https://theses.gla.ac.uk/

Theses Digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

STUDIES ON THE STEROL AND LIPID COMPOSITION OF BILE

BY

RONALD ANDREW McALLISTER

A Thesis submitted to the University of Glasgow
for the degree of Master of Science
in the Faculty of Science

University Department of Surgery,
Western Infirmary,
Glasgow.

MARCH, 1972.

ProQuest Number: 10647207

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 10647207

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ACKNOWLEDGMENTS

I should like to take this opportunity of thanking most sincerely Professor A.W. Kay for his encouragement and advice at all stages during the preparation of this thesis. I should also like to thank Professor R.M.S. Smellie for the time he spent in helpful discussion at various stages of this work. An investigation of this nature would not have been possible without the co-operation of surgical colleagues in obtaining specimens from patients under their care and for the preparation of animal models. In particular, I thank Mr. C. MacKay for his help and time spent in giving freely of his knowledge in this field. My thanks also to Mr. D. Smith, Mr. R. Quin and Mr. K. Calman, who have also helped at various stages and to Mr. G. Donald and his staff for the illustrations. Finally, I am indebted to the Technical Staff of the Department for their excellent work, and to Miss Joanna Young for her care in typing the manuscript.

ABBREVIATIONS

B.S.	Bile Salts
Chol.	Cholesterol
E.	Extinction
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacetate
NAD	Nicotinamide-Adenine Dinucleotide
nm	nanometer
n.s.	not significant
p	Probability
PLP	Phospholipids
S.D.	Standard Deviation
S.E.	Standard error of the mean
t.l.c.	Thin-layer Chromatography
Tris	Tris (hydroxymethyl) aminomethane
U.	Unit of soluble Insulin B.P.

TABLE OF CONTENTS

INTRODUCTION

Man is peculiarly susceptible to gallstone disease and as his living standards increase, so does the incidence. It has been estimated that there are 15 million people in the United States with gallstones (Ingelfinger, 1968). There is a geographical variation in the chemical composition of gallstones. In Western countries these are found to consist mainly of cholesterol with small amounts of bile pigments (Rains, 1964a). In Eastern countries, stones tend to be composed of calcium bilirubinate (Maki, Sato, Yamaguchi and Saito, 1964).

Despite much investigation, the aetiology remains obscure. There is a vast literature that abounds with hypotheses, and this has been recently reviewed by Bouchier (1968, 1971) and Small (1970). The difficulty of resolving the cause of gallstone disease appears to lie in the multiplicity of factors involved in stone formation.

In recent years, evidence has been accumulating to suggest that cholesterol gallstone formation is associated with physicochemical changes in bile, involving the three principal solids namely cholesterol, phospholipids and bile salts (Hoffman and Small, 1967; Admirand and Small, 1968). Basically the problem is why cholesterol, which is normally held in aqueous solution in bile, becomes precipitated and thereby involved in the genesis of gallstones.

Analysis of gallbladder bile shows that the water content is approximately 90 per cent, and the remainder solid material. The major solid components present are taurine and glycine conjugated bile salts,

cholesterol and phospholipids. About 60 per cent of the solids are composed of bile salts, 20 per cent phospholipid that is mainly lecithin, and 4 to 5 per cent cholesterol (Schersten, Nilsson and Cahlin, 1970). The bile acids present are cholic, chenodeoxycholic and deoxycholic, conjugated as already stated in the glyco- and tauro-forms. How this high content of cholesterol is kept in solution in bile has interested a number of investigators for the past hundred years, and Rains (1962) has reviewed some of these earlier studies. More recently Small and his colleagues have studied cholesterol solubilisation in model systems composed of the main biliary components, bile salts, lecithin, cholesterol and water. These workers (Small, Bourges and Dervichian, 1966; Hoffman and Small, 1967) have defined the role played by the conjugated bile salts and lecithin in the formation of mixed micelles with cholesterol, the mechanism whereby the sterol is held in aqueous solution in bile.

The importance of the ratio of cholesterol to bile salts plus lecithin in the solubilisation process as first reported by Isaksson (1954) has been well established (Admirand and Small, 1968). The physicochemical characteristics of aqueous mixtures of these three components at a total solid concentration of 5-20 per cent, depends upon the relative amounts of each. Thus an increase in cholesterol or a reduction in bile salts and/or lecithin would tend to result in the precipitation of cholesterol (Admirand and Small, 1968).

Physicochemical data obtained from these model systems have provided

much information on the micelles and their cholesterol-holding capacity (Small, Bourges and Dervichian, 1966; Admirand and Small, 1968). The information obtained from these in vitro studies has been of great value in elucidating the cholesterol solubilising process in bile from patients, and the physicochemical conditions which result in the deposition of cholesterol. The determination by analysis of the three main biliary components, bile salts, cholesterol and lecithin in the bile from normals and patients with gallstones, and the use of phase diagrams by Admirand and Small (1968) has aided a definition of 'abnormal' bile. This is now considered to be one that is saturated or supersaturated with cholesterol. These workers (Admirand and Small, 1968) were able to delineate bile of normal patients, and those with cholesterol gallstones by this device.

Man, apparently has the defect of having bile in which the micelles are normally already nearly saturated with cholesterol (Bouchier and Freston, 1968). The potential, additional cholesterol-holding capacity has been estimated to be 70 mg./100 ml. (Nakayama, 1969). This might explain the high incidence of cholesterol gallstone disease in countries with the so-called high standard of living, that is usually concomitant with a high consumption of carbohydrates and saturated fats. In other mammals, for example the dog, which is practically immune to gallstone disease (Nakayama and Miyake, 1966), the additional cholesterol-holding capacity of the bile has been estimated to be about 170 mg./100 ml. (Nakayama, 1969). By plotting the relative compositions of normal human and dog hepatic and gallbladder bile on the phase diagram of

Admirand and Small (1968), dog's bile falls well within the micellar zone, whereas that of humans occupies the outer part of the micellar zone (Nakayama, 1969).

Studies by Small and Rapo (1970) and Vlahcevic, Cooper Bell and Swell (1970) have demonstrated that the hepatic bile of patients with gallstones is supersaturated with cholesterol. Small and Rapo (1970) studied American Indians, and Vlahcevic et.al. (1970) United States residents, both of whom are known to have a higher incidence of gallstone disease than United Kingdom residents. There was therefore a need for further investigation and this comprises Part 2 of the present thesis, where it is shown that the hepatic bile in patients with gallstones in this country is saturated but not supersaturated with cholesterol.

Although the studies just referred to suggest that a hepatic factor is involved in the aetiology, theoretically the abnormal bile could be due to an excess of cholesterol, or a reduction in the bile salts or lecithin content. In attempting to elucidate the mechanism involved some workers have claimed that a diminished pool of bile salts occurs, consequently less bile salts are available for solubilising cholesterol. Others have claimed that interruption of the enterohepatic circulation of bile salts is the determining factor in cholesterol precipitation. Both of these will be considered. The bile salt pool is held mainly in the gallbladder. Using an isotopic dilution method, Vlahcevic, Cooper Bell, Buhac, Farrar and Swell (1970) measured the bile salt pool in normal and gallstone patients. They found that in the patients with gallstones, the pool size was 46 per cent lower than normals. Since

the bile salts are determining factors in the biliary secretion of phospholipids (Nilsson and Schersten, 1969; Nilsson, 1970), the lowered bile salt pool would also result in a diminished level of phospholipid, the two factors involved in the cholesterol solubilisation process. These workers (Vlahcevic et.al., 1970) therefore suggested that a diminished bile salt pool was an important factor in the aetiology.

On purely mechanical grounds, one could envisage that the presence of a single or multiple gallstones in the gallbladder, providing they were large enough could in themselves take up enough space and thereby reduce the pool size. This was studied by measuring the pool size in dogs before and after cholecystectomy and comprises Part 3d of the present thesis. From the data obtained it is suggested that the diminished pool size in patients with cholelithiasis may be a result and not a cause of the disease.

As detailed in a later section of this thesis (p. 52) 95 per cent or more of the bile salts delivered to the intestinal lumen are reabsorbed into the portal circulation, and returned to the liver, where they are re-excreted in the bile. This is the so-called enterohepatic circulation of bile salts, and alterations in this process predispose to changes in bile composition. The effect of interruption of the enterohepatic circulation in patients after cholecystectomy was investigated by Thureborn (1962) by draining bile from a T-tube in the common bile duct. He showed that a diminished secretion of bile salts and lecithin occurred 1-3 hours after interruption, the cholesterol

secretion remaining for the most part unchanged.

Later Small (1968) plotted Thureborn's (1962) data on triangular co-ordinates and showed that when the enterohepatic circulation of these patients was intact, the relative composition of the bile fell within the micellar zone. When, however, the enterohepatic circulation was broken as described above, within 2 hours, the hepatic bile composition was such that it fell well outside the micellar zone.

The findings of Thureborn (1962) were confirmed and extended in the studies of Nilsson and Schersten (1969). These workers showed that following interruption of the enterohepatic circulation, the changes effected in bile composition resulted in crystallisation of cholesterol. This was reversed by duodenal infusion of conjugated bile salts, with return of the bile composition to approximately normal values. This response to the infusion of bile salts indicated, as already mentioned, that bile salts in the enterohepatic circulation govern biliary secretion of phospholipids.

The studies of Dowling, Mack and Small (1969 and 1970) have placed further emphasis on the importance of the effect of interruption of the enterohepatic circulation on biliary composition. Using the rhesus monkey, these workers have shown that the biliary levels of bile salts and phospholipids fall as a result of interruption of the enterohepatic circulation, the bile becoming supersaturated with cholesterol. However after prolonged interruption, solubilisation of the sterol in the bile was again effected due to an increase in biliary phospholipids. Nilsson (1970) has suggested that this may be a possible reason why

gallstones are uncommon in monkeys.

As described in more detail in Part 3c of this thesis, during the enterohepatic circulation 95 per cent of the bile salts are reabsorbed by the ileum. In surgical operations that involve removal of this area of the bowel, the patient's enterohepatic circulation is interrupted and the pool size reduced. These patients have been shown by Heaton and Read (1969) to have a much greater incidence of gallstones. There is thus considerable evidence that interruption of the enterohepatic circulation predisposes to the formation of abnormal bile. The experimental evidence cited has been obtained under abnormal conditions. Of particular interest therefore is the suggestion that a functional interruption of the enterohepatic circulation due to delay in gallbladder emptying may take place during fasting, pregnancy and during the progesterone phase of the menstrual cycle (Schersten, Nilsson and Cahlin, 1970). This might explain the greater incidence of the disease in females.

Gallstones are thought to form within the gallbladder. Whether this organ is involved passively or actively is unknown. On the one hand it may simply serve as a cul de sac from which water is absorbed, or on the other hand it may produce changes in the chemical composition of bile such that cholesterol tends to come out of solution. It is obvious however that the rudimentary stone must be held long enough in the gallbladder for growth to take place. The mechanism of retention might be stasis (Rains, 1962; Thureborn, 1965) and/or binding of the elements of the future gallstone in the gallbladder wall (Womack, Zeppa and Irvine,

1963). These latter workers implicated mucus as the binding and nucleating agent, as did Nilsson (1970). They also demonstrated the presence of mucopolysaccharides in the nucleus and matrix of sections of stones stained with Schiff's leucofuschin.

Bouchier and Cooperband (1967) showed that considerable amounts of mucous substances, as measured by their hexosamine content, were present in the bile of patients with gallstones. It is uncertain however whether this is a secondary feature of the presence of stones. They also showed (Bouchier and Cooperband, 1967), that normal bile cohtained small amounts of mucous substances. Bouchier (1971) has made the interesting suggestion that these large glycoprotein molecules might sequester bile salts thereby reducing the amount available for cholesterol solubilisation. A reduction in the bile salts content in gallbladder bile in situ, as a result of their absorption by an inflamed gallbladder wall is suggested by the work of Andrews, Schoenheimer and Hrdina (1932). This would also result in a more lithogenic bile. That the inflammatory changes need not be due to bacteria is suggested by the work of Caldwell and Levitsky (1967) and Frey, Thorpe and Abrams (1968). The former showed that the mechanism involved in the formation of gallstones in mice fed a cholesterogenic diet was by absorption of bile salts through the inflamed gallbladder wall. The latter workers, provided evidence that gallstone formation in mice produced by dietary means resulted in both germ-free and conventional animals. In addition to the factors that have been discussed above, there are a number of physiological ones that alter the flow of bile, or delay emptying of the gallbladder. Many of

these have been defined and are well documented (Bouchier, 1968; Brooks, 1969). The hormones produced in the upper part of the gastrointestinal tract influence bile secretion. This has been shown to be increased by gastrin (Gregory and Tracy, 1964), secretin (Douglas, Watts, Jablonski and Owen, 1969) and cholecystokinin (Amer, 1969). In addition gastrin is a mild, and cholecystokinin a potent, stimulator of gallbladder contraction. Neural mechanisms are also involved. Stimulation of the vagus results in choleresis (Tanturi and Ivy, 1938), whereas vagotomy prevents it (Fletcher and Clark, 1969).

Gallstone disease has been reported to be commoner in patients who have had gastric surgery (Majoor and Suren, 1947; Krause, 1963; Lundman, Orinius and Thorsen, 1964). As discussed later, other workers have denied any association. In reviewing the extensive literature, Fletcher and Clark (1968) reached a verdict of 'not proven', a view also reached essentially by Bouchier (1970) who suggested that further study was necessary. From what was discussed in the previous chapter, it is apparent that gastric surgery might interfere with bile flow and composition. The fact that in the Professorial Unit of the Western Infirmary, Glasgow, some 200 patients per year undergo vagotomy for the treatment of duodenal ulcers, reinforces the necessity for a carefully controlled study of bile composition in such patients, backed with experiments on animal models. It was therefore proposed to study the chemical composition of the bile with particular reference to the cholesterol, bile salts and phospholipid contents, of patients before and after vagotomy, and this is reported in Part I of the present thesis.

In addition, experiments were conducted using dogs with chronic biliary fistulae. These were used to investigate the effect of vagal stimulation by insulin hypoglycaemia on the production and composition of bile.

The investigations to be reported followed four main lines of enquiry.

In Part I, a study was made of the sterol and lipid composition of bile in gallstone patients and in patients with duodenal ulcer. The duodenal ulcer patients were further studied to assess the effect of vagotomy on the composition of bile.

In Part 2, bile obtained from (1) the common bile duct, and (2) the gallbladder in patients with gallstones was studied to investigate the hypothesis that cholesterol gallstones are due to the production by the liver of bile which is supersaturated with cholesterol.

In order to elaborate the above studies, an animal model was developed. Chronic biliary fistulae were created in dogs and the following studies were made.

1. The composition of hepatic and gallbladder bile.
2. The size and circulation rate of the bile salt pool.
3. The effect of cholecystectomy on the bile salt pool size.

These sections comprised Part 3 of this thesis. In Part 4, dogs with chronic biliary fistulae were used to study the effect of insulin hypoglycaemia on bile secretion and composition.

MATERIALS AND METHODS

ANALYTICAL METHODS

The cholesterol, phospholipid, and total bile salt content of the bile samples were determined by the following procedures :-

1. CHOLESTEROL

Cholesterol was determined by a slight modification of the method of Sperry and Webb (1950). Since the sterol is present in normal and pathological human bile in the free unesterified form only (Isaksson, 1953-1954; Neiderhiser, Roth and Webster, 1966; Van der Linden and Norman, 1967), a saponification step was omitted. The same applied to canine bile (Norman, 1965). The procedure used, differed from the original Sperry and Webb (1950) method for serum cholesterol in that precipitation of proteins, and extraction of cholesterol was effected with isopropanol in place of the acetone-ethanol (1:1) used by these workers. This permitted extraction of the sterol from bile samples to be carried out at room temperature. The efficacy of this solvent for serum cholesterol has been well established (Leffler, 1959; Levine and Zak, 1964).

After removal of proteins by centrifugation, the free cholesterol in a 3 ml. aliquot of the supernatant was precipitated as the digitonide, and the cholesterol content of the purified precipitate determined by the Liebermann-Burchard reaction. Separation of the sterol as the digitonide is a necessary step in the analysis, since tauro- and glycochenodeoxycholic acids give colours in the reaction (Jones, 1968), and bilirubin also reacts, giving a colour several times more intense

than that produced by the same amount of cholesterol (Tonks, 1967).

PRECISION OF THE METHOD FOR BILE

Cholesterol for use as standard in these determinations was obtained from the British Drug Houses, and had an analytical specification according to Young and Mears (1968) and Muelling and Copeland (1967).

In day to day analysis of the bile samples, standard solutions of cholesterol in glacial acetic acid were used and, in addition, a solution of the sterol at a concentration of 0.3 mg. per 3 ml. of isopropanol was put through the procedure, and served as a control of the efficacy of the precipitation step with digitonin. Thirty-seven of these determinations were taken at random from the results obtained, and gave a mean of 0.293 mg. with a S.D. of $\pm$ 0.01109 and S.E. of $\pm$ 0.00185.

In Figure I p. 13a is shown the correlation obtained between duplicate cholesterol determinations on 103 samples of human bile, and a similar series on 35 samples of dog's bile is shown (Figure 2 p. 13b).

As will be seen from the data reported later the cholesterol content of hepatic bile from the animal models was frequently of the order of 0.2 - 0.5 m-mole/litre.

In the initial stages of the present investigation, it was felt that a more sensitive method than that of Sperry and Webb (1950) might be useful for determining cholesterol on the occasions when only small

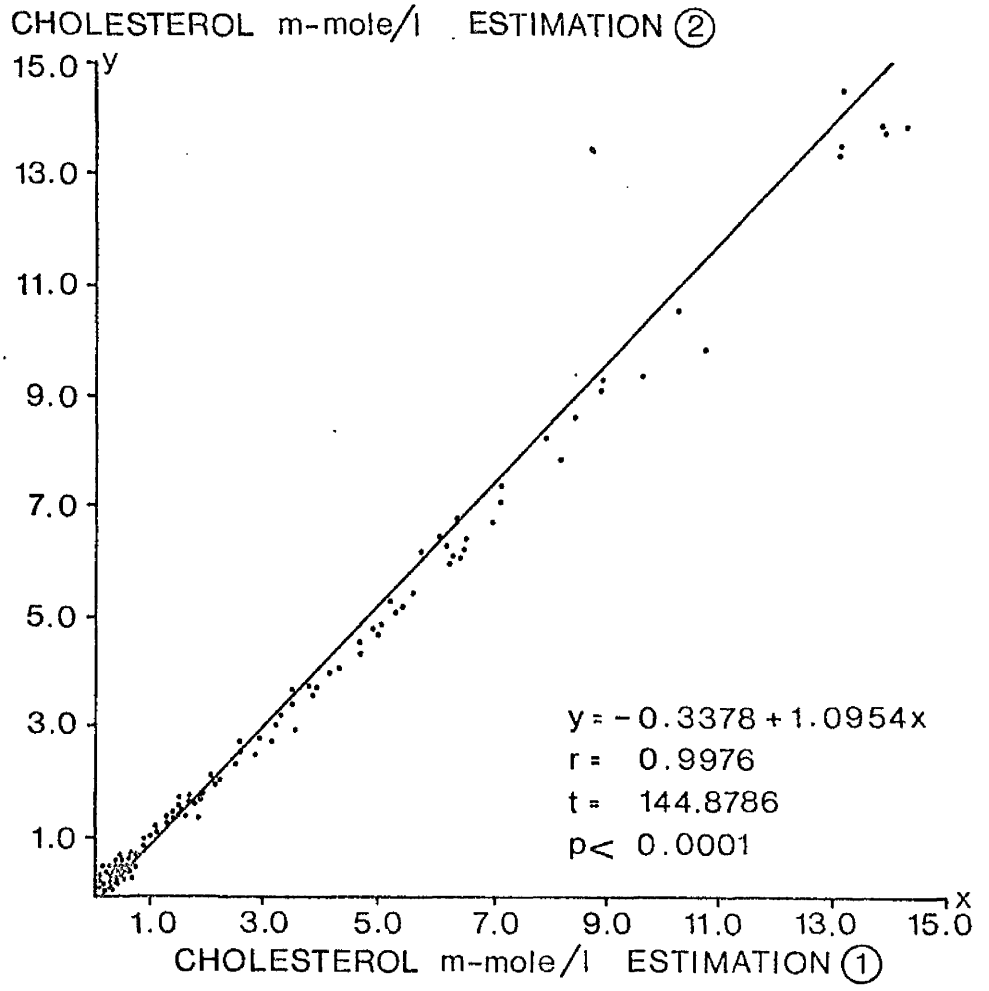


FIGURE 1.

