the 5% level of significance to determine if the variance in each paired value was poolable or not. Student's t test was then used to determine whether the 2 mean values being compared were significantly different at the stated level of significance. Significant differences were observed in a number of bile acids as shown in Table XI.

Examination of the data shows glycine conjugates to constitute the major portion of bile acids in the gallbladder and hepatic bile of both the non-pregnant and pregnant animals. Of these, GDC was present in the highest concentration in the gallbladder and hepatic bile of both groups of animals, while GCDC was the second most abundant bile acid. Among the glycine conjugates, GLiC had the lowest concentration. (Table XII). The absolute concentrations of the individual glycine conjugates were approximately the same in the hepatic biles of the pregnant and non-pregnant animals, while they were higher in the gallbladder bile of the pregnant than in the non-pregnant animals.

The total unconjugated dihydroxy bile acids (present as UDC, CDC and DC) were next in abundance in the gallbladder and hepatic bile of both groups. Of these DC was present in highest concentration in both gallbladder and hepatic samples.

C had approximately the same absolute concentration in the gallbladder and hepatic bile of the non-pregnant and pregnant animals. In the gallbladder bile of both groups, LiC was present in the same absolute concentration, but its concentration was twice as great in the hepatic bile of the pregnant animals, as in that of the non-pregnant.

Total taurine conjugates were present in small but significant amounts in all samples of bile. The absolute concentrations were higher in the gallbladder bile of the pregnant than of the non-pregnant group, but were about the same in the hepatic bile of the 2 groups.

The % distribution of bile acids (i.e. concentration of individual bile acids to total bile acids) is shown in Table XIII. This determination provides a more accurate parameter for comparison of the bile composition since the concentration of bile acids in the gallbladder depends on the length of stay in that organ and therefore on the degree of concentration, factors which are variable and uncontrollable. On statistical analysis of this data, a number of significant differences were observed in bile composition, particularly among the hepatic samples.

The % concentrations of GDC and GCDC in the 4 cases where these acids were individually determined are shown in Table XIV. No statistical differences were seen in the comparison of the paired values of these acids.

The concentrations of cholesterol and Lipid-phosphorous, the gallbladder bile volume and the bile flow rate are shown in Table XV. The mean absolute concentration of cholesterol was higher in the gallbladder

TABLE XII

Absolute Concentrations of Individual Glycine Conjugates

(μ moles/ml) in Bile

Bile Acid	Non-pregnant		Pregnant	
	Gallbladder	Hepatic	Gallbladder	Hepatic
GC	10.27	0.51	15.28	0.79
GDC	114.19	3.89	134.55	3.42
GCDC	35.69	1.49	53.14	1.08
GLiC	7.54	0.43	4.80	0.55

TABLE XIII

Bile Composition, % Distribution of Bile Acids

Bile Acid	Non-pregnant		Pregnant		
	Gallbladder	Hepatic	Gallbladder	Hepatic	
GC	6.4±1.0	6.3±1.3	9.2±2.3	8.1±2.8	
GDC+GCDC	71.2±3.2	63.3±5.3	69.7±6.1	52.0±6.1	xx
GLiC	4.3±0.8	5.3±0.7	2.7±0.7	5.3±0.9	
Total glycine conjugates	79.8±3.7	74.9±2.8	81.7±3.9	65.4±5.7	xxx
Total taurine conjugates	3.0±0.6	2.0±0.4	5.1±1.0	3.8±0.8	
C	2.6±0.4	2.8±0.6	2.9±1.5	2.2±0.5	
UDC	1.8±0.3	2.9±0.8	1.5±0.4	4.0±0.9	x
CDC	2.0±0.3	2.0±0.4	0.7±0.2	4.1±0.8	xx
DC	2.9±0.3	4.7±0.8	2.0±0.4	4.3±0.6	xxx
Total di-OH Acids	6.7±0.7	9.8±1.6	4.2±1.0	12.3±1.9	xxx
LiC	2.8±0.3	4.1±0.7	3.5±1.0	7.0±1.2	xxx
Unknown Rf 0.15	2.1±0.2	3.1±0.8	1.9±0.3	3.5±0.4	xx
Unknown Rf 0.72	3.0±0.2	3.8±0.7	2.2±0.5	6.9±2.3	