CORRELATION OBTAINED BETWEEN DUPLICATE CHOLESTEROL DETERMINATIONS

ON 103 SAMPLES OF HUMAN BILE

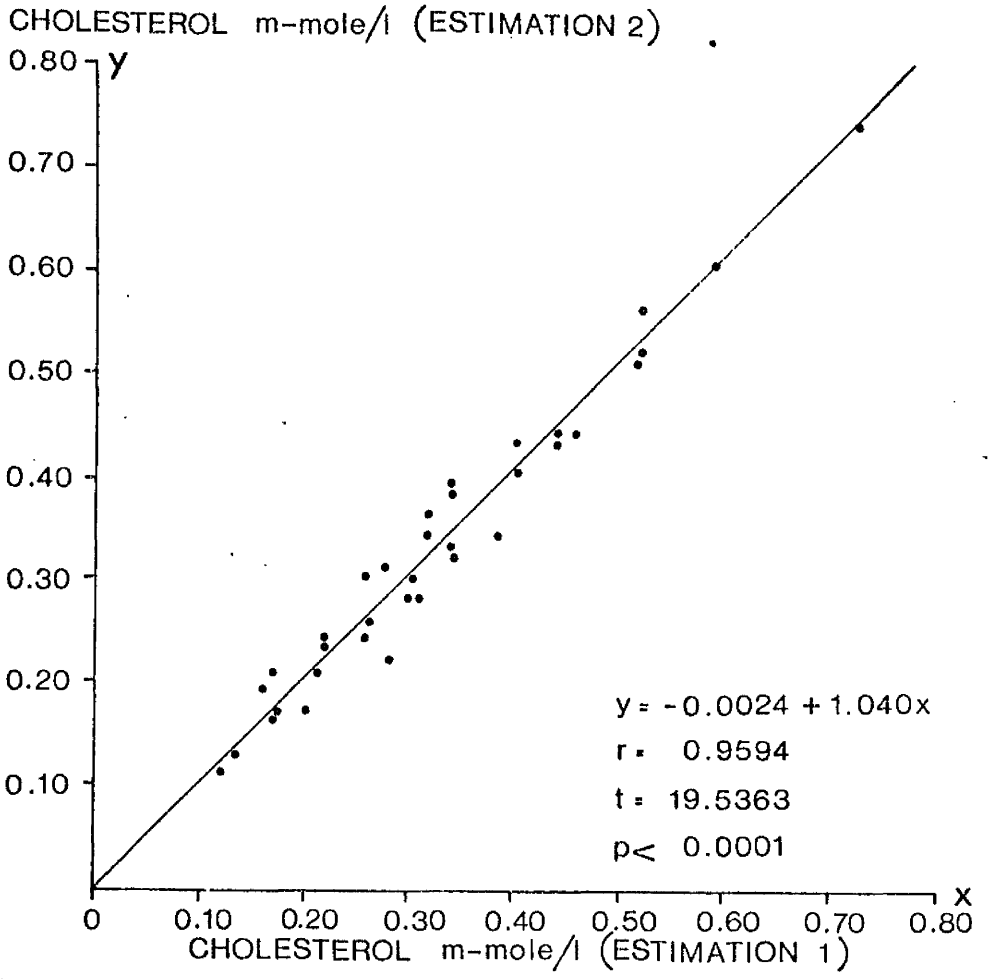


FIGURE 2.

CORRELATION OBTAINED BETWEEN DUPLICATE

CHOLESTEROL DETERMINATIONS ON 35 SAMPLES OF DOG'S BILE

amounts of human hepatic, or canine hepatic bile could be obtained.

The Kiliani (1945) reaction for cholesterol, using Fe^{3+} and sulphuric acid was known to give several times more colour with the sterol than did the Liebermann-Burchard reaction, and had been applied in a number of procedures to the determination in serum. These methods have been reviewed by Tonks (1967). Several of these, particularly the so-called direct methods, in which no prior separation of the sterol was carried out, could not be applied to bile. For instance, either bilirubin (Martinek, 1966) or bile salts (Martinek, 1966; Tonks, 1967) interfered in the reaction at the level of these compounds found in serum.

Although Brown, Zlatkis, Zak and Boyle (1954) showed that the reaction could be applied to cholesterol digitonide, the present author considered that considerable problems might arise in the application of this non-specific colour reaction to the determination in bile, particularly since it had been shown (Tonks, 1967) that different batches of isopropanol caused significant changes in the calibration curves, and the same worker had also noted a similar effect with glacial acetic acid, in the method of Zak, Luz and Fisher (1957).

In examining alternative methods, it was considered that a sensitive procedure might be developed, based on the determination of the carbohydrates in the side chain at $C_{(3)}$ in cholesterol digitonide. In Figure 3 p. 14a is shown the structural formula of digitonin. As will be seen the side chain referred to is composed of two molecules of glucose, two molecules of galactose, and one molecule of xylose.

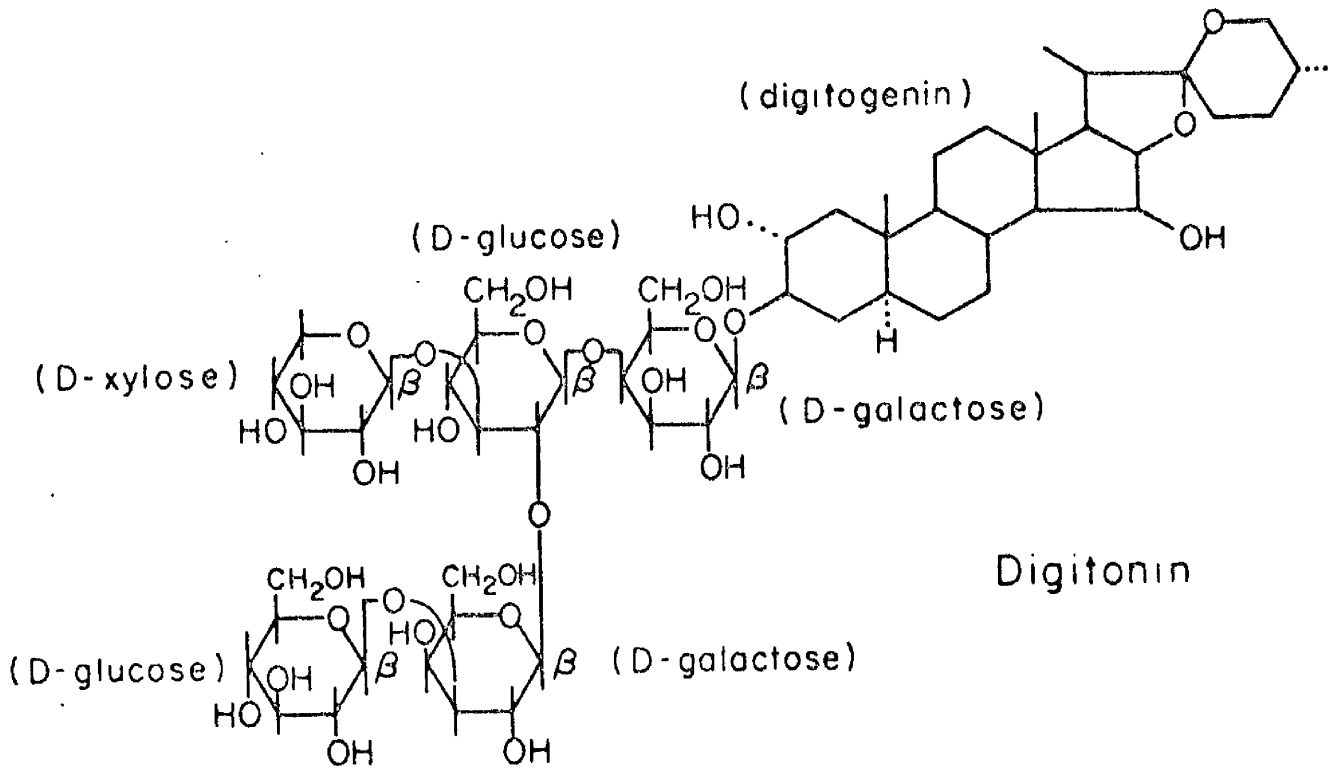


FIGURE 3.

STRUCTURAL FORMULA OF DIGITONIN

On the basis of this 4:1 ratio of hexose to pentose, it was decided to attempt to determine the hexose moieties by means of the cysteine-sulphuric acid reaction (Dische, 1947).

The literature showed that previous workers had described titrimetric or colorimetric methods for the determination of cholesterol digitonide based on the pentasaccharide nature of the side chain. Okey (1930) oxidised the complex with silver chromate and determined the excess chromate iodometrically, and Rappaport and Klapholz (1933) described a method based on the same principle. Feichtmeir and Bergerman (1953), and Zak and Zlatkis in the same year reported that the digitonide could be determined by the anthrone reaction, the carbohydrates reacting directly with the colour reagent. These colorimetric methods, and later modifications (Beher and Anthony, 1954; Jonnard and Scalera, 1956; Webster, Nichols and Chaikoff, 1959) required careful control of the unstable reagent to avoid erratic results. In 1960, Vahouny, Mayer, Roe and Treadwell improved the method by stabilising the reagent with thiourea.

Purification of the cholesterol digitonide in order to remove free digitonin, although realised to be necessary by Okey (1930) was either omitted in the earlier methods or the purification step was unsatisfactory. Webster et.al. (1959) claimed that a single reprecipitation of the digitonide from 50 per cent methanol removed all of the free saponin. Vahouny et.al. (1960) washed the precipitate twice with boiling acetone-water (1:1 v/v) a procedure found effective in the present study. Later, Vahouny, Borja, Mayer and Treadwell (1960) described a more rapid

procedure, based on the use of aluminium chloride as a gathering agent in the digitonide precipitation step, followed by removal of free digitonin by recrystallising twice from methanolic-aluminium chloride.

Although the anthrone reaction is a sensitive one, and capable of application in the present context, it seemed of interest to determine whether the cysteine-sulphuric acid reaction could be applied to the estimation of cholesterol digitonide.

DEVELOPMENT OF A PROCEDURE FOR THE DETERMINATION OF 3β-HYDROXYSTEROLS IN BILE AND TISSUE EXTRACTS

EXPERIMENTAL

Reagents:-

Glacial acetic acid, sulphuric acid, * hydrochloric acid, isopropanol, methanol, acetone, diethyl ether, digitonin, and D (+) glucose were Analar grade (British Drug Houses). Ethanol (Burroughs B.P. grade) was redistilled. D (+) xylose, D (+) galactose, and L-cysteine hydrochloride were reagent grade (British Drug Houses). Cholesterol, with the specification already referred to (p. 13) was purchased from the British Drug Houses. 5α-cholestan-3β-ol was obtained from Steraloids Limited.

*The present author (McAllister, 1971) showed that Fe^{3+} was an inhibitor of the cysteine-H_2SO_4 reaction with sedoheptulose, consequently acids used in the reaction should be low in iron.

Preparation of Cholesterol Digitonide:-

The digitonide was prepared by mixing 3 ml. of a solution of the sterol (300 µg./3 ml. isopropanol) with 1.5 ml. of digitonin (1 per cent w/v in methanol) on a vortex mixer, followed by 1.5 ml. of water. After again mixing, precipitation was effected by standing overnight in the dark. The digitonide was centrifuged down and the supernatant removed. The tube was drained over filter paper. The cholesterol digitonide was then washed free of digitonin by the method of Vahouny, Mayer, Roe and Treadwell (1960). For this purpose, 2 ml. of acetone-diethyl ether (1:2 v/v) was added, and the precipitate resuspended with a fine glass rod. The tube was then centrifuged for 10 minutes at 3000 r.p.m. and the supernatant removed. The tube was drained over filter paper, then 2 ml. of boiling acetone-water (1:1 v/v) was added. After gently mixing with a fine glass rod, the mixture was again centrifuged. The supernatant was removed, and the washing process with boiling acetone-water repeated. After again centrifuging the supernatant was removed, and the tube drained over filter paper. The precipitate was then dissolved in 1 ml. of glacial acetic acid, with the aid of heat by placing for a few minutes in a boiling water bath. 0.1 ml. of this solution was equivalent to 30 µg. cholesterol.

For studies of the reaction with free digitonin, a solution containing 159 µg. of the compound in 0.1 ml. of glacial acetic acid was used. On the basis of a 1:1 complex of cholesterol and digitonin (Windaus, 1909) this amount was equivalent to 50 µg. of cholesterol.

Standard Solution of Carbohydrates:-

This consisted of the calculated equivalents of carbohydrates present in 159 ug. of digitonin, and contained, 46.58 µg. D (+) glucose, 46.58 µg. D (+) galactose, and 19.4 µg. D (+) xylose in 0.2 ml. of water. The solution was prepared afresh by dilution of a stock solution.

Procedure

Dische (1962) has reviewed the various modifications of the cysteine-sulphuric acid reaction for carbohydrates. This depends upon the formation of furan-type aldehydes when cooled solutions of the carbohydrates are treated with strong sulphuric acid. The addition of cysteine and other thiols, results in the formation of coloured products with, in some cases, characteristic absorption spectra.

For the following experiments, the procedure of Dische and Danilchenko (1967) was used.

In glass-stoppered test tubes was placed 0.1 ml. of the solution of free digitonin (159 ug.) in glacial acetic acid, 0.12 ml. of cholesterol digitonide also in glacial acetic acid and equivalent to 36 µg. of cholesterol, and 0.2 ml. of the carbohydrate mixture. To the latter was added 0.1 ml. of glacial acetic acid. The contents of all tubes were then made to a volume of 0.5 ml. with water. A blank consisting of 0.1 ml. of glacial acetic acid was treated likewise. To each was added 0.5 ml. of 1.5N hydrochloric acid, and the tubes placed in crushed ice for ten minutes. Five ml. of 86 per cent v/v sulphuric acid in water

was then added to each tube at intervals. The contents of the tubes were shaken after 1 minute, transferred to tap water for 2 minutes, then into a boiling water bath for exactly 3 minutes. After cooling to room temperature, 0.1 ml. of cysteine hydrochloride solution (freshly prepared 3 per cent w/v in water) was added to each, the contents of the tubes vigorously shaken and allowed to stand at room temperature.

RESULTS AND DISCUSSION

Cholesterol digitonide, digitonin and the mixture of D (+) glucose, D (+) galactose and D (+) xylose all gave an intense yellow colour in the reaction similar to that described for free hexoses by Dische and Danilchenko (1967). The product of the reaction with the three solutions was unstable. Measurements of the extinction at 414 nm. showed a slow decrease until after 18 hours at room temperature the colour system stabilised, and remained so for a further 3 hours at room temperature. These changes are shown in Figure 4 p. 19a.

After 20 hours at room temperature, the solutions were transferred to cuvettes (path length, 1 cm.) and scanned over the wavelength range 350-500 nm. in a Pye-Unicam SP. 1800 recording spectrophotometer (Pye Unicam Instruments, Cambridge, England) with the blank in the reference cell.

As shown in Figure 5 p. 19b, the extinction curves almost paralleled one another in the range 380-445 nm. and gave maxima at 410-420 nm. This is in agreement with the findings of Dische (1955) for free

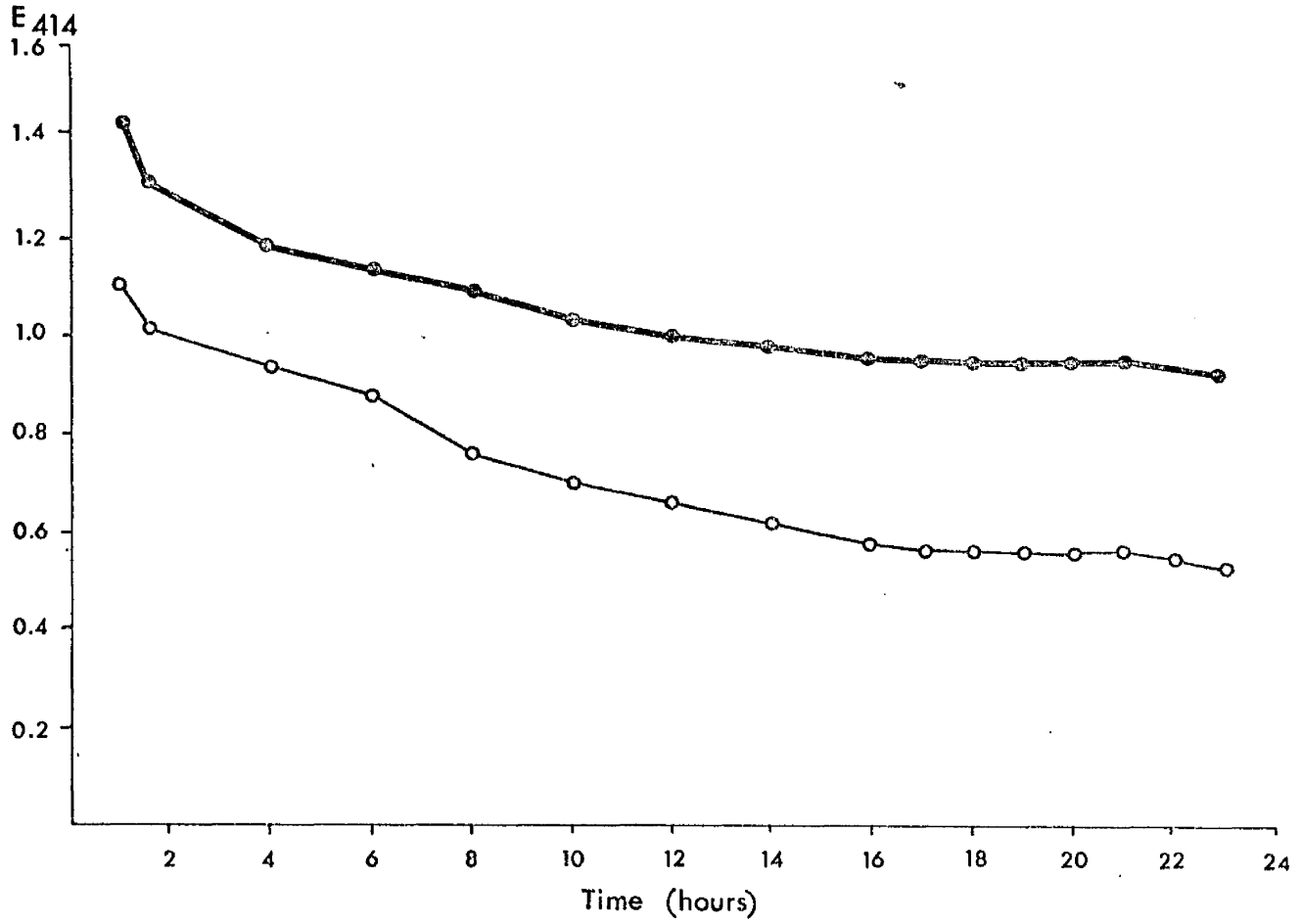


FIGURE 4.

EFFECT OF TIME OF STANDING AT ROOM TEMPERATURE

ON THE REACTION WITH 159 µg. FREE DIGITONIN (o-o-o)

AND 30 µg. OF CHOLESTEROL AS THE DIGITONIDE (o-o-o)

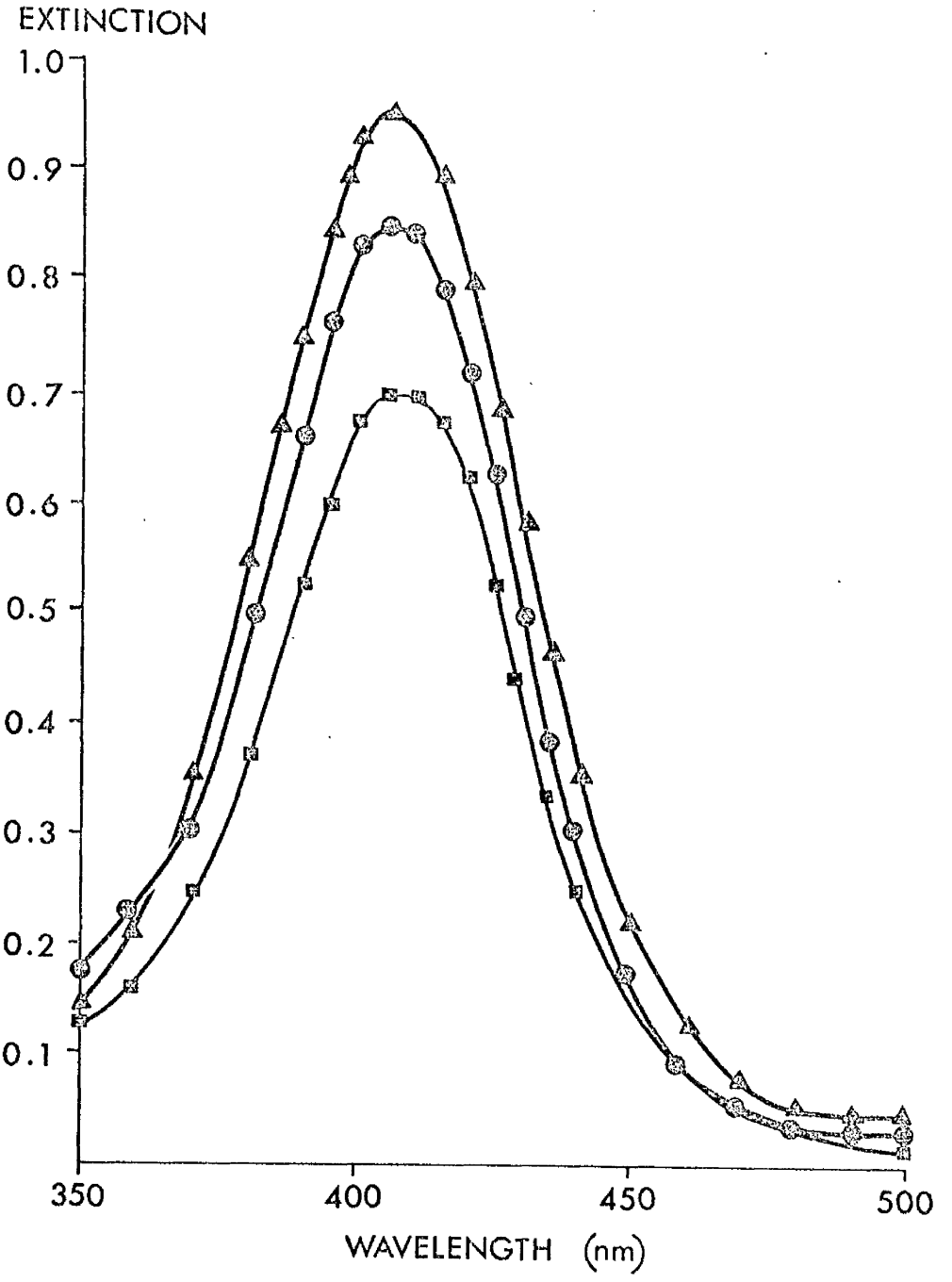


FIGURE 5.

Extinction curves of the products of the cysteine-sulphuric acid reaction with 159 µg. digitonin (▲–▲): the calculated equivalents of galactose, glucose and xylose present in 159 µg. digitonin (●—●): and cholesterol digitonide equivalent to 36 µg. cholesterol (■--■). Readings made after 20 hours at room temperature.

hexoses in an earlier version of the procedure used here (Dische and Danilchenko, 1967). Although as stated above D (+) xylose was present, the reaction conditions are such that the pentose does not react (Dische and Danilchenko, 1967). When 38.8 µg. D (+) xylose that is twice the amount present in 159 µg. of digitonin was put through the reaction the extinction at 414 nm after 20 hours at room temperature was only 0.017. It was concluded that the hexose moieties in free digitonin and cholesterol digitonide were reacting, and that the close correlation obtained with the saponin, and the equivalent amounts of the free hexoses (Figure 5 p. 19b) in the reaction indicated that cholesterol did not influence it.

When different amounts of cholesterol were taken and put through the whole procedure, there was a linear relationship between extinction and concentration up to 30 µg. of the sterol (McAllister, Crook, Smith and Mackay, 1972).

In Table I p. 20a is shown the agreement obtained with bile samples in which the cholesterol content was determined by the modified Sperry and Webb (1950) procedure and the proposed method. There was a highly significant correlation between the two methods ($r=0.999$). In general, the cysteine-sulphuric acid reaction tends to give either similar or slightly higher readings that reach the level of statistical significance (mean difference = $0.061 \pm$ S.E. 0.0229, $p < 0.02$). This might be due to the presence of saturated 3β-sterols that have been shown to be present in very small amounts in hepatic and gallbladder bile (Cook, 1958).

Since the method depends upon determination of digitonin, all

TABLE I.

COMPARISON OF RESULTS OF CHOLESTEROL DETERMINATION

BY THE MODIFIED SPERRY & WEBB PROCEDURE AND THE PROPOSED METHOD

SAMPLE	CHOLESTEROL m-mole/l.	
	LIEBERMANN-BURCHARD REACTION	CYSTEINE-H_2SO_4 REACTION
1	8.08	8.14
2	7.27	7.24
3	3.49	3.57
4	9.80	9.96
5	5.56	5.49
6	19.29	19.47
7	8.89	8.78
8	4.55	4.57
9	0.86	0.93
10	0.45	0.47
11	0.31	0.29
12	0.57	0.59
13	0.72	0.68
14	1.67	1.83
15	2.26	2.32
16	16.14	16.30
17	1.40	1.60
18	10.30	10.51
19	6.25	6.41
20	15.91	15.84
MEAN	6.19	6.25
S.E.	1.29	1.30
t value (paired t-test)	2.735	
p	< 0.02	

digitonin-precipitable sterols will react. Further since 5α-cholestan-3β-ol does not give a colour with the Liebermann-Burchard reaction, the difference between results obtained by the proposed method, and that using the Liebermann-Burchard reaction can be used as a measure of saturated 3β-sterols. The proposed method was found to be about ten times more sensitive than the Sperry and Webb (1950) method, and gave also a sixfold gain in sensitivity over methods using the anthrone reaction. Fuller details are given in the paper by McAllister et.al. (1972) included with this thesis.

2. BILE SALTS

Total conjugated bile salts were determined enzymatically using a modification by Admirand and Small (1968) of the procedure of Hurlock and Talalay (1957) for the micro-determination of steroid hormones and their metabolites having 3α, and/or 3β and 17β-hydroxyl groups in their molecule.

Bile was extracted with methanol at room temperature and the proteins removed by centrifugation. Dilutions of the samples were such that 0.1 ml. of the methanolic extract contained about 0.1 to 0.25 μmole bile acids.

For the assays, which were conducted at 24^{o}C the reaction mixture contained, 0.1 ml. extract, 1 ml. tris EDTA buffer, pH 9.5 (0.2 M tris in 0.001 M EDTA), 1.5 ml. hydrazine hydrate (approximately 1M at pH 9.5) and 0.3 ml. 0.005M NAD. The extinction at 340 nm. was measured in 1 cm. path-length cuvettes using an SP 1800 recording spectrophotometer

fitted with an automatic cell changer (Pye Unicam, Cambridge) against a blank consisting of the above reagents and 0.1 ml. methanol in place of the bile extract. The initial reaction usually stopped after less than one minute. Thereafter, 0.1 ml. of the NAD-linked hydroxysteroid dehydrogenase, containing both the 3α- and 3β (or 17β)-enzyme (EC 1.1.1.50 and EC 1.1.1.51, Worthington Biochemical Corporation Freehold New Jersey) was added. The enzyme preparation contained approximately 1 mg./ml. of the steroid dehydrogenase preparation in 0.03M tris-HCl pH 7.2 containing 10^{-3} M EDTA. 0.1 ml. was added to the test solution and the blank. The increase in extinction of 340 nm. was then recorded, with the blank in the reference cell. Depending upon the concentration of bile salts present, and the activity of the enzyme preparation, the reaction usually stopped after about 25 minutes.

Standard solutions containing 0.24 and 0.12 μmoles sodium taurocholate in 0.1 ml. methanol were assayed with each batch of the test samples. Figure 6 p. 22a shows the linearity between concentration and extinction at 340 nm. and shows the close agreement found between the calculated extinctions and those found in practice. Fifty of the values found for the 0.24 μmole standard gave a mean of 0.24 μmoles with a S.D. = 0.0055 and S.E. = 0.0008.

3. PHOSPHOLIPIDS

The major phospholipid of human bile is lecithin, with traces of lysolecithin and phosphatidyl ethanolamine (Isakkson, 1951; Phillips, 1960; Spitzer, Kyriakides and Balint, 1964). In confirming the

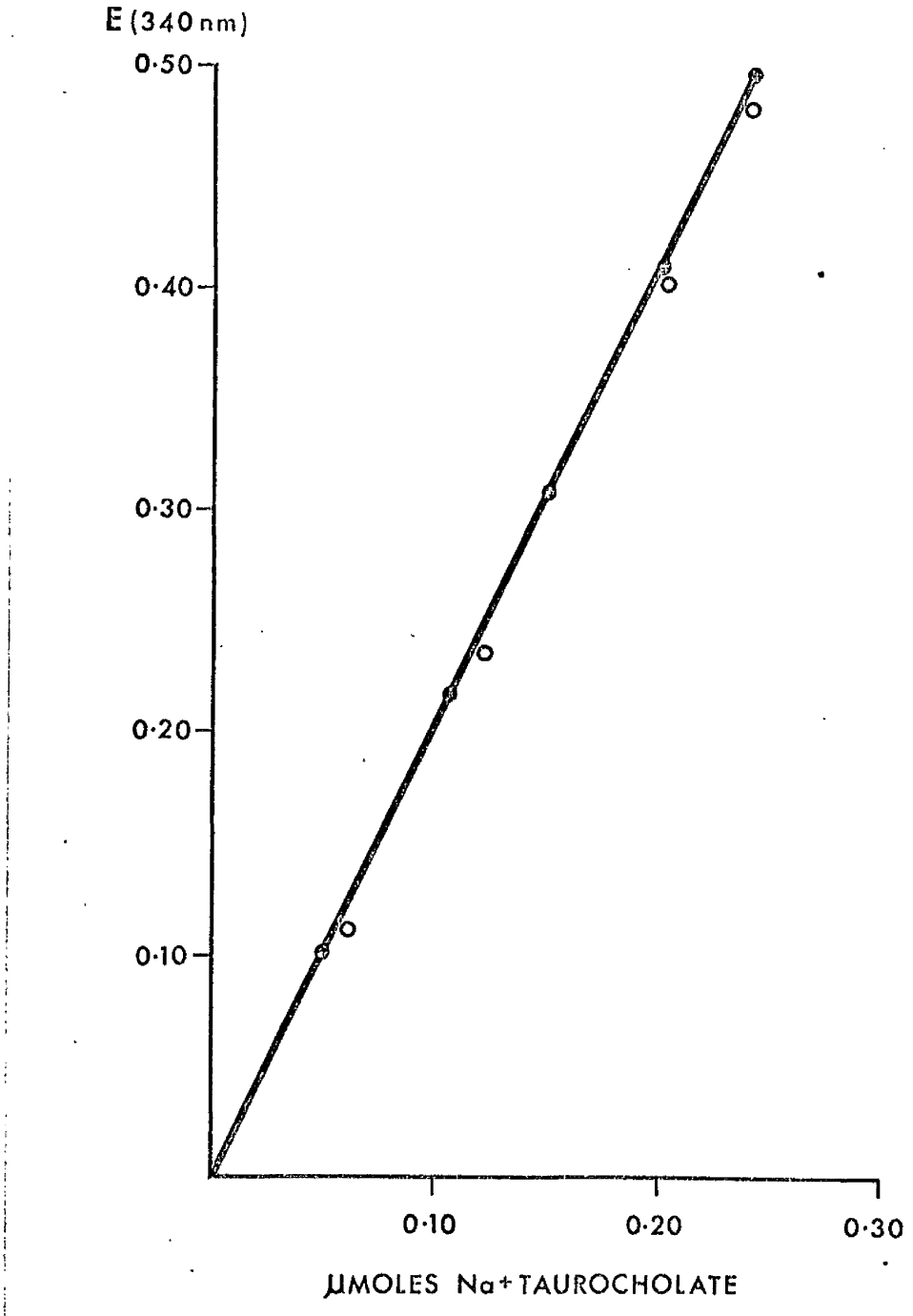


FIGURE 6.

Relationship between extinction at 340 nm. and concentration of sodium taurocholate in the hydroxysteroid dehydrogenase reaction. The calculated extinctions (o-o-o) are shown together with those obtained by measurement (•-•-•).

reports by these workers, Anderson and Bouchier (1969) showed also that lysolecithin is present in traces in lithogenic bile. In the gallbladder bile of the dog, Tompkins, Burke, Zollinger and Cornwell (1970) showed that lecithin was also the major constituent of the phospholipid fraction.

In the present study, phospholipids in human and canine bile was determined by the method of Bartlett (1959). Values in terms of phosphorus obtained by this method were multiplied by 25 to convert to terms of lecithin.

Under the conditions of the method, human and canine bile was found to contain no phosphorus, other than that extractable with lipid solvents. Eleven samples of human and four of canine gallbladder bile that had been extracted with boiling ethanol-diethyl ether (3:1 v/v) and the phosphorus determined in an aliquot of the filtrate, gave results in agreement with duplicate samples that had been diluted with water, and the phosphorus content determined on an aliquot. As shown in Table 2 p. 23a there was a highly significant correlation ($r = 0.990$) and no significant difference between the two sets of data (Mean difference = $0.804 \pm$ S.E. 0.6072 $p < 0.3$).

Precision of the Method for Bile:-

Figures 7 p. 24a and 8 p. 24b respectively show the correlation obtained in duplicate analysis of 112 human and 57 canine bile samples. As a check on the precision of the analyses, at weekly intervals samples of a control serum (Baxter Laboratories, London) with a known lecithin

TABLE 2.

THE LECITHIN CONTENT OF GALLBLADDER BILE AS DETERMINED WITH AND WITHOUT EXTRACTION BY LIPID SOLVENTS

Results in terms of m-moles/litre

	SAMPLE	EXTRACTED	UNEXTRACTED
HUMAN	A	23.24	21.36
	B	27.12	32.87
	C	21.78	20.94
	D	17.90	18.43
	E	18.74	18.11
	F	19.37	18.05
	G	50.52	53.29
	H	24.40	27.22
	I	39.37	42.40
	J	57.01	58.24
	K	7.24	6.91
DOG	A	8.68	9.18
	B	25.61	22.56
	C	11.56	13.62
	D	36.72	38.14
	MEAN	25.95	26.75
	S.E.	3.85	4.09
	t value (paired t-test)	1.324	
	p	< 0.3	

content were extracted with ethanol-diethyl ether (3:1 v/v) by a standard procedure (Varley, 1964). The phosphorus content of aliquots of the filtrate were then analysed by the method used for bile samples. Results were entered in a quality control system, and served as a check on reagents, the digestion process and other parameters.

4. ANALYSIS OF GALLSTONES

Gallstones removed at operation were washed with water, and dried for several days on filter paper in an oven at 37°C. The physical characteristics and type according to Rains (1964b) were noted. If the stone (or stones) was sufficiently large it was divided in half. If a number of stones were present, a representative sample was taken, or more than one from those analysed. For this purpose, the stone or part of the stone was ground in a pestle and mortar, and approximately 70 mg. placed in a weighed centrifuge tube. The exact weight of the sample was then determined. Ten ml. of chloroform-methanol (2:1 v/v) was added, the tube well shaken, then allowed to stand for about 1 hour at room temperature with intermittent shaking. The contents of the tube were then centrifuged, and the supernatant removed. The residue was then heated at 85°C to constant weight. The cholesterol was determined on an aliquot of the extract. From the data obtained values for 'Total-chloroform-methanol soluble material'; cholesterol and 'Insoluble material' were calculated and expressed as a percentage.

In some instances, particularly where the cholesterol content was

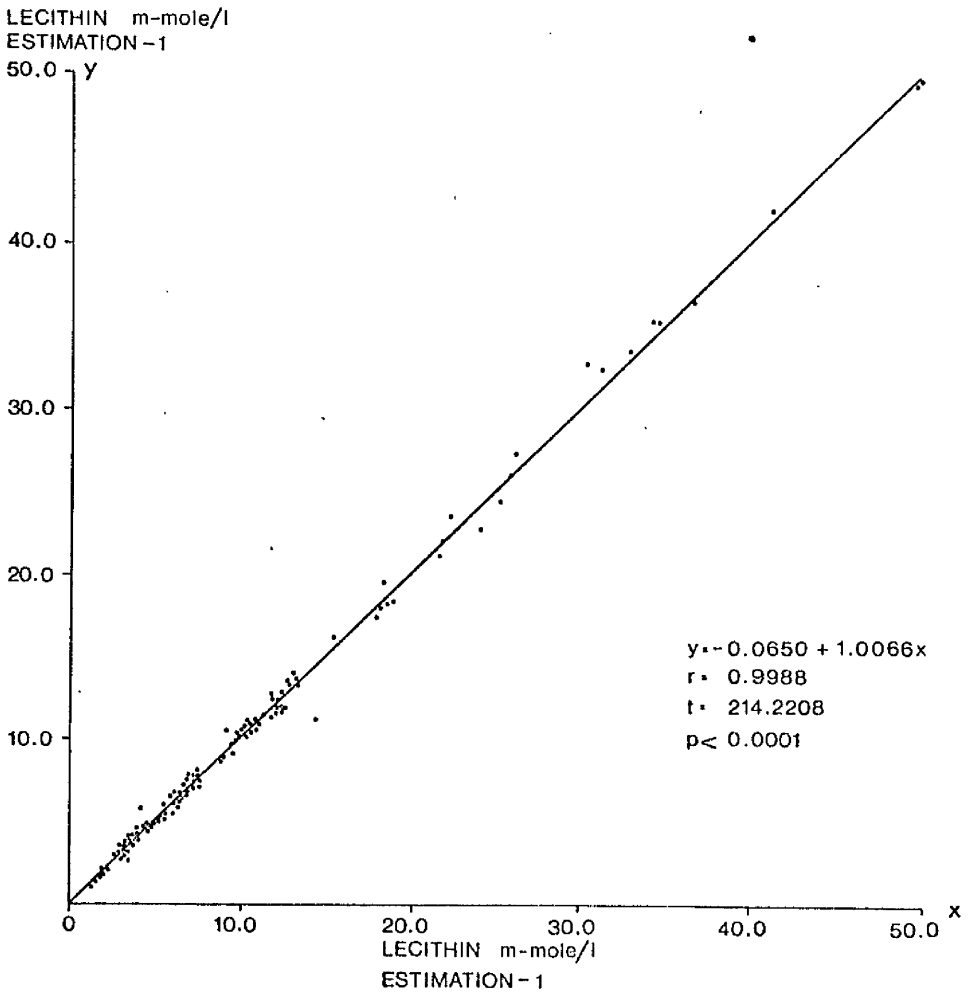


FIGURE 7.

CORRELATION OBTAINED IN DUPLICATE DETERMINATIONS OF THE LECITHIN CONTENT OF 112 SAMPLES OF HUMAN BILE

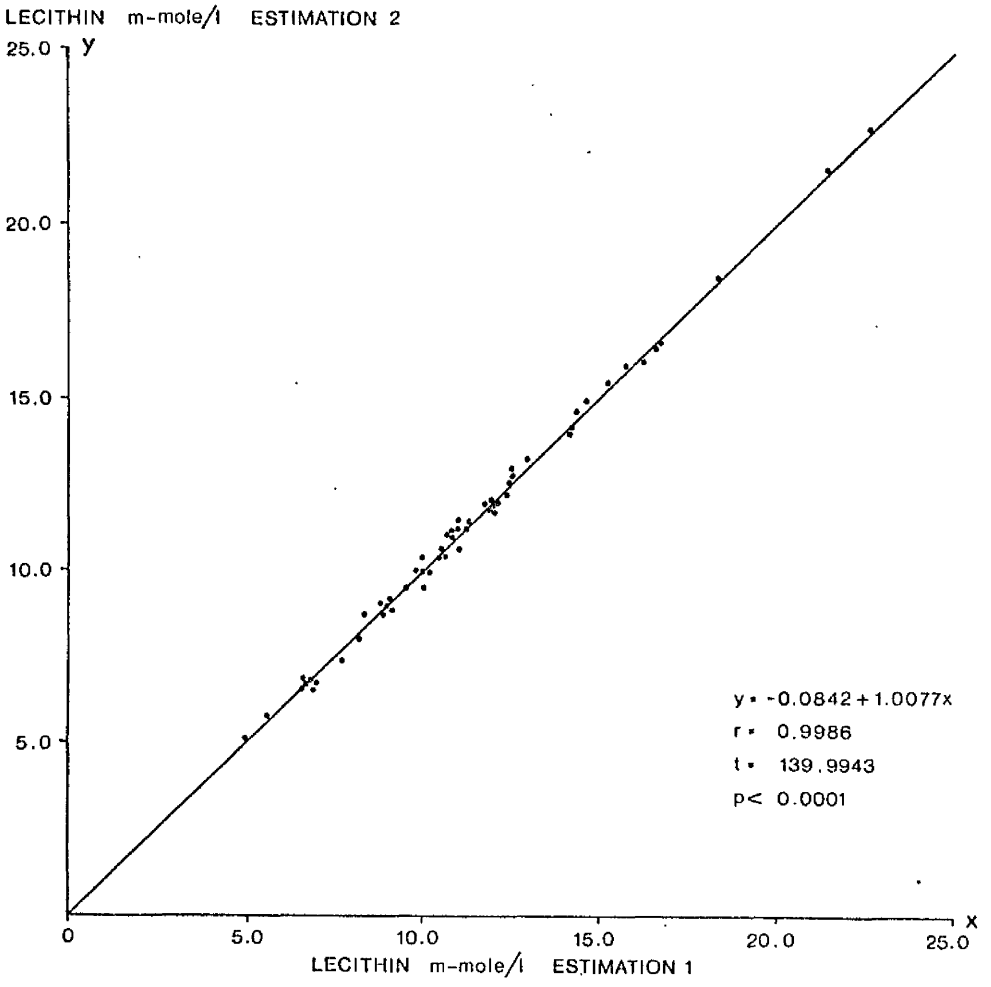


FIGURE 8.