Significance of difference between values in paired samples of gallbladder and hepatic bile

x $p < 0.025$
xx $p < 0.01$
xxx $p < 0.005$

bile of the pregnant group than in that of the non-pregnant while they were about the same in the hepatic bile of both groups Lipid-phosphorous concentrations of gallbladder and hepatic bile were higher in the pregnant animals than in the non-pregnant. The volume of gallbladder bile was greater in the pregnant animals, while the bile flow rate was slightly greater in the non-pregnant. However, the only statistically significant difference seen was in the comparison of bile volumes.

TABLE XIV

GDC and GCDC in Bile (% of Total Bile Acids)

	Non-pregnant		Pregnant	
	Gallbladder	Hepatic	Gallbladder	Hepatic
GDC	54.7±2.0*	47.1±6.3	53.4±6.9	36.7±3.7
GCDC	17.7±1.8	18.6±2.6	22.5±3.5	11.6±3.3

* values are mean and S.E.

Table XV CHOLESTEROL AND LIPID-PHOSPHOROUS CONCENTRATIONS, BILE VOLUME AND BILE FLOW RATE IN NON-PREGNANT AND PREGNANT RABBITS.

	NON-PREGNANT						PREGNANT						
Animal No.	Cholesterol mg/100ml		Lipid Phosphorous µg/ml		Gallbladder bile volume ml.	Hepatic bile flow rate ml/hr.	Animal No	Cholesterol mg/100ml		Lipid Phosphorous µg/ml		Gallbladder bile volume ml.	Hepatic bile flow rate ml/hr.
	gb	hep.	gb	hep.				gb	hep.	gb	hep.		
1	82.0	1.6	<0.025	<0.025	0.55	11.0	1	72.0	4.8	1.25	0.07	1.7	12.5
2	30.0	1.5	<0.025	<0.025	0.50	8.4	2	52.0	0.8	1.7	0.17	1.1	10.4
3	20.0	1.1	<0.025	0.05	0.80	17.5	3	72.0	5.0	1.9	0.5	3.9	2.8
4	20.0	1.4	<0.025	<0.025	0.62	20.6	4	71.3	4.9	1.0	0.11	1.4	6.4
5	9.6	-	0.2	-	1.1	8.8	5	57.4	4.0	1.7	0.13	2.0	8.5
6	155.0	11.5	3.5	0.05	0.9	9.3	6	65.3	0.8	1.4	0.44	1.1	23.0
Mean ±SE	52.8±21.0	3.4±1.8	0.6±0.5	0.04±0.004	0.75±0.009	12.6±1.9		65.0±3.2	3.4±0.8	1.5±0.1	0.24±0.1	1.9±0.04*	10.6±2.6

Significance of difference between values in paired samples

* p <0.001

CHAPTER IV

DISCUSSION AND CONCLUSION

Much research has been done on various aspects of bile acid metabolism in the rabbit (19, 36, 71, 103, 104). These investigations however, have been carried out mainly on fistula bile collected for various lengths of time, after surgery. Direct comparison of bile acid composition of hepatic and gallbladder bile has not previously been reported, neither have comparisons been made between the cholesterol, lipid-phosphorous or bile acid content of non-pregnant and pregnant rabbits.

The results presented here, are in agreement with those of Gregg and Poley (19), in demonstrating that in the normal rabbit the major bile acid is GDC. Gregg and Poley (19), also demonstrated the presence of GCDC in rabbit bile. The present results confirm this finding, and in fact this acid was found to be the second most important acid quantitatively. In addition, its metabolites UDC and LiC were found at all times in both the gallbladder and hepatic bile. The relative concentration of GCDC was found to be higher in the gallbladder bile than in the hepatic bile. This was more pronounced in the pregnant animals. The increase could be accounted for by decreases in the relative concentration of GLiC, LiC and the unconjugated dihydroxy acids. That the relative concentration of GCDC was higher in the gallbladder bile than hepatic bile suggests that some GCDC may be formed

in the intestinal tract Gregg and Poley (19), detected GCDC in fistula bile despite prolonged drainage, and argue that this acid is directly synthesized by the liver and not formed by bacterial degradation. The results here provide evidence that bacterial degradation of other bile acids may indeed provide a complementary route of GCDC formation. Another possible explanation could be that the other bile acids are reabsorbed to a greater extent than GCDC, resulting in an increased absolute concentration of this acid.

The total LiC (free and conjugated) was lower in the gallbladder bile than in the hepatic bile. The explanation for this is not obvious, but it is possible that bacterial oxidation occurs in the intestinal tract. Bergstrom (32), has pointed out that intestinal microorganisms may actually incorporate bile acids such as lithocholate, or bind them so tightly that they are unavailable for reabsorption; or on the other hand deconjugation and dehydroxylation can reduce the polarity of bile acids to a point where they are less readily absorbed. Such mechanisms could accout for the lower relative concentration of LiC in gallbladder during the enterohepatic circulation of bile acids. Similarly, and evidently more so in the pregnant animals, the relative decrease in the unconjugated dihydroxy acids and the increase in glycine conjugates in going from the hepatic to gallbladder bile, suggest that some measure of conjugation may occur in the intestine. However, this finding might theoretically be due to greater selective reabsorption of unconjugated bile acids than of conjugated acids. The marked similaritities in absolute

bile acid concentrations of hepatic bile and in the relative bile acid concentrations of gallbladder bile, between non-pregnant and pregnant animals, argue against the possibility that pregnancy produces a change in bile acid composition which leads to stone formation. In addition, the lack of difference in bile flow rate between pregnant and non-pregnant animals, indicates that bile metabolism and production was unchanged during pregnancy. In mice fed a gallstone -inducing diet, an increase in bile flow rate was associated with stone formation (81). However, the volume of gallbladder bile was found to be significantly higher in the pregnant animals. This is most readily explained by stasis or incomplete emptying of the gallbladder. It is interesting to note that these observations have been considered as causative factors in the etiology of gallstones (8,66).

Although there was a somewhat higher mean cholesterol concentration in the gallbladder bile of the pregnant animals, this was not found to be statistically significant. In man, pregnancy is marked by pronounced hyperlipemia, with respect to cholesterol, total phospholipids and total lipids. Moreover, some investigators have indicated that in man, there may be a reduced concentration of cholesterol and phospholipid in bile during pregnancy (102), while others have suggested that the cause of the hyperlipemia of pregnancy in man is a change in liver function with a retention of bile (102). These findings do not seem applicable in the case of the rabbit. Even so, with the higher mean cholesterol concentration in the gallbladder bile of the pregnant animals, there was also a higher

mean lipid-phosphorous concentration in both the gallbladder and hepatic biles of these animals. The essential role of phospholipids in the solubilization of cholesterol is well known and has already been discussed (Chapter 1). Thus, even though more cholesterol may be secreted into the bile and concentrated by the gallbladder, the higher phospholipid concentration would increase the cholesterol-holding capacity of gallbladder bile.

The finding that in the pregnant animals the relative concentration of glycine-conjugated dihydroxy acids (GDC and GCDC),GLiC, LiC and total dihydroxy conjugated acids (DC,CDC, and UDC) were lower in the gallbladder bile than in the hepatic bile,is of theoretical interest, regarding the observation that the rabbit is known to be resistant to gallstones in its natural state. Studies of the macromolecular complexing of bile have shown that the polarity of DC is less than that of C and even less than that of CDC and that the lower polarity of micelles containing large amounts of DC favours sedimentation phenomena in bile and leads to stone formation (19). Therefore an increase in the relative concentration of the more non-polar dihydroxy acids especially DC should contribute to sedimendation phenomena and, hence to gallstone formation, since dihydroxy acids have a higher saturation ratio for cholesterol than the trihydroxy conjugates. In this study a decrease in the relative concentration of dihydroxy acids in the gallbladder bile of pregnant animals is observed, a situation which favours the formation of micelles more effective in solubilizing cholesterol. There has also been speculation that in the rabbit the macromolecular complexes might be expected to

contain principally DC (19), since this is the main bile acid in the rabbit. In this study, GCDC was found to be the second principal bile acid. This acid might therefore contribute to a large extent to micelle formation, and having a lower saturation ratio than DC, its contribution in the micellar complexes is likely to increase the efficiency of these complexes in solubilizing cholesterol.