CORRELATION OBTAINED IN DUPLICATE DETERMINATIONS

OF THE LECITHIN CONTENT OF 57 SAMPLES OF CANINE BILE

low, or the one rare occasion where stones of a different type were found in the same gallbladder, thin layer chromatography of chloroform-methanol (2:1 v/v) extracts was carried out by the method of Nakayama (1969). For this purpose, extracts were loaded on silica gel plates 300 mμ thick. The plates were developed in three separate solvent systems to separate conjugated bile acids, free bile acids cholesterol, free fatty acids and cholesterol esters. By replacing the isopropyl ether-iso-octane, acetic acid (2:1:1 v/v) used as the second solvent system by Nakayama (1969) to separate free bile acids with the upper phase of the solvent system toluene, glacial acetic acid, water (50:50:10 v/v) of Ganshirt, Koss and Morianz (1960) a better resolution of the mixture was obtained.

Figure 9 p.26 shows a thin layer chromatogram of three different types of stones found in the same gallbladder. At present, one such example has been found in stones from 104 patients. Nakayama (1969) shows that human pigment stones contain conjugated and free bile acids, and the above example offers some confirmation of this.

SAMPLING AND STORAGE OF BILE

Campbell and Burton (1949), Tera (1960) and Rains (1964) have demonstrated that gallbladder bile in vivo is never completely homogenous, and tends to stratify into layers. In studies of the chemical composition of gallbladder bile from patients, the analyst's problems begin with the surgeon ensuring that all of the bile is removed from the gallbladder.

Figure 9. Thin layer chromatogram on silica gel G of chloroform-methanol (2:1 v/v) extracts of different gallstones from the same patient.

A. Cholesterol gallstone (98.86 per cent w/w cholesterol).

B. Pigment stones (3.3 per cent w/w cholesterol).

C. Mixed cholesterol-pigment stones (54.2 per cent w/w cholesterol).

D. Mixed standards. Dihydroxycholanic acids comprised chenodeoxycholic and deoxycholic acids. Glycodihydroxycholanic acids comprised glycodeoxycholic and glycochenodeoxycholic acids. Taurodihydroxycholanic acids comprised taurochenodeoxycholic and taurodeoxycholic acids.

Solvents: The plate was developed to 5 cm. from the origin in butan-1-ol-acetic acid-water (10:1:1 v/v) to separate conjugates. After drying at room temperature, the plate was developed in the upper phase of the solvent system, toluene-acetic acid-water (50:50:10 v/v) to a height of 10 cm. from the origin to separate free bile acids. Again after drying, the plate was developed to 17 cm. from the origin in petroleum ether (boiling range 40-60°C) - butan-2-one-acetic acid (95:4:1 v/v).

Detection Agent: 10 per cent w/v phosphomolybdic acid in ethanol. Heated for five minutes at 110°C.

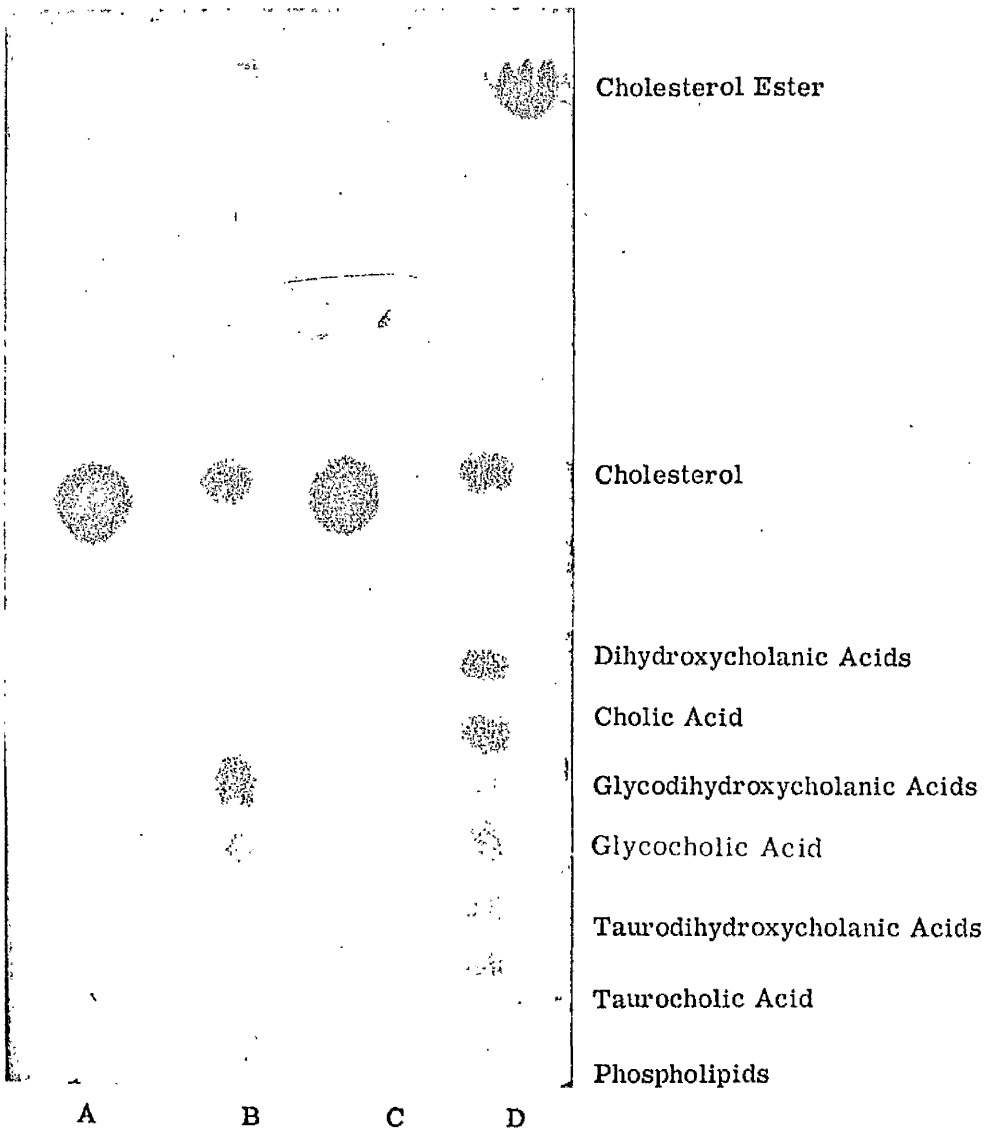


FIGURE 9.

Thin layer chromatogram on silica gel G of chloroform-methanol (2:1 v/v) extracts of different gallstones from the same patient.

In the work reported in Parts 1 and 2 of this thesis, gallbladder bile samples were obtained only from patients admitted for elective cholecystectomy (Mr. C. MacKay).

Cholesterol crystals tend to deposit from the bile of patients with gallstones, when the samples are kept at room temperature. The phenomenon has been studied by Tera (1963). He found that after 4 hours, the cholesterol content of the lower layer was about twice that of the supernatant. Very vigorous shaking of the samples is therefore necessary prior to sampling. As noted by Isaksson (1953-1954), particularly viscous samples sometimes resist even mechanical shaking. Of particular importance in this respect are the observations of Womack, Zeppa and Irvine (1963). These workers showed that the mucus phase of gallbladder bile contained on the whole, much greater amounts of cholesterol than the aqueous phase. In some of these, differences of the order of 900 mg./100 ml. were noted. Very viscous samples of bile have always presented a sampling problem, and in the present studies these were homogenised.

Gallbladder bile from patients with gallstones sometimes contains the so-called 'sand' composed of fine fragments of stones. As would be expected this causes falsely elevated cholesterol values. Phospholipid values are also affected. Centrifuging of these samples to remove this material also brings down cholesterol that is already in the precipitable state. In the present author's opinion, the investigator should exclude such samples from his series as has been done in the studies reported here. The presence of microcrystals of cholesterol in the gallbladder

bile of patients with cholelithiasis has been studied in the centrifuged deposit by Juniper and Burson (1957), and Admirand and Small (1968). In the former's series, 12.4 per cent contained cholesterol crystals, and 34.5 per cent calcium bilirubinate plus cholesterol. The latter workers found that the gallbladder bile from 28 of the 66 cases examined contained microcrystals of cholesterol, incidence of 42.4 per cent. Surprisingly, these workers refrigerated their samples on receipt, then warmed them to room temperature before centrifuging them. They did not state at what temperature the samples were refrigerated at, but as shown below, if gallbladder bile from patients with cholelithiasis is frozen at -20°C, then centrifuged major quantities of cholesterol are found in the deposit.

EFFECT OF STORAGE AT -20°C ON THE CHOLESTEROL CONTENT OF GALLBLADDER BILE

In Table 3 p.29a data is presented on the effect of storage at -20°C on the cholesterol content of a series of gallbladder bile samples from patients with cholelithiasis. The first determination was made within 1 week of the specimen being received in the laboratory. During this time they were kept at -20°C. Some months later as indicated, they were thawed out at room temperature, and well mixed. The cholesterol content was determined, and at the same time, the remainder of the sample was centrifuged at 3000 r.p.m. for 10 minutes. The cholesterol content of the supernatant was then determined.

RESULTS AND DISCUSSION

The results of these experiments are shown in Table 3 p. 29a. It will be seen that after storage of the specimens for the times shown there was a slight increase in the mean cholesterol content (mean difference 1.06 m-moles/l.). This was statistically significant ($p < 0.05$) but was not considered to be of practical significance. This result was interpreted to mean that cholesterol was coming out of micellar solution as a result of freezing and thawing. The difference found between the two series was therefore considered to be due to sampling errors, as a result of uneven distribution of the crystals in the specimens. That cholesterol crystals were being formed in the bile samples was then shown by centrifuging the samples and determining the cholesterol content of the supernatants. This is also shown in Table 3 p. 29a where it will be seen that a marked drop in the cholesterol content occurred after centrifugation.

Some time after this work was completed, the authors attention was drawn to a paper by Thistle and Schoenfield (1971) in which they noted that duodenal bile that was supersaturated with cholesterol deposited crystals of the sterol as a result of freezing and thawing.

EXPRESSION OF RESULTS

Isaksson (1953-1954), and Crawford (1955) suggested that components of bile as determined by analysis, should be expressed as a percentage

TABLE 3.

EFFECT OF STORAGE AT -20°C ON THE PRECIPITATION OF CHOLESTEROL

FROM GALLBLADDER BILE VALUES IN M-MOLES CHOLESTEROL/L.

SPEC.	1st DETERMINATION*	2nd DETERMINATION AFTER STORAGE AT -20°C ##	VALUE FOUND AFTER CENTRIFUGING
A	9.80	12.63 (2)	6.11
B	14.48	15.32 (7)	4.98
C	11.73	12.16 (7)	5.37
D	18.10	21.80 (7)	12.76
E	6.25	7.03 (7)	3.36
F	10.30	14.09 (7)	5.92
G	7.27	7.60 (6)	5.12
H	1.45	1.70 (6)	0.39
I	8.08	9.78 (6)	4.09
J	9.70	8.96 (6)	4.79
K	12.49	13.40 (6)	4.94
L	10.62	10.01 (3)	6.00
M	6.17	5.84 (3)	2.73
MEAN	9.726	10.793	5.12
S.E.	1.200	1.446	0.804
t value (paired t-test)	2.4361		7.021
p	< 0.05		< 0.001

* = Analysis within one week of obtaining specimen

= Figure in parenthesis represents months at -20°C between 1st and 2nd Determinations.

of the total solids. This, they pointed out, would obviate variables due to the concentrating power of the gallbladder. In addition, it would also eliminate the effect of the stratification that occurs in gallbladder bile in vivo (p. 25) and which causes variations in the composition of the sample as taken by the surgeon by needle aspiration, at operations other than cholecystectomy (Isaksson, 1953-1954).

In the data to be presented, where applicable, the results of the determinations of cholesterol, bile salts and lecithin, were expressed as a percentage of the sum of the three (i.e. 'total solids'). These were then represented as single points on the ternary phase diagrams of Admirand and Small (1968). In Figure 10 p. 31a which is illustrative, the relative composition of a hypothetical bile samples containing 85 per cent bile salts, 10 per cent phospholipid and 5 per cent cholesterol is shown. In the rather similar phase diagram shown for example in Figure 11 p. 36a the irregular line represents maximum cholesterol solubilisation. The area below this is the micellar zone. This was determined by Admirand and Small (1968) using quaternary model systems composed of all possible combinations of bile salts, lecithin and cholesterol, at concentrations of 10 per cent solids and 90 per cent water. As discussed later, these workers determined the relative compositions of gallbladder bile, and plotted the results as described. When the samples contained microcrystals of cholesterol, the points fell well outside the zone of maximum cholesterol solubilisation. Specimens of bile without microcrystals fell on or very near the line. Normal biles on the other hand fell within the micellar zone. A cautionary note on

these determinations has already been made in the present thesis (p. 28).

It will have been noted that the selection of a 90 per cent water content in the model systems of Admirand and Small (1968) as discussed above, may appear somewhat arbitrary. These workers chose this value since it represented the mean water content of 91 samples of gallbladder bile, 25 of which had no history of biliary tract disease. It was shown however that the limits of the micellar zone were the same at 5, 10 and 20 per cent levels of solids in the model systems. At concentrations of 3 per cent solids, changes were found in the solubilisation of the cholesterol. The limits of cholesterol solubilisation were therefore not considered to be applicable to very dilute biles (Admirand and Small, 1968).

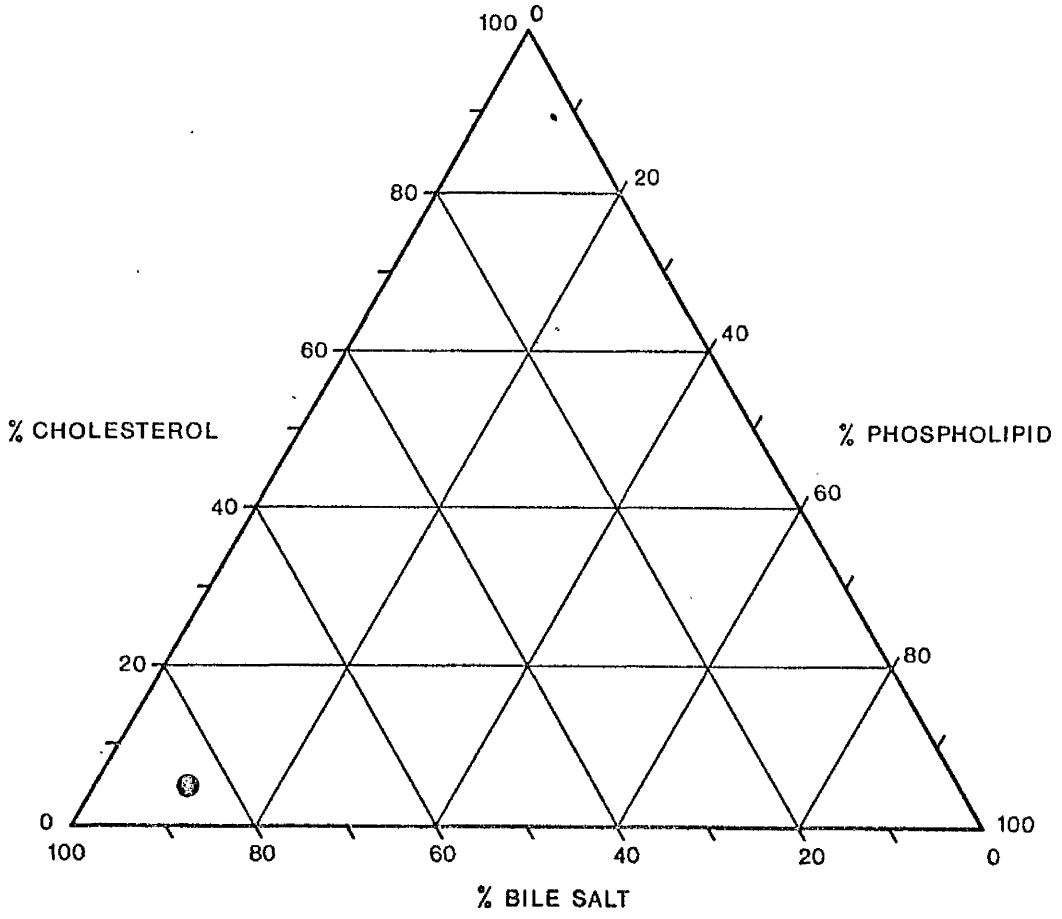


FIGURE 10.

PHASE DIAGRAM AS USED BY ADMIRAND AND SMALL (1968)

The dot shown represents the position of a bile sample with a hypothetical composition of 85 per cent bile salts, 10 per cent phospholipid, and 5 per cent cholesterol.

PART I.

THE STEROL AND LIPID COMPOSITION OF GALLBLADDER BILE IN PATIENTS WITH GALLSTONES AND IN DUODENAL ULCER PATIENTS BEFORE AND AFTER VAGOTOMY

The first suggestion that there might be a causal relationship between gallstone disease and gastric surgery, came from the clinical studies of Majoor and Suren (1947). Since then, several confirmatory reports have appeared (Krause, 1963; Griffiths and Holmes, 1964; Lundman, Orinius and Thorsen, 1964). Others have denied that there is such a relationship (Chapa and Engel, 1959; Turunen and Antila, 1964). In reviewing the considerable literature on the subject, Fletcher and Clark (1968) stated that the relationship was not proven, a view also reached essentially be Bouchier (1970) who suggested that further investigation was necessary.

Many of the previous investigations both of clinical material, and in animal models, have been concerned with the flow of bile, and its ionic composition. Few have determined the chemical composition of the bile with respect to its cholesterol, phospholipid and bile salt content, the determining factors in gallstone formation.

As discussed in a previous part of this thesis (p. 3) dog's gallbladder bile is at a low level of saturation with cholesterol, and will dissolve human gallstones in vivo. Barnett and Hilbun (1966) found that the rate of dissolution of human gallstones in the gallbladder of the dog was greatly reduced if the animal was vagotomised. This could not be confirmed by Loeb and Nicoloff (1968). Later, Tompkins, Kraft and Zollinger (1970), in agreement with Barnett and Hilbun (1966), showed that the dissolution of human gallstones in the gallbladder of the dog, decreased markedly after complete vagotomy.

In contrast to previous workers, these authors analysed bile from their experimental animals, and found a marked decrease in the phospholipid/cholesterol ratio when compared with controls. In dogs, Fletcher and Clark (1969) found no changes occurred in the phospholipid and cholesterol content of hepatic bile after vagotomy, but found that there was a reduction in bile salt/cholesterol ratios. This fall in biliary cholate has been challenged by Bouchier (1970) who considered that it required confirmation.

In view of the paucity of data on the chemical composition of bile with respect to the three main solid components after vagotomy, there was an obvious need for a carefully controlled study in patients before and after vagotomy.

The aim of the study here reported was two-fold. Firstly, to compare the relative composition of gallbladder bile in gallstone patients and in duodenal ulcer patients, and secondly to establish whether vagotomy had any effect on the relative composition of gallbladder bile.

PROCEDURES

Bile samples were obtained by duodenal intubation (Mr. D. Smith) following the intravenous injection of cholecystokinin (1 Unit/Kg. body weight). All samples were rapidly frozen after collection. The cholesterol, bile salt and lecithin contents were determined by the procedures already described.

The initial phase of the investigation was to establish that bile samples obtained by duodenal intubation were representative of gallbladder bile. This was investigated in 9 patients with gallstones all of whom had radiologically functioning gallbladders. Prior to operation bile samples were obtained as described above. Later at elective cholecystectomy (Mr. C. MacKay), before removal of the gallbladder, bile was obtained by aspiration.

Bile samples were also obtained by duodenal intubation from 10 duodenal ulcer patients prior to operation. Eight to ten days after vagotomy and drainage, the duodenum was again intubated and further specimens of gallbladder bile obtained for analysis. Completeness of vagotomy was determined by the Insulin Test (Hollander, 1946).

RESULTS

In Table 4 p. 35a is shown the relative composition of bile obtained from the 9 gallstone patients by duodenal intubation, and at operation. It can be seen that there is no statistically significant difference between the samples as regards the bile salts, phospholipid and cholesterol content. From the data presented it was concluded that the specimens obtained by duodenal intubation were representative of gallbladder bile.

In Table 5, p. 35b shows the relative composition of bile obtained pre-operatively by duodenal intubation from the gallstone patients and

9 GALLSTONE PATIENTS

RELATIVE COMPOSITION OF BILE

	Cholesterol %	Bile Salt %	Phospholipid %
Duodenal intubation	8.8 ± 1.5	69.4 ± 3.3	21.8 ± 2.2
Operative aspiration	6.0 ± 1.2	73.2 ± 2.0	20.8 ± 1.4
p	n.s.	n.s.	n.s.

TABLE 4.

THE RELATIVE COMPOSITION OF BILE OBTAINED BY DUODENAL INTUBATION AND OPERATIVE ASPIRATION FROM 9 GALLSTONE PATIENTS

Values shown represent the means ± S.E. (n.s. = not significant)

DUODENAL BILE

RELATIVE COMPOSITION

	Cholesterol %	Bile Salts %	Phospholipid %
Gallstone patients	8.8 ± 1.6	69.4 ± 3.3	21.7 ± 2.2
Pre-op D. U. patients	4.9 ± 0.4	72.6 ± 3.1	22.5 ± 2.9
p	<0.05	n.s.	n.s.

TABLE 5.

THE RELATIVE COMPOSITION OF DUODENAL BILE FROM PREOPERATIVE GALLSTONE PATIENTS AND PREOPERATIVE DUODENAL ULCER PATIENTS

Values shown represent the means ± S.E. (n.s. = not significant)

duodenal ulcer patients. It will be seen that this comparison of the two groups shows that bile from patients with gallstones contains significantly more cholesterol than did the bile from the duodenal ulcer patients. No significant difference in the bile salts and phospholipid contents were found in the two groups. The data were plotted on triangular co-ordinates as previously described. Figure 11 p. 36a shows this, with the means plus or minus one standard error, plotted. It will be seen that both values lie within the micellar zone. Bile from patients with gallstones however is seen to lie nearer to the limit of cholesterol solubility.

In Table 6 p. 36b is shown the relative compositions of bile from the duodenal ulcer patients, obtained pre and post-operatively. It can be seen that there is no significant change in the relative amounts of bile salts, cholesterol and phospholipid in the samples obtained after vagotomy and drainage. When the data were plotted on triangular co-ordinates, the relative compositions of both are seen (Figure 12 p. 37a) to lie well within the micellar zone.

DISCUSSION

Bile obtained by duodenal intubation, and aspiration after the injection of cholecystokinin was shown to be similar in sterol and lipid composition to gallbladder bile from the same patient. This finding confirms the recent report by Vlahcevic, Cooper Bell, Juttijudata and Swell (1971). These workers showed that the relative composition of the sterol and lipid in

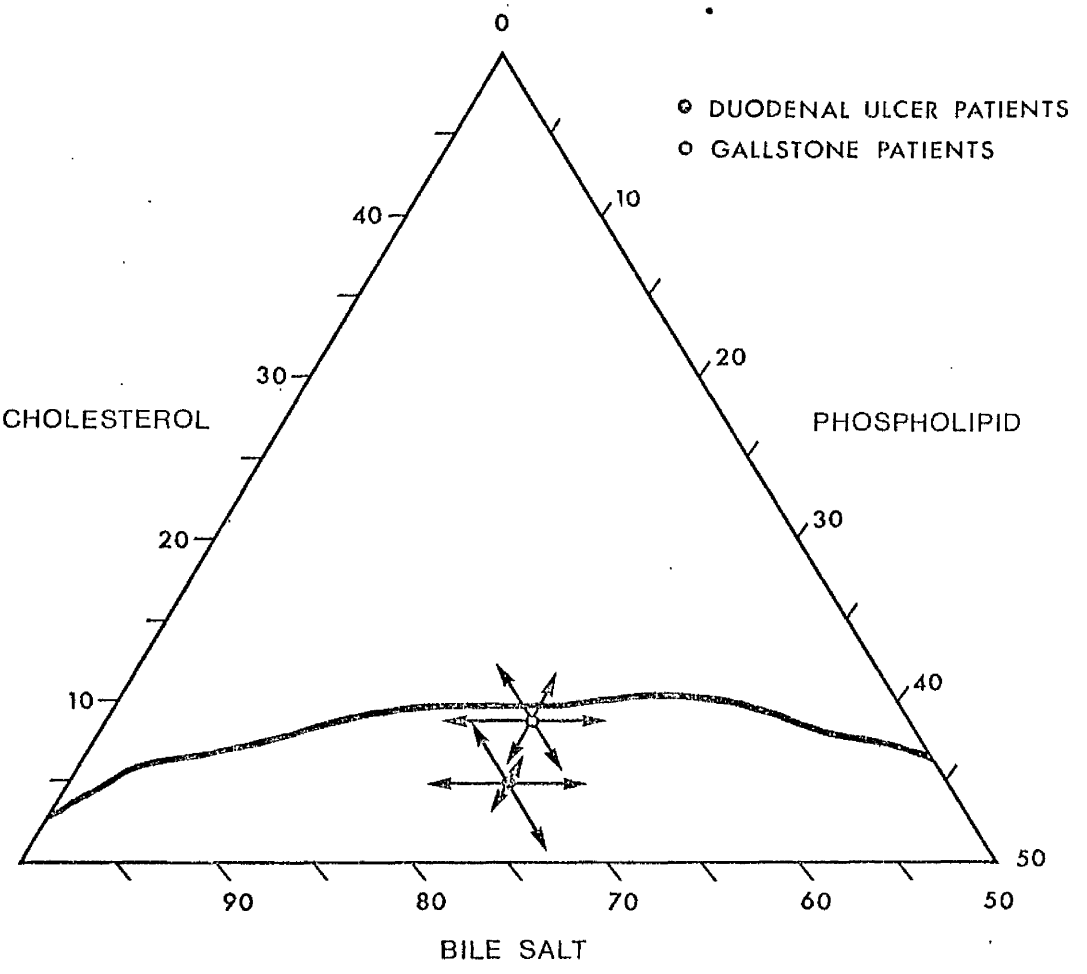


FIGURE 11.

THE MEAN RELATIVE COMPOSITION OF DUODENAL BILE OBTAINED FROM 9 GALLSTONE PATIENTS AND 10 DUODENAL ULCER PATIENTS, BOTH IN THE PREOPERATIVE PHASE

Values shown represent the means $\pm$ S.E.

DUODENAL BILE

RELATIVE COMPOSITION

	Cholesterol %	Bile Salt %	Phospholipid %
Pre V + D	4.9 ± 0.4	72.6 ± 3.1	22.5 ± 2.9
Post V + D	5.3 ± 0.6	75.0 ± 2.4	19.7 ± 2.0
p	n.s.	n.s.	n.s.

TABLE 6.

THE RELATIVE COMPOSITION OF DUODENAL BILE FROM PRE- AND POSTOPERATIVE DUODENAL ULCER PATIENTS

Values shown represent the means ± S.E. (n.s. = not significant)

duodenal bile could be used as an indicator of lithogenic bile.

Bile samples obtained by duodenal intubation are more dilute than gallbladder bile, due to the presence of pancreatic and intestinal secretions. This dilution effect was obviated, as discussed in a previous section of this thesis (p. 29) by expressing the concentrations as a percentage of the total solids. In order to minimise the conversion of lecithin to lysolecithin by lecithinase in the pancreatic secretion, the bile samples were frozen as soon as possible after collection. It was considered however that any breakdown of lecithin in this manner would not greatly influence the phospholipid determination since this was based on the measurement of the total phosphorus present. Further, the difference in the molecular weights of lecithin and lysolecithin was not sufficiently great to greatly alter the results, when the values for total phosphorus were converted by calculation into terms of m-moles of lecithin. While this work was in progress however, Thistle and Schoenfield (1971) showed that by means of t.l.c. methods that bile obtained by duodenal intubation following the injection of cholecystokinin, contained only lecithin, lysolecithin could not be detected.

Although the findings reported here show that there was no significant change in the relative composition of bile eight to ten days after vagotomy, this may well be too short a period for any of the supposed effects on bile composition to take place. For this reason, these studies are being continued at a later date after operation on these patients. Here the possibility arises, as has been pointed out by

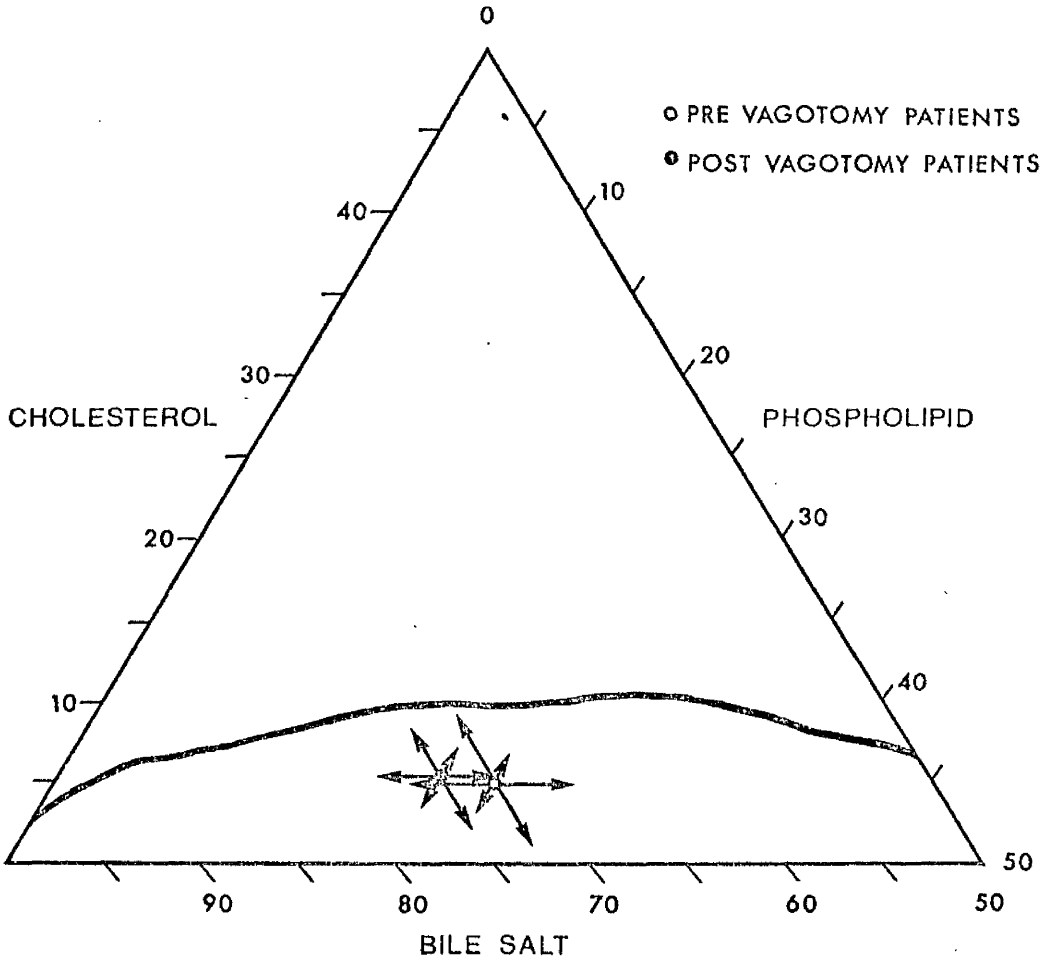


FIGURE 12.

THE MEAN RELATIVE COMPOSITION OF DUODENAL BILE FROM 10 PRE- AND POST OPERATIVE DUODENAL ULCER PATIENTS

Values shown represent the means $\pm$ S.E.

Horwitz and Kirson (1965) that the incidence of gallstone disease would be expected to increase irrespective of whether the patient had undergone vagotomy or not, since the incidence increases with the age of the individual.

It might also be argued that any supposed increase in the incidence of gallstone disease as a result of vagotomy will also be influenced by the incidence of the disease in the population as a whole. As has been pointed out by Bouchier (1970) information on the incidence of gallstone disease has been for the most part obtained at autopsy. There are no reliable figures on the incidence in the living population. In the study presented here, patients with gallstone disease were used as controls, and a clear delineation, based on the relative composition of bile samples, was made between the two groups. With the establishment that bile representative of gallbladder bile can be readily obtained by duodenal intubation, studies of the composition of bile from normal controls are now being carried out.

PART 2.

THE STEROL AND LIPID COMPOSITION OF HEPATIC AND GALLBLADDER BILE IN PATIENTS WITH GALLSTONES

Small and Rapo (1970) and Vlahcevic, Cooper Bell and Swell (1970) have reported that cholesterol gallstones are associated with the production by the liver of bile which is supersaturated with cholesterol with respect to phospholipid and bile salt. Small studied American Indians, a population known to have a significantly higher incidence of gallstone disease than the general population of the United States of America (Comess, Bennett & Burch, 1967). Vlahcevic et.al. (1970) studied a group of patients in the Veterans Administration Hospital, Richmond, Virginia and it is known that there is a higher incidence of gallstone disease in North America than in Great Britain (Lieber, 1952; Gross, 1929). For these reasons it was considered important to establish whether or not patients resident in the United Kingdom, and suffering from gallstone disease, produced hepatic bile which was supersaturated with cholesterol.

MATERIALS AND METHODS

Nineteen patients (2 males and 17 females) admitted to the Western Infirmary, Glasgow for elective cholecystectomy were studied. The ages ranged from 20 to 72 years, the mean being 48 years. Each patient had a functioning gallbladder demonstrated pre-operatively by oral cholecystography. At operation specimens of gallbladder and hepatic bile were aspirated. After opening the abdomen a needle was inserted into the fundus of the gallbladder and the contents completely aspirated. At a later stage in the operation a specimen of bile was obtained from the common bile duct, either by direct needle puncture

or by aspirating through a catheter inserted via the cystic duct. A determined attempt was made by the surgeons to avoid contamination of hepatic bile with gallbladder bile. The specimens of bile were then brought to the laboratory without delay and stored at -20°C until analysed. The cholesterol, phospholipids and bile salts content of the samples were determined by the procedures described earlier. Stones removed at operation were washed and dried. When the gallstone was large a representative cross-section of the stone was studied, but where the gallstone was small a representative whole stone was analysed by the procedure already described. Only those patients whose gallstones were composed of at least 50 per cent cholesterol was included in the present study.

RESULTS

The concentrations of cholesterol, phospholipid and bile salt in gallbladder and hepatic bile in the 19 patients are listed in Table 7 The mean total solid contents of gallbladder and hepatic bile were 9.0% and 3.8% respectively. The cholesterol content of the gallstones is also included. Gallbladder bile will be seen to contain relatively more bile salt and relatively less phospholipid and cholesterol than hepatic bile, the difference reaching the level of statistical significance in each case, viz. $P < 0.005$ for bile salt, $P < 0.02$ for phospholipid and $P < 0.001$ for cholesterol using a paired T-test. The mean relative compositions of gallbladder and hepatic bile, plotted on triangular co-ordinates as described by Admirand and Small (1968) are

TABLE 7.

THE COMPOSITION OF GALLBLADDER AND HEPATIC BILE IN PATIENTS WITH GALLSTONES

Patient	GALLBLADDER BILE						HEPATIC BILE						Gallstone
	Concn. m.mole/l.			Relative Composn. %			Concn. m.mole/l.			Relative Composn. %			
	B.S.	PLP	Chol.	B.S.	PLP	Chol.	B.S.	PLP	Chol.	B.S.	PLP	Chol.	% Chol.
J.S.	157.0	25.93	10.34	81.2	13.4	5.4	91.5	11.68	9.14	81.5	10.4	8.1	87.17
A.S.	183.0	41.60	13.82	76.8	17.4	5.8	124.0	31.01	14.12	73.3	18.3	8.3	89.25
M.A.	116.0	24.20	6.36	79.1	16.5	4.3	40.0	9.97	3.54	74.8	18.6	6.6	92.80
J.McC.	100.0	18.17	6.96	79.9	14.5	5.6	55.0	13.14	4.53	75.7	18.1	6.2	66.98
M.P.	200.0	49.60	20.22	74.1	18.4	7.5	37.5	13.31	6.58	65.3	23.2	11.5	68.19
J.G.	35.0	12.25	5.00	67.0	23.4	9.6	35.0	14.42	3.84	65.7	27.1	7.2	54.52
D.T.	76.0	22.92	5.50	72.8	21.9	5.3	33.5	9.23	2.46	74.1	20.4	5.4	90.30
J.K.	206.0	49.87	18.86	75.0	18.2	6.9	27.5	13.06	6.10	58.9	28.0	13.1	93.00
M.B.	140.0	26.60	13.26	77.8	14.8	7.4	76.0	18.45	10.26	72.6	17.6	9.8	93.39
A.K.	53.0	6.91	3.21	84.0	10.9	5.1	46.0	10.78	5.20	74.2	17.4	8.4	78.29
C.W.	32.0	10.77	3.29	69.5	23.4	7.1	40.0	11.47	3.49	72.8	20.9	6.4	82.18
D.R.	107.07	34.17	9.80	70.9	22.6	6.5	33.04	19.64	5.56	56.7	33.7	9.5	93.36
J.B.	85.85	28.22	14.48	66.8	22.0	11.3	39.07	17.60	9.80	58.8	26.5	14.7	91.58
M.F.	69.93	28.52	11.73	63.5	25.9	10.6	15.43	7.24	4.28	57.3	26.9	15.9	84.23
J.McC.	148.07	37.00	18.10	72.9	18.2	8.9	17.85	11.73	6.95	48.9	32.1	19.0	93.22
A.P.	95.98	38.82	10.30	66.1	26.8	7.1	36.08	8.91	5.29	71.8	17.7	10.5	90.91
A.B.	80.06	23.94	9.70	70.4	21.1	8.5	82.48	26.43	9.47	69.7	22.3	8.0	55.88
A.McD.	147.00	10.76	13.92	85.6	6.3	8.1	23.40	4.87	2.93	75.0	15.6	9.4	88.18
M.B.	207.00	42.15	19.41	77.1	15.7	7.2	16.00	13.47	4.08	47.7	40.1	12.2	98.52
MEAN	117.84	28.02	11.28	74.24	18.49	7.27	45.76	14.02	6.19	67.09	22.89	10.01	83.79
S.D.	55.95	12.94	5.42	6.23	5.24	2.08	28.56	6.38	3.07	9.68	7.22	3.62	13.05
S.E.	12.84	2.97	1.24	1.43	1.20	0.49	6.55	1.46	0.71	2.28	1.70	0.85	3.08

shown in Figure 13 p. 42a. Gallbladder bile lies within the zone of cholesterol solubility whereas hepatic bile is saturated but not supersaturated with cholesterol.

Table 8 p. 42b shows the ratios of the three main solid constituents of bile. There is an increase in all three ratios, bile salt/cholesterol, bile salt/phospholipid and phospholipid/cholesterol, in gallbladder bile compared with hepatic bile, but only in the case of bile salt/cholesterol does this difference reach a high level of statistical significance.

DISCUSSION

Admirand and Small (1968) showed that gallbladder bile from patients with gallstones was saturated with cholesterol whereas that from patients without gallstones was unsaturated. Small and Rapo (1970) and Vlahcevic et.al. (1970) then showed that in patients with cholesterol gallstones, the liver produced bile which was supersaturated with cholesterol and postulated that this was the primary aetiological factor in the production of cholesterol gallstones. The data here presented show that cholesterol gallstones can be found in the presence of hepatic bile which is saturated but not supersaturated with cholesterol. It might be argued that the method used for obtaining the specimens resulted in contamination of hepatic bile with gallbladder bile thus masking any supersaturation of the former. If this was the case, however, it is surprising that gallbladder bile was undersaturated with cholesterol and not saturated. This, therefore, does not explain

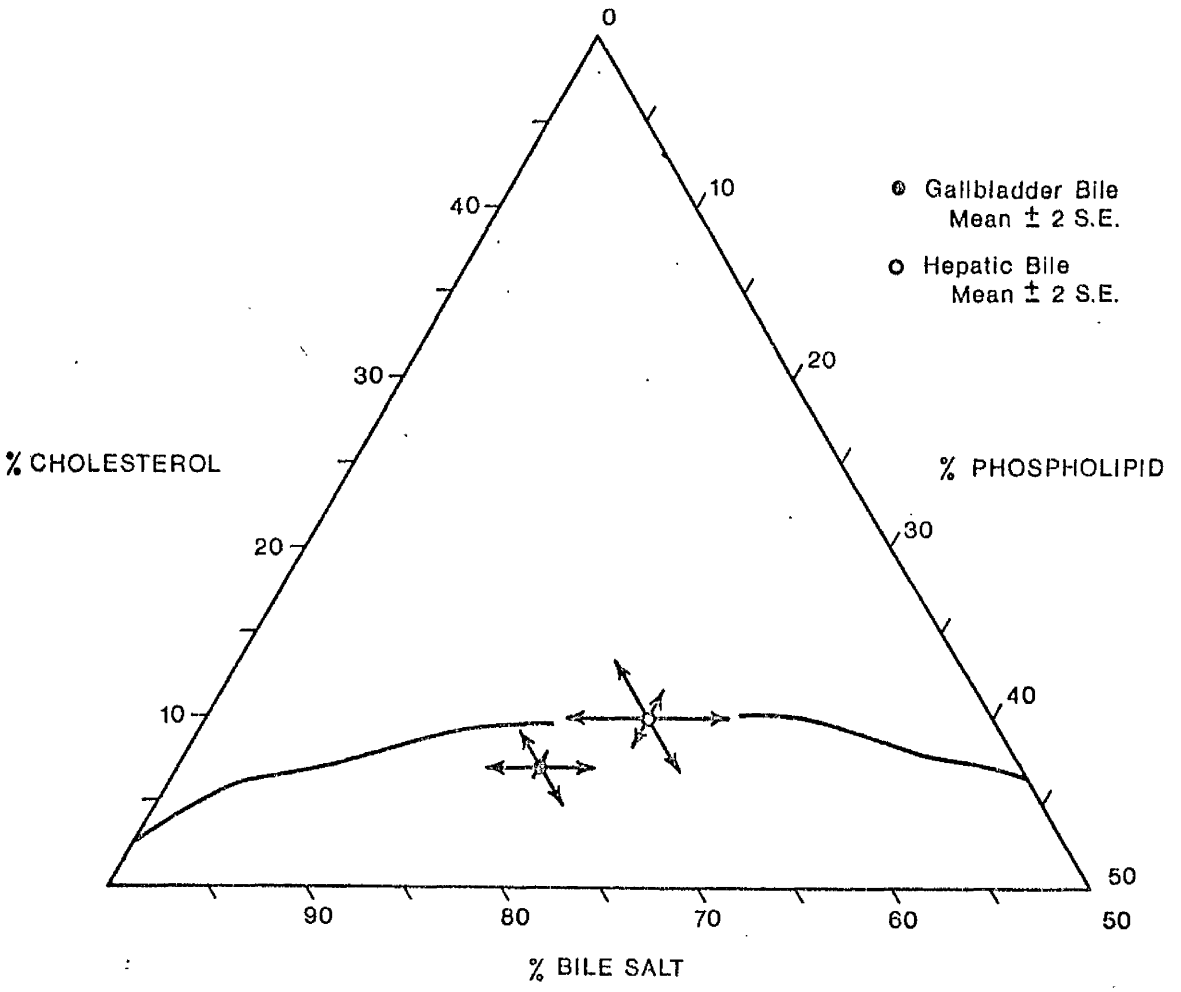


FIGURE 13.