An important criterion which is regarded as being of major importance in gallstone formation, is the bile acid to cholesterol ratio of gallbladder bile. Table XVI shows the bile acid to cholesterol ratios in the bile of pregnant and non-pregnant rabbits. In both the gallbladder and hepatic bile of the 2 groups, the ratios were high, far exceeding the critical ratio at which cholesterol precipitation takes place. Low bile acid to cholesterol ratios, concomitant with gallstone formation have been reported (73,93). Schoenfeld and Sjovall (193) found a high cholesterol concentration in association with a lowered bile acid concentration in groups of guinea pigs which developed stones, and confirm this as being a contributory factor in stone formation. The bile acid to cholesterol ratios were about the same in the non-pregnant and pregnant rabbits providing no further evidence that in pregnancy there is any gross alteration in the bile acid and cholesterol content of bile that might cause stone formation.

An interesting observation brought out by this study, is the marked similarity between the bile composition of rabbit gallbladder bile and that of the ox, another herbivore (58). In both these species, the gallbladder bile contains small amounts of cholesterol and phospholipids

TABLE XVI

Bile Acid /Total Cholesterol (Molar ratios) in Pregnant and non-pregnant Rabbits.

Animal No.	Non-Pregnant						Animal No.	Pregnant					
	Cholesterol μ moles/ml		Total Bile acid μ moles/ml		Bile acid cholesterol molar ratio			Cholesterol μ moles/ml		Total bile acid μ moles/ml		Bile acid cholesterol moles ratio	
	gb	hep	gb	hep	gb	hep		gb	hep	gb	hep	gb	hep
1	2.12	0.04	134.7	9.9	63.5	247.5	1	1.9	0.12	230.4	10.5	121.3	87.5
2	0.8	0.04	113.4	7.0	141.8	175.0	2	1.3	0.02	236.1	9.0	181.6	450.0
3	0.5	0.03	145.3	6.2	290.6	206.7	3	1.9	0.01	124.4	9.0	65.5	900.0
4	0.5	0.04	181.3	9.1	362.6	227.5	4	1.8	0.13	108.5	9.4	60.3	72.3
5	0.3	-	348.6	7.2	1162.0	-	5	1.5	0.40	303.0	16.0	202.0	160.0
6	4.0	0.3	205.1	10	51.3	33.3	6	17	0.02	204.2	9.0	120.1	450.0

gb- gallbladder

hep - hepatic

with moderate amounts of bile acids. Examination of the cholesterol complexing fraction of ox bile reveals small amounts of cholesterol complexed with large amounts of bile salts and phospholipids (58). One may therefore expect the macromolecular complexes of rabbit bile to be similarly composed. Cholesterol is less easily precipitated from bile containing complexes of this type, which probably accounts to some extent for the rare occurrence of gallstones in the rabbit. This situation is in direct contrast to that of the human gallbladder bile for instance, where large amounts of cholesterol is complexed with moderate amounts of bile salts and phospholipids. In this case, cholesterol is more easily precipitated, forming cholesterol-rich stones.

In conclusion, this study is in agreement with that of Large et al (90), that neither hepatic nor gallbladder bile appear to be altered during pregnancy in any way that might induce stone formation. Although experimental work done on animals may not be wholly applicable to man, the present study disputes the concept that pregnancy induces changes in bile metabolism and composition which would favour gallstone formation. Thus other mechanisms must be invoked in order to explain the association between gallstones and pregnancy. At best, one may only speculate that the mechanisms operating in the bile metabolism of rabbit, especially those in pregnancy, which make this species resistant to gallstone formation, are lacking or reduced in man.

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