THE RELATIVE COMPOSITION OF GALLBLADDER AND HEPATIC BILE IN PATIENTS WITH GALLSTONES

TABLE 8.

RATIOS OF THE 3 MAIN BILIARY COMPONENTS

IN GALLBLADDER-HEPATIC (CBD) BILE

	Bile Salt / Cholesterol		Bile Salt / Phospholipid		Phospholipid / Cholesterol	
	GB	CBD	GB	CBD	GB	CBD
MEAN	11.01	7.71	4.63	3.35	2.64	2.43
S.D.	3.44	3.10	2.57	1.53	0.80	0.80
S.E.	0.79	0.69	0.59	0.35	0.18	0.18
t value (Paired t-test)	4.97		2.41		1.12	
p	< 0.001		< 0.05		> 0.2	

the discrepancy between the findings and those of Small and Rapo, and Vlahcevic and his colleagues.

Other workers have reported information from gallstone patients showing that the hepatic bile is not supersaturated with cholesterol. Thureborn (1962) studied the composition of hepatic bile from patients one week after cholecystectomy and when his data were plotted on triangular co-ordinates by Small (1968) the relative composition was found to lie within the zone of cholesterol solubility. Burnett (1965) reported data on the composition of gallbladder and hepatic bile obtained at cholecystectomy and when this data were plotted on triangular co-ordinates by Small (1968) the relative composition of both lay within the micellar zone. Sarles et.al. (1971) studied the effect of diet on the composition of hepatic bile in 4 patients with indwelling T-tubes following cholecystectomy for cholesterol gallstones. Only when the patients were on a high calorie high protein diet was there a tendency for the relative composition of bile to lie outwith the zone of cholesterol solubility.

Bile supersaturated with cholesterol has also been found in patients with no evidence of gallstones. Nakayama and van der Linden (1970) studied gallbladder bile obtained at laparotomy from 20 patients with no evidence of hepatobiliary disease. In 12 of these 20 patients the relative composition lay outwith the zone of cholesterol solubility. Vlahcevic et.al. (1970) in their studies describing the presence of supersaturated hepatic bile in patients with cholesterol gallstones, reported data from 2 patients without evidence of gallstones. In both

these patients the relative composition of gallbladder and hepatic bile lay outwith the micellar zone. They suggest that these patients may form gallstones in the future but this would seem to be begging the question.

The data here presented show that the bile salt:cholesterol ratio is significantly greater in gallbladder bile than it is in hepatic bile. This could be due to secretion of bile salt by the gallbladder mucosa and/or cholesterol coming out of solution. There is no evidence that the relatively unspecialised cells of the gallbladder mucosa can secrete bile salt and it would seem much more likely that cholesterol comes out of solution. The factor or factors responsible for the precipitation of cholesterol from mixed micelles of bile salt, phospholipid and cholesterol remain to be elucidated. It may be that the presence of mucus (Bouchier & Cooperband, 1967), protein, infection (Rains, 1962) or stasis (Thureborn, 1965) in bile renders the micelles unstable so resulting in the precipitation of cholesterol. The present findings suggest that this precipitation can occur from hepatic bile which is saturated but not supersaturated with cholesterol suggesting that supersaturation of hepatic bile is not the sine qua non for cholesterol gallstone formation.

SUMMARY

Nineteen patients, each with a radiologically functioning gallbladder, who were undergoing cholecystectomy were studied. At operation specimens of bile were aspirated from the gallbladder and from the common bile duct and the concentrations of bile salt, phospholipid and cholesterol estimated. The cholesterol content of the stones was estimated. The relative composition of gallbladder bile lay within the zone of cholesterol solubility on the phase diagram whereas hepatic bile was saturated but not supersaturated with cholesterol. This suggests that supersaturation of hepatic bile is not the *sine qua non* for cholesterol gallstone formation.

PART 3.

THE ANIMAL MODEL: DEFINITION OF NORMAL VALUES

In patients, it is extremely difficult to obtain accurate measurement of the volume of bile secreted by the liver, and thereby acquire information on the secretion rates of biliary constituents. Some investigators have studied the hepatic bile from patients with indwelling T-tubes. This approach can be criticised since diversion of bile flow itself alters the composition (Thureborn, 1965; Nilsson, 1970). In order to study the secretion rate of bile constituents in some of the experiments to be reported here, an animal model was required.

Dowling, Mack and Small (1970) have described a model in the rhesus monkey. The disadvantage of this preparation is that the animals are expensive, and difficult to handle, requiring restraining cages during the experiments. For use in the present investigations, dogs with chronic biliary fistulae were created by Mr. C. MacKay and Dr. J. Crook.

At operation, bile was obtained from the gallbladder by needle aspiration and kept frozen for subsequent analysis. The gallbladder was then removed. The common bile duct was ligated. A T-tube was inserted into the common bile duct, and another tube placed in the duodenum. Both tubes were connected to a cannula that was attached externally to the abdominal wall. This preparation permits (a) that no anaesthetic would be required during collection, since these are known to alter biliary physiology, (b) a fistula that would ensure total diversion of the bile and (c) an intact enterohepatic circulation.

Figure 14 p. 48a shows the completed preparation in diagrammatic form, the cannula referred to being a modification of that described by Fletcher and Corry (1968) is shown in Figure 15 p. 48b.

When not under experimental conditions, the animals after recovery from the operation were maintained on commercial dog meat and water ad lib. Six mongrel dogs, weighing between 18 and 20 kg. were prepared and used in the following experiments.

EXPERIMENTAL

(a) Comparison of the Bile Salts, Cholesterol and Phospholipid Content of Gallbladder and Hepatic Bile

After an 18 hour fast, the animals were placed on Pavlov stands. Their gastric cannulae were opened to prevent gastric juice from entering the duodenum, with subsequent release of secretin. The perspex cap of the biliary cannula was removed and approximately 2 ml. of bile collected. Samples were kept frozen (-20°C) for subsequent determination of cholesterol, phospholipids and total bile salts by the procedures already described.

RESULTS AND DISCUSSION

In Table 9 p. 49a is shown a comparison of the composition of gallbladder bile obtained at operation, with that of hepatic bile obtained several days after operation with the enterohepatic circulation

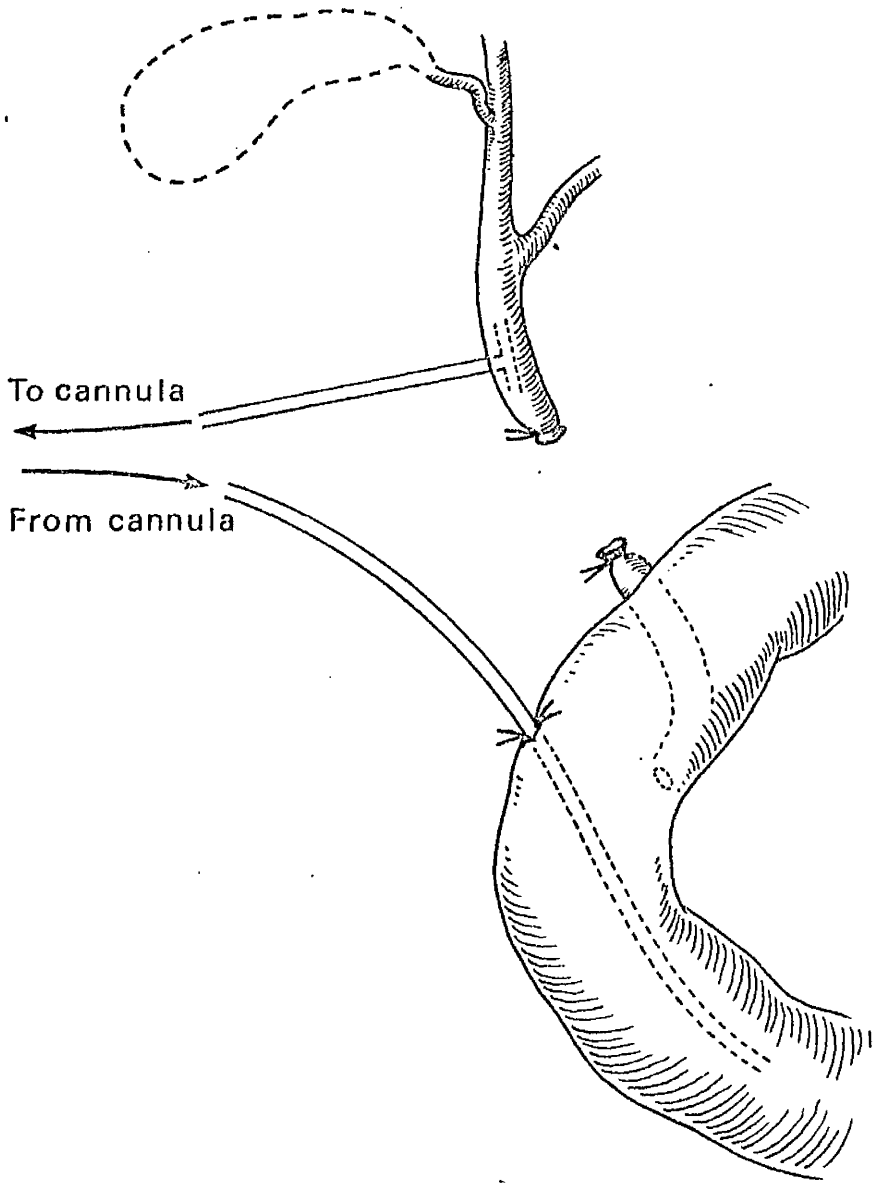


FIGURE 14.

Diagram of chronic biliary fistula preparation in the dog showing T-tube in the common bile duct, and duodenostomy tube. The ligated and divided common bile duct is also shown.

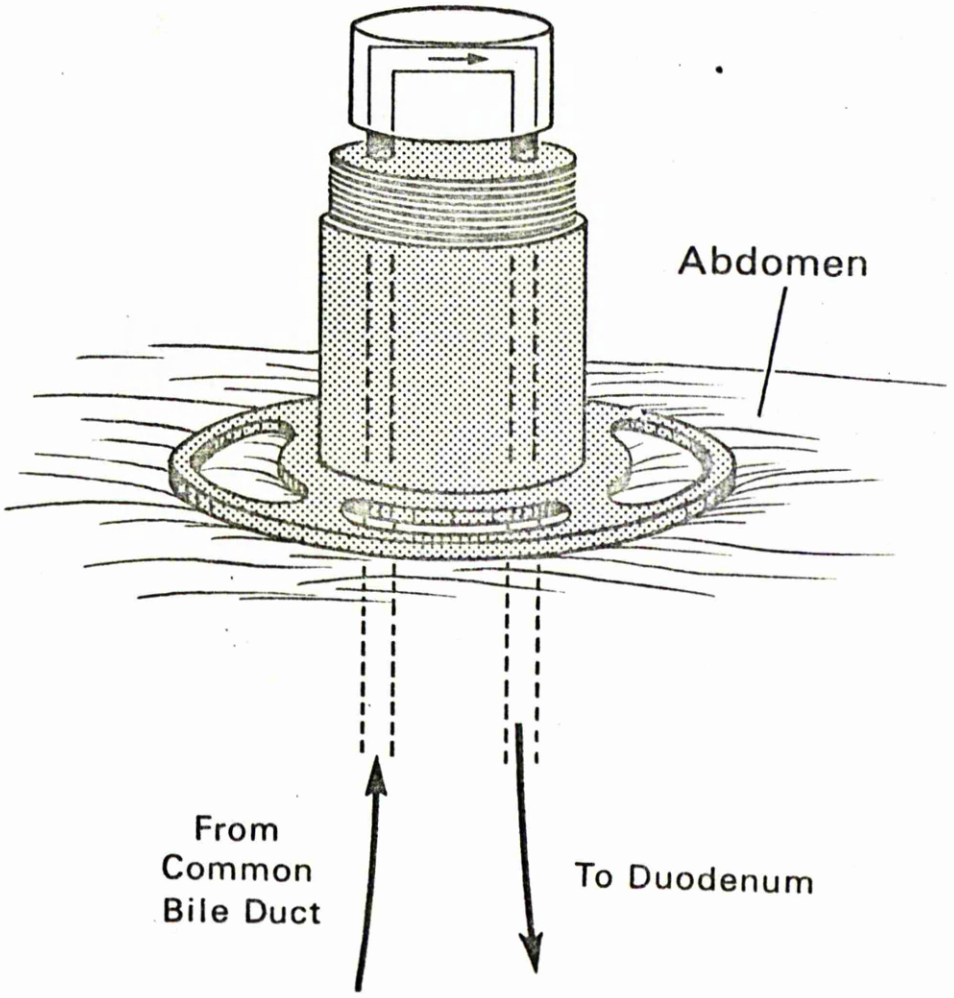


FIGURE 15.

MODIFIED BILIARY CANNULA OF FLETCHER AND CORRY (1968)

intact. These values represent the mean plus one standard error, of bile from 6 dogs. From this data it will be seen that dog's gallbladder bile as obtained at operation contains significantly more bile salts, and less phospholipid than hepatic bile, when calculated as a percentage of the total solids. There was no significant difference in the cholesterol content of hepatic and gallbladder bile, when the results of the analysis were expressed in this way.

In Figure 16 p. 49b is shown the relative compositions plotted on triangular co-ordinates. From this it will be seen that the values for both gallbladder and hepatic bile samples lie well within the zone of cholesterol solubility. This is in agreement with the findings of Nakayama (1966, 1969) who found dog's gallbladder bile to be very unsaturated with respect to cholesterol.

Compared to that of humans, the hepatic and gallbladder bile of the dog contains low levels of cholesterol (Isaksson, 1951; Adlersberg and Sobotka, 1958) and some mention has already been made in the present thesis (p. 3) to the differences in the cholesterol-holding capacity of the bile in the two species.

In further studies, the cholesterol content of gallbladder bile from 19 dogs was found to range from 1.01 - 3.42 m-moles per litre (mean 1.82 $\pm$ S.D. 0.62). This is in good agreement with the values reported by Tompkins, Burke, Zollinger and Cornwell (1970) who found a range of 1.099 - 2.536 m-moles/litre (mean 1.75 $\pm$ S.D. 0.489) for cholesterol in the gallbladder bile from 7 dogs. Nakayama (1969)

TABLE 9.

THE STEROL AND LIPID COMPOSITION OF DOG'S GALLBLADDER AND HEPATIC BILE

IN THE BASAL FASTING STATE

	CHOLESTEROL		PHOSPHOLIPID		BILE SALTS		TOTAL SOLIDS
	m-mole/l.	% Comp.	m-mole/l.	% Comp.	m-mole/l.	% Comp.	m-mole/l.
GALLBLADDER BILE	2.31 ± 0.49	0.62 ± 0.15	57.11 ± 2.26	15.19 ± 0.69	318.79 ± 10.18	84.19 ± 0.68	378.21 ± 8.11
HEPATIC BILE	0.52 ± 0.04	0.57 ± 0.05	19.46 ± 0.92	22.18 ± 1.29	70.02 ± 5.36	77.02 ± 1.28	90.01 ± 5.90
p Value	$p < 0.001$	Not Sig.	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	

* All values represent the mean results with 6 dogs ± S.E.

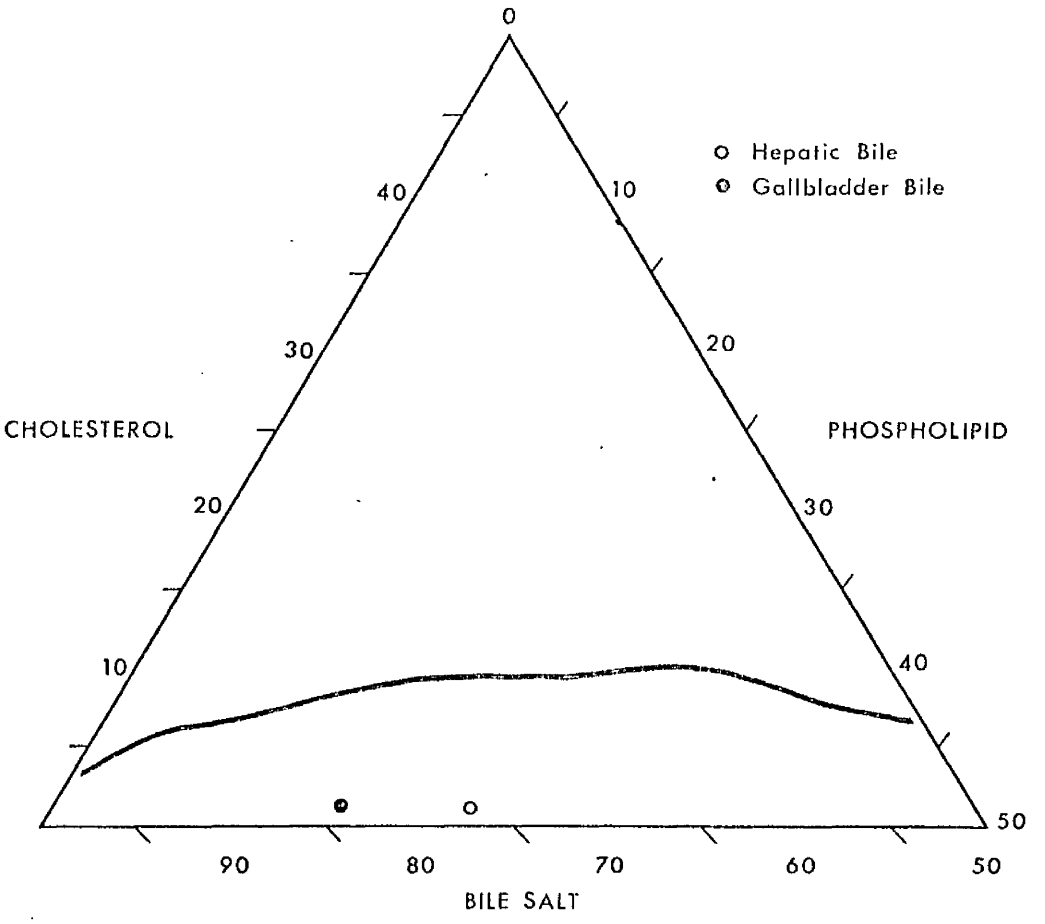


FIGURE 16

THE RELATIVE COMPOSITION OF CANINE HEPATIC AND GALLBLADDER BILE PLOTTED ON TRIANGULAR CO-ORDINATES

Values represent the mean results obtained with 6 dogs.

reported higher values. In his series of 10 dogs, he found the mean cholesterol content of gallbladder bile to be 3.54 m-moles/litre (S.D. ± 1.93).

The values shown in Table 9, for phospholipid in gallbladder and hepatic bile, are also in agreement with those found by other workers. In the aforementioned studies, Tompkins et.al. (1970) reported the mean phospholipid content of gallbladder bile to be 59.20 m-moles/litre (± S.D. 7.97). In his series, Nakayama (1969) found a mean of 66.66 m-moles/litre (± S.D. 26.25) in gallbladder bile from 10 dogs. The phospholipid content of dog's hepatic bile has been reported (Nakayama and Johnston, 1960) to be 17.96 m-moles/litre (± S.D. 5.49), this value representing the mean of samples from 4 dogs. It has not been possible to compare the present findings of bile salts, with those of previous workers, mainly because of lack of data, and where such determinations have been made (Nakayama and Johnston, 1960; Nakayama, 1969), gas liquid chromatography has been used which does not measure conjugates.

(b) <u>The Effect of Sampling on the Bile Salts, Cholesterol and Phospholipid Content of Hepatic Bile</u>

As already described, samples taken for analysis represented 10 per cent of the volume of bile collected. It was necessary to determine whether this degree of sampling affected the volume or composition of the hepatic bile: an essential criteria for later studies of the effect of various stimuli on the secretion and composition of the bile.

To evaluate this, bile was collected as before at 15 minute intervals, and the volumes measured. Four of the samples were pooled to give adequate amounts for analysis and data on specimens collected over hourly periods, and ten per cent retained for analysis, the remainder being returned to the animal via the duodenostomy tube. The collection was continued for 5 hours. In all 6 dogs were studied.

RESULTS

The data presented in Table 10 p. 51a shows the results of 10 per cent sampling of the hepatic bile on the volume, and the cholesterol, bile salts and phospholipid content over the 5 hour period. These values are the mean of the data from the 6 dogs that were studied. It will be seen that none of the parameters noted above were altered significantly by the sampling procedure. This is further emphasised in Figure 17 p. 51b where it is shown that the output of bile salts remains constant over this period.

Dowling et.al. (1970) have reported that 10 per cent sampling of hepatic bile in the rhesus monkey does not affect bile composition. It is now reported that the same is true in the dog.

TABLE 10.

THE EFFECT OF TEN PER CENT SAMPLING ON THE COMPOSITION OF HEPATIC BILE*

HOUR	VOL. ml/hr.	CHOLESTEROL	PHOSPHOLIPID	BILE SALTS	TOTAL SOLIDS
FIRST	15.9 ± 1.5	0.52 ± 0.04	19.46 ± 0.092	70.02 ± 5.36	90.07 ± 5.90
SECOND	15.0 ± 1.3	0.48 ± 0.04	19.12 ± 1.41	68.45 ± 6.16	88.05 ± 7.29
THIRD	14.5 ± 1.0	0.49 ± 0.05	20.88 ± 1.27	70.39 ± 4.77	91.77 ± 5.49
FOURTH	14.6 ± 1.3	0.45 ± 0.04	18.92 ± 0.88	67.83 ± 3.83	85.86 ± 4.80
FIFTH	15.7 ± 1.1	0.52 ± 0.05	19.59 ± 0.80	73.93 ± 5.78	94.03 ± 6.26

* All values represent the mean results with 6 dogs ± S.E.

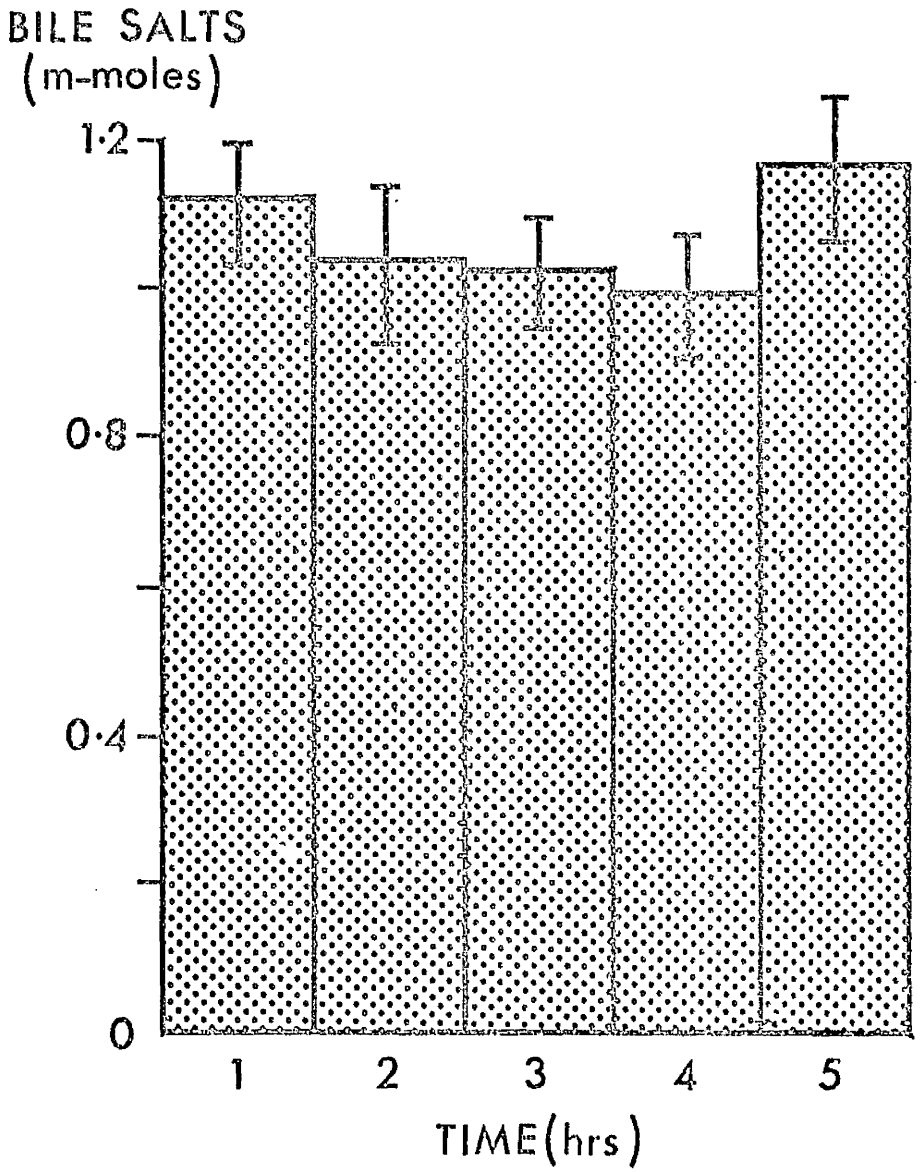


FIGURE 17.

THE EFFECT OF TEN PER CENT SAMPLING ON THE OUTPUT OF BILE SALTS OVER A FIVE HOUR PERIOD

Values represent the mean of results with six dogs ± S.E.

(c) The Bile Salt Pool Size and Circulation in the Dog

Bile acids are synthesised from cholesterol in the liver. The two primary bile acids produced are cholic and chenodeoxycholic, and these are excreted as the glycine and taurine conjugates. In the intestine the primary bile acids are dehydroxylated at $C_{(7)}$ by intestinal bacteria with the formation of deoxycholic acid and lithocholic acid from their precursors cholic and chenodeoxycholic acids respectively. Reabsorption of deoxycholic acid takes place but lithocholic acid is not absorbed to any great extent, and is excreted in the faeces. Deoxycholic acid is returned to the liver and re-excreted in the hepatic bile. Bile therefore contains primary and secondary bile acids, the former constituting about 80 per cent of the total. In the dog, the bile acid pattern is similar, but cholic acid predominates (Nakayama, 1969).

In man, about 95 per cent of the bile salts are reabsorbed in the intestinal lumen, returned to the liver and re-excreted in the bile. About 5 per cent are excreted in the faeces, and in order to maintain the steady state, this amount is balanced by new bile acid synthesis in the liver. This constitutes the enterohepatic circulation, and is shown diagrammatically in Figure 18 p. 52a. The bile salt pool can be considered to be the total of the circulating bile salts.

The total bile salt excretion, pool size, and synthesis has only been previously measured by a direct method in the monkey (Dowling et.al. 1970). For the present investigation it was necessary to measure this in the fasting cholecystectomised dog, and to determine the bile salt

ENTEROHEPATIC CIRCULATION of BILE SALTS

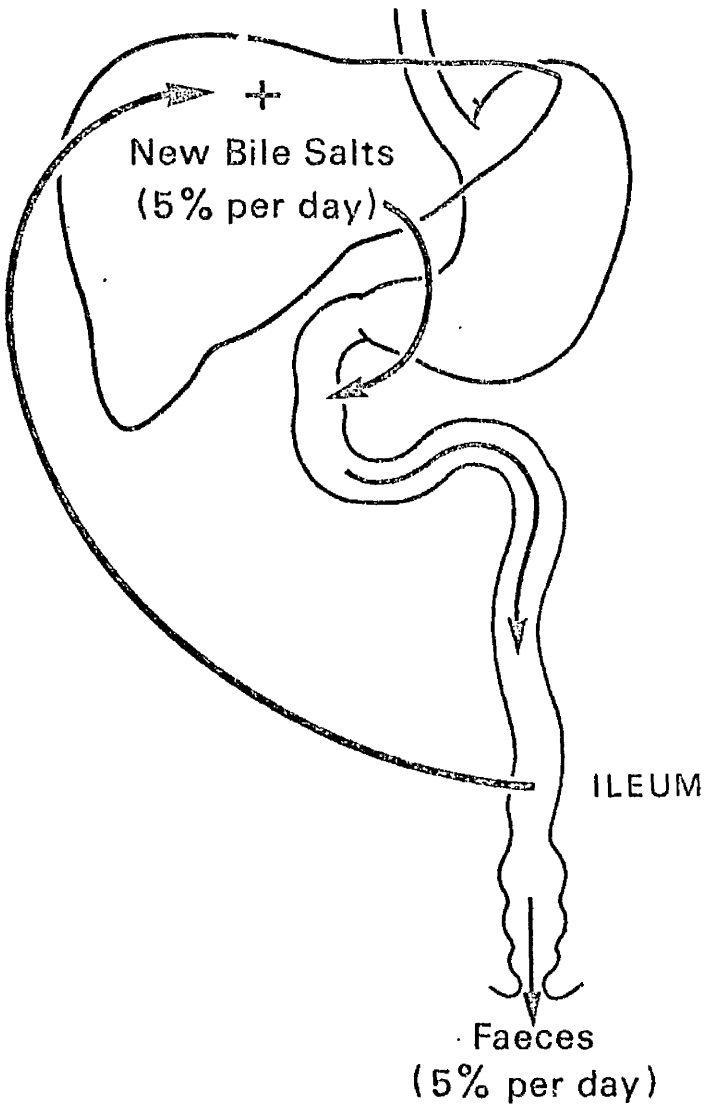


FIGURE 18.

ENTEROHEPATIC CIRCULATION OF BILE SALTS (DIAGRAMMATIC)

pool size and circulation in this species.

PROCEDURE

Firstly the bile salt pool size was measured in 6 cholecystectomised dogs by the wash-out technique of Dowling et.al. (1970). Prior to testing, the enterohepatic circulation was maintained intact. After a 12 hour fast, the animals were placed on Pavlov stands, their biliary cannulae opened, and bile collected at hourly intervals for a total of 15 hours. Ten per cent of each sample was retained for analysis, and at the end of the experiment, the remainder of the bile was returned to the animal via the duodenostomy tube. The experiment was conducted a total of twelve times using the 6 dogs under study. The total bile salts content of the samples was then determined as described previously, and the bile salts excretion calculated in terms of m-moles/hour.

RESULTS AND DISCUSSION

The data shown in Figure 19 p. 53a represents the means excretion of bile salts in the 6 dogs measured a total of twelve times. It will be seen that following interruption of the enterohepatic circulation the bile salt output fell rapidly, reaching a basal state after the fourth hour. After about 7 hours, the output began to rise again, and this represents an increase in bile salts synthesis by the liver (Dowling et.al., 1970). The bile salt pool is therefore that amount excreted over the 4 hours, before the steady state is reached. From this data, it was calculated that the

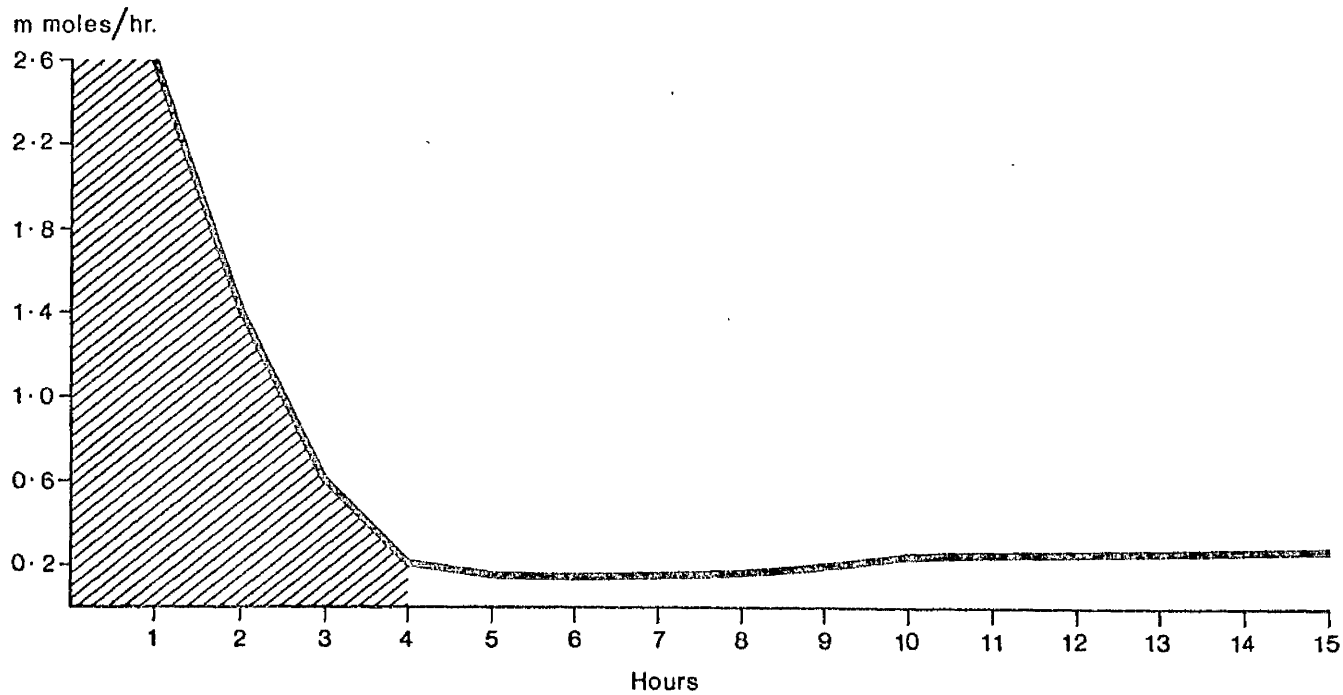


FIGURE 19.

BILE SALT EXCRETION IN THE CHOLECYSTECTOMISED DOG DURING INTERRUPTION OF THE ENTEROHEPATIC CIRCULATION

Values shown represent the mean of twelve results obtained with six dogs.

mean bile salts pool in the dog was 4.6 m-moles ± S.E. 0.3. In the fasting cholecystectomised dog, the mean bile salt output was found to be 1.2 m-moles per hour, that is 28.8 m-moles per diem. Thus with a bile salt pool size of 4.6 m-moles, the bile salt pool must circulate about six times. The bile salt pool is therefore very large when compared with man (3.5 g.) and monkey (1.14 m-moles). It also recirculates fewer times than in primates (Dowling et.al., 1970). This may reflect the eating habits of the species, as experimental dogs tend to be fed once per diem, which is consumed within minutes, whereas primates tend to eat several times a day.

(d) The Bile Salt Pool Size in the Dog, Before and After Cholecystectomy

It has already been noted in a previous part of this thesis (p. 4) that Vlahcevic et.al. (1970) found a lowered bile salt pool in patients with gallstones, when compared with that of normals. These workers used [^{14}C]-cholic acid to determine the pool size by an isotopic dilution method, and Bouchier (1971) has questioned the validity of this, claiming that it might not be representative of the bile salts participating in the enterohepatic circulation.

It was considered that the presence of stones in the gallbladder might reduce the storage capacity, and so alter the bile salt pool. It seemed of interest therefore to study the effect of cholecystectomy as a means of reducing pool size, and compare this with the pool size measured before removal of the gallbladder. The following experiments

were conducted in the dog.

PROCEDURES

In this study, 4 dogs with chronic biliary fistulae but with intact gallbladders were prepared (Mr. C. MacKay and Dr. J. Crook). After recovery from the operation, the bile salt pool size was determined by the method which has already been described (Part 3c). This was measured a total of 18 times in the 4 animals under study. The animals then underwent cholecystectomy, and the pool size was again measured after complete recovery from the operation. This was determined 15 times after cholecystectomy.

RESULTS AND DISCUSSION

Figure 20 p. 55a shows the mean bile salt pool size before and after cholecystectomy. It will be seen, that this was 6.6 $\pm$ S.E. 0.7 prior to removal of the gallbladder, and 4.6 $\pm$ S.E. 0.3. This latter value was similar to that found in the 6 cholecystectomised dogs used in the previous study (Part 3c). Comparison of the two series of results in the experiment under discussion showed that there was a significant ($p < 0.01$) reduction in the pool size as a result of cholecystectomy (Table 11 p. 55b).

As discussed previously, a diminished pool size would also result in a decrease in phospholipids (Nilsson, 1970), a situation that would predispose to cholesterol coming out of solution. Vlahcevic et.al.

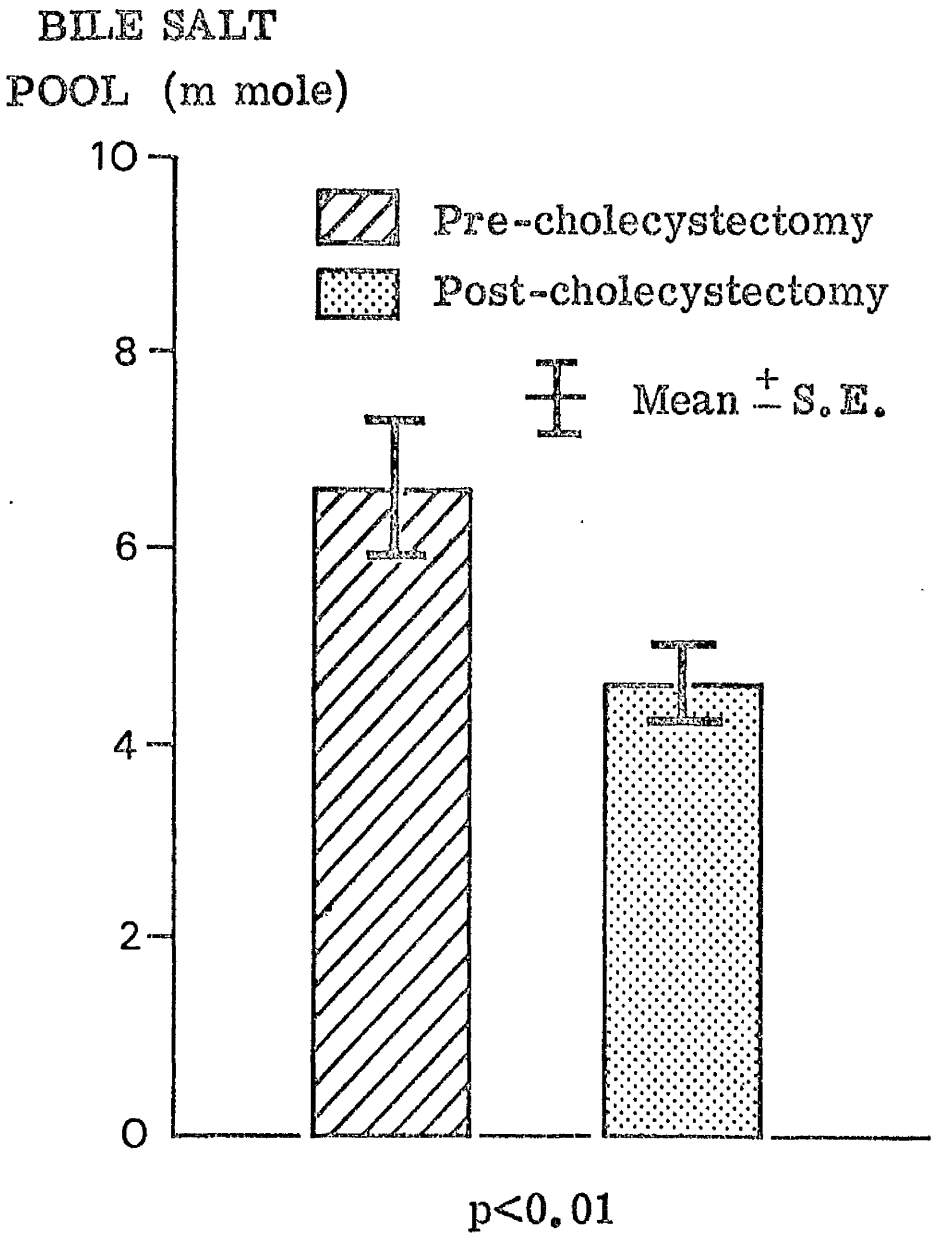


FIGURE 20.

THE BILE SALT POOL SIZE BEFORE AND AFTER CHOLECYSTECTOMY

Values shown represent the means of eighteen measurements before cholecystectomy, and fifteen measurements after cholecystectomy.

TABLE 11.

EFFECT OF CHOLECYSTECTOMY ON THE BILE SALT POOL IN THE DOG

	PRE-CHOLECYSTECTOMY		POST-CHOLECYSTECTOMY
DOG	POOL SIZE (m-moles)	DOG	POOL SIZE (m-moles)
A	8.10 9.09 9.79 5.84 4.95 - -	A	4.71 5.94 3.72 5.77 4.18 4.43 4.92
B	11.25 10.92 9.50 - -	B	5.58 2.93 2.87 2.48 5.10
C	1.54 7.20 2.89 2.08 3.51 4.40	C	3.16 - - - - -
D	7.03 6.37 8.75 4.79	D	7.33 5.10 - -
	MEAN 6.60 S.E.M. 0.70 p	< 0.01	4.6 0.3

(1970) in the aforementioned studies postulated that a diminished pool size could be a major contributing factor to the production of abnormal hepatic bile in patients with gallstones. To accept this as a major initiating factor in gallstone formation requires further support. The findings presented here indicate that a reduction in the storage space in the extra hepatic biliary tree significantly reduces the bile salt pool size. It is therefore suggested, that the diminished pool size in patients with gallstones may be a result, and not a cause of the disease.

SUMMARY

The bile salt pool size was measured in the dog by a direct method before and after cholecystectomy. With the gallbladder intact, the pool size was 6.6 m-moles $\pm$ S.E. 0.7 and after cholecystectomy it was 4.6 m-moles $\pm$ S.E. 0.3. These values were significant.

From the data presented, it is suggested that a diminished bile salt pool in patients with gallstones may be a result, and not a cause of the disease.

PART 4.

THE EFFECT OF VAGAL STIMULATION BY INSULIN HYPOGLYCAEMIA ON THE SECRETION, AND STEROL AND LIPID COMPOSITION OF BILE IN THE DOG

In Part I of this thesis, it was shown that in a group of duodenal ulcer patients there was no significant alteration in the relative composition of bile in the eight to ten days immediately after vagotomy. One of the problems in the study of bile composition in patients is that information can only be obtained about the relative composition of bile, as there is no simple and safe way to measure total bile flow. Therefore, no information can be obtained about the rate of secretion of the various components of bile. In order to elaborate on the above clinical observations, dogs with chronic biliary fistulae were studied.

Before investigating the effects of vagotomy on the sterol and lipid composition of bile in the dog, it was decided firstly to study the effect of vagal stimulation. The method chosen for stimulating the vagus was the production of hypoglycaemia by the intravenous injection of insulin (Hollander, 1946).

Tanturi and Ivy (1938) in aforementioned studies, were the first to show that cervical stimulation of the vagus increased bile flow in the dog. This was confirmed by Fritz and Brooks (1963) who showed that vagal stimulation by means of insulin hypoglycaemia caused a marked choleresis in conscious dogs, and that the insulin response could be blocked by atropine. Baldwin et.al. (1965) studied patients with indwelling T-tubes following cholecystectomy, and found that insulin administration produced a significant increase in bile flow and that this could be blocked by atropine. The findings of Fritz and Brooks and Baldwin et.al. suggested that insulin choleresis was brought

about via the vagus.

The choleretic effect of insulin hypoglycaemia in dogs was also demonstrated by Geist and Jones (1971). These workers however failed to block the response with atropine, suggesting that the action of insulin was extra-vagal, for example by acting directly on the hepatic ductules, or indirectly by releasing another choleretic agent such as glucagon or gastrin. The suggested implication of gastrin in the process was based on previous studies by Jones and Brooks (1967) who showed that in dogs, removal of the pyloric antrum reduced the choleresis induced by insulin hypoglycaemia, indicating that gastrin might be implicated in the response.

Geist and Jones (1971) also showed that vagotomy did not prevent insulin choleresis. Powell, Miller and Brooks (1965) however found that sham-feeding, i.e. exposing the animal to the sight of food, had little effect on bile secretion, but the expected marked increase in gastric acidity took place.

These conflicting reports may be in part explained by the number of different experimental approaches employed. Some workers studied humans and some experimental animals. In the latter instance these had been conscious or anaesthetised preparations. In addition, some had used cholecystectomised animals, and others with intact gallbladders. Perhaps more important, some had studied animals with the enterohepatic circulation intact, or animals that were depleted of bile salts, or infused with bile salts during the experiments.

In the experiments now to be reported, dogs with chronic biliary fistulae have been used. This preparation has been described in Part 3 of this thesis, and permits total diversion of bile flow, yet maintains an intact enterohepatic circulation since 90 per cent of the bile is returned to the animal during the experiments.

METHODS

Six mongrel dogs weighing between 15 and 20 kg. were used. Chronic biliary fistulae were created (Mr. C. MacKay and Dr. J. Crook) by a procedure that has been described previously (Part 3).

After a three week recovery period, the animals were placed on Pavlov stands. The gastric cannula was opened to divert gastric juice from entering the duodenum. The biliary cannula was then opened, and bile allowed to drain into graduated tubes. The volume of bile was measured at 15 minute intervals, and 10 per cent of each sample retained. Four such samples were pooled to give specimens representing one hourly periods, and sufficient material for subsequent analysis. As was described previously (Part 3b) sampling of this order did not affect the composition of bile. The remainder of the samples was returned to the animal via its duodenostomy tube. In these experiments, no single animal was studied more than twice per week.

Bile samples were rapidly frozen, and kept so until analysed. Total bile salts, phospholipids and cholesterol were determined by the procedures already described. In addition, blood samples were taken

for the determination of blood glucose, by the method of Asatoor and King (1954).

Following a basal hour of collection, the animal was given insulin (0.4 U/kg.)* as a single intravenous injection, and the collection of bile continued for three hours. During this period, bile was collected and sampled as described above. In addition, one hour after the injection of insulin a blood sample was taken for blood glucose determination. The response to insulin was measured 15 times in the six animals under study.

A second series of experiments were then carried out two days later. The same regime was followed. After a basal hour of collection of bile from the fasting animal, insulin (0.4 U/kg.) was given intravenously together with atropine (0.04 mg./kg.) in a single 10 ml. bolus. Bile collections were then continued for a further three hours. This effect was measured eight times in the six animals under study.

Further studies were then made. In these experiments, after a basal hour of collection from the fasting animal, insulin was given intravenously at the same dose level, then one hour later, atropine (0.04 mg./kg.) was given by the same route. Bile samples were collected for three hours after the initial injection of insulin. This response was measured eleven times in the six animals.

* Soluble Insulin B.P. (Burroughs Wellcome & Co., London).

RESULTS

Blood glucose levels:

The mean blood glucose level in the six dogs in the fasting state was 94.66 mg. per 100 ml. ± S.D. 10.42. One hour after insulin, this fell to a mean of 30.4 ± S.D. 1.2 mg. per 100 ml.

After stimulation of the vagus by insulin hypoglycaemia, there was a significant increase in bile flow, that reached a peak level in the second hour after the stimulus, returning to normal in the third hour. This is shown in Figure 21 p. 66a. The data presented in Figures 22, 23 and 24, show the significant reduction that occurred in the concentrations of phospholipids, cholesterol and bile salts.

That the changes in concentrations were due to a water choleresis induced by insulin are shown in Figures 25, 26 and 27, where the mean output of the three solid components are shown. As will be seen, there was no significant change in bile salt output after insulin. The secretion of phospholipids and cholesterol showed little or no change, the p value in the third hour after insulin being only < 0.05.

When insulin (0.4 U/kg.) and atropine (0.04 mg./kg.) were given together in one single intravenous injection there was no significant increase in bile flow showing that atropine blocked insulin choleresis. This is shown in Figure 28 p. 68a. Again this caused a significant reduction in the concentrations of all three solids, the maximum being achieved during the second hour after the injection of insulin and atropine. These changes are shown in Figures 29, 30 and 31. The

administration of insulin and atropine together also effected a significant reduction in the output of phospholipids and bile salts (Figures 32 and 34). Cholesterol output was not affected to the same extent. As shown in Figure 33, the values were significant at the 0.05 level in the second and third hour following the injection.

When the experiments were repeated eleven times in the six animals under study by the intravenous injection of insulin (0.4 U/kg.) followed one hour later by atropine (0.04 mg./kg.) by the same route, there was a partial block of the choleresis induced by insulin hypoglycaemia. This is shown in Figure 35. As will be seen in Figures 36, 37 and 38, a significant fall in the concentration of phospholipids, cholesterol and bile salts occurred. The reduction in the concentration of cholesterol however did not reach a significant level ($p < 0.01$) until the first hour after the administration of atropine.

As shown in Figures 39, 40 and 41, the secretion of phospholipids, cholesterol and bile salts was inhibited due to atropine blockade. In contrast to that found when insulin and atropine were given together there was a significant reduction ($p < 0.01$) in the secretion of cholesterol. In general, a significant decrease occurred in the output of all three solids in the second hour following atropine administration.

Table 12, p. 71b, shows the relative composition of bile during these experiments. The results show that in the second hour after the administration of insulin there was a significant decrease in

cholesterol and phospholipids, and an increase in bile salts that also reached a significant level ($p < 0.001$). This effect was blocked by atropine either when it was given together with insulin, or one hour after insulin.

DISCUSSION

In these studies, vagal stimulation by means of insulin hypoglycaemia produced a significant increase in bile flow with an accompanying fall in the concentrations of bile salts, phospholipids and cholesterol. The net result was to produce no significant change in the output of these three solids. Insulin therefore produces a water choleresis.

To shed some light on the mechanism of insulin choleresis, atropine was given along with insulin. This abolished the choleretic effect of insulin but there was a significant fall in the concentrations and outputs of bile salts, phospholipids and cholesterol. This suggests that the action of insulin is mediated via the vagus and also that atropine itself inhibits the output of these three bile solids.

When atropine was given one hour after insulin the choleretic effect of insulin is seen to occur but to be abolished by the administration of atropine. It is also seen that in the fourth hour of the experiment, i.e. the second hour after the administration of atropine there is a significant decrease in the output of all three solids, thus confirming the conclusion that insulin acts via the vagal pathway, and atropine itself inhibits the output of cholesterol, phospholipids and bile salts.

These findings are in keeping with the work of Fritz and Brooks (1963) who showed that insulin choleresis in dogs was abolished by cutting the vagus nerves. They are however contrary to the findings of Geist, Jones and Hall (1970) who were unable to prevent insulin choleresis by atropine administration. Part of the reason for these discrepancies may be the different experimental models used. Geist et.al. artificially maintained the circulation of bile salts by the intravenous infusion of sodium taurocholate, whereas in the present investigation the enterohepatic circulation was maintained by returning 90 per cent of the bile to the duodenum. It has already been shown in Part 3 of this thesis, that removal of 10 per cent of the bile secreted produces no change in the bile volume or bile salt secretion.

The action of atropine in producing inhibition of the secretion of bile salts, phospholipids and cholesterol has not been reported previously and is at present being studied in our laboratory. It is possible that this effect is mediated by producing an alteration in hepatic haemodynamics rather than by a direct action on the hepatocyte.

Dog bile as has already been mentioned contains very little cholesterol and the relative composition lies far within the micellar zone of cholesterol solubility. Vagal stimulation by insulin administration caused a significant increase in bile salts and phospholipids relative to cholesterol, and this effect was blocked by atropine. It remains to be shown what the effect of removing vagal activity has on the relative composition of bile.

THE EFFECT OF VAGAL STIMULATION BY INSULIN HYPOGLYCAEMIA ON BILE FLOW AND CONCENTRATIONS OF PHOSPHOLIPIDS, CHOLESTEROL AND BILE SALTS

Values shown are the mean $\pm$ S.E. of 15 determinations in the six dogs under study. $_{o}$ = $p < 0.05$; $_{oo}$ = $p < 0.01$; $_{ooo}$ = $p < 0.001$

FIGURE 21: Hepatic bile output

FIGURE 22: Phospholipid concentrations

FIGURE 23: Cholesterol concentrations

FIGURE 24: Bile salt concentrations

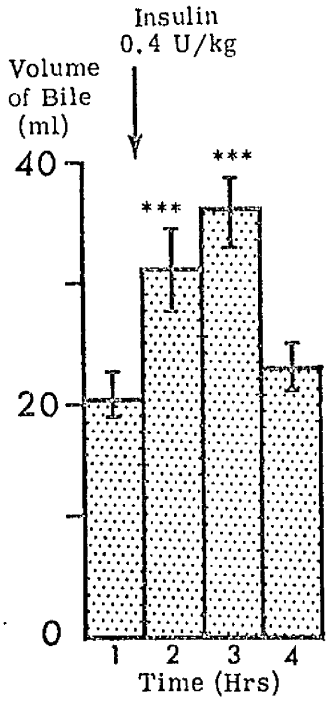


FIGURE 21.

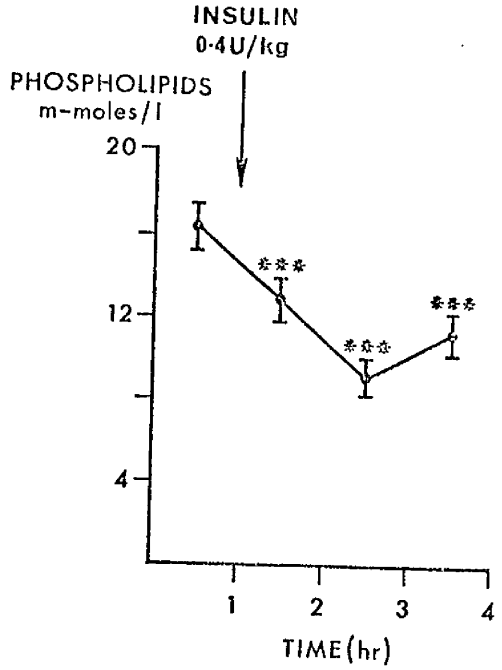


FIGURE 22.

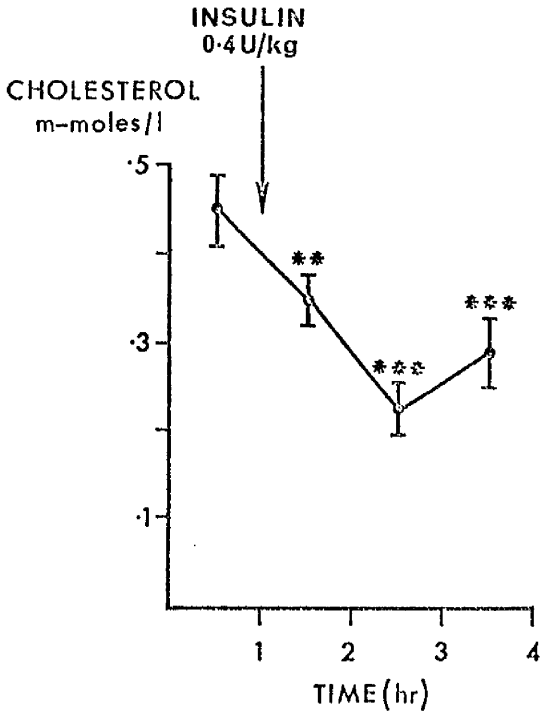


FIGURE 23.

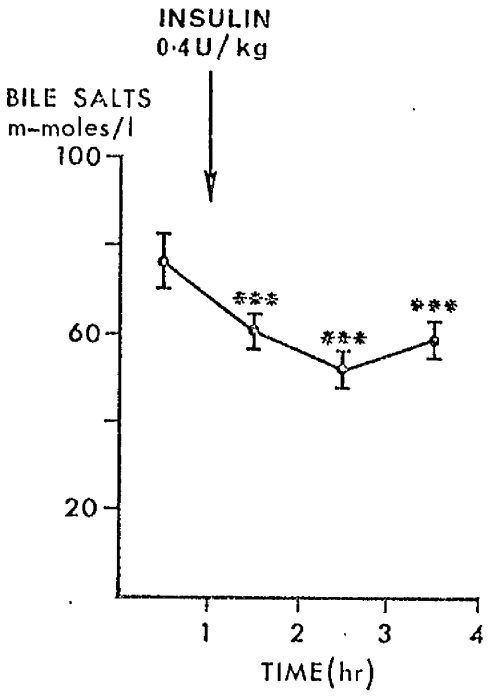


FIGURE 24.

THE EFFECT OF VAGAL STIMULATION BY INSULIN HYPOGLYCAEMIA ON THE OUTPUT OF PHOSPHOLIPIDS, CHOLESTEROL AND BILE SALTS

Values shown are the mean $\pm$ S.E. of 15 determinations in the six dogs under study. $\circ$ = $p < 0.05$.

FIGURE 25: Phospholipid output over the four hour period

FIGURE 26: Cholesterol output over the four hour period

FIGURE 27: Bile salt output over the four hour period

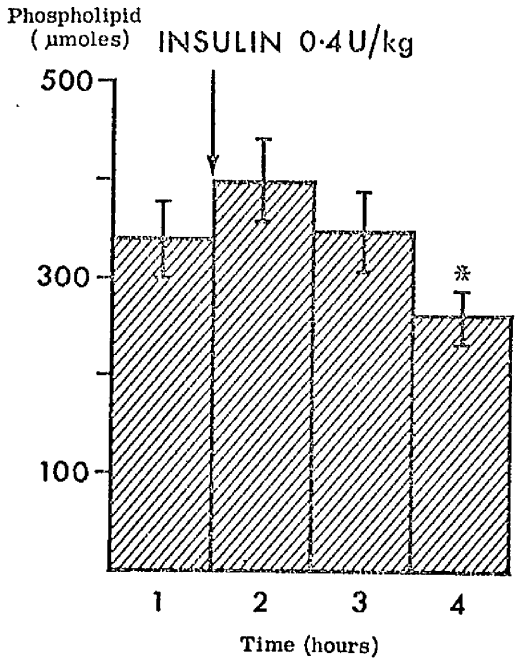


FIGURE 25.

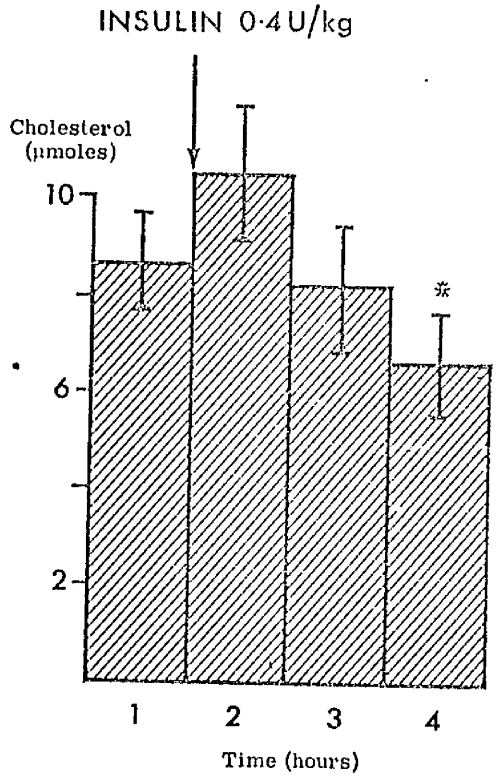


FIGURE 26.

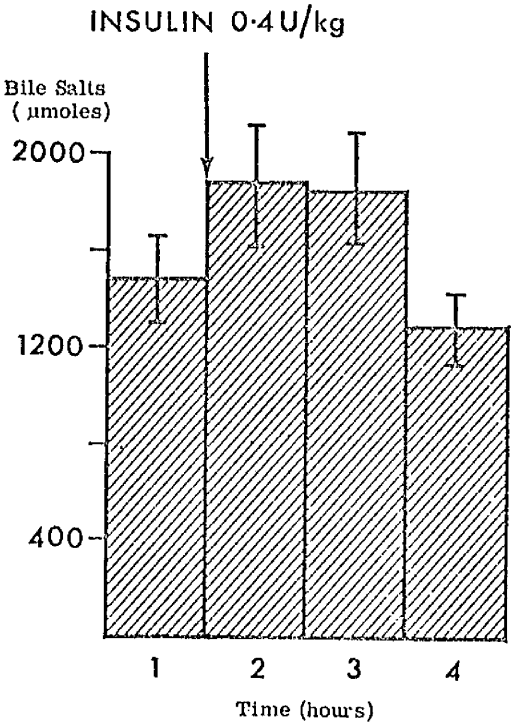


FIGURE 27.

THE EFFECT OF INSULIN AND ATROPINE TOGETHER ON BILE FLOW AND CONCENTRATIONS OF PHOSPHOLIPIDS, CHOLESTEROL AND BILE SALTS

Values shown are the mean of 8 determinations on the six dogs under study. $^{\circ}$ = $p < 0.05$; $^{\circ\circ}$ = $p < 0.01$; $^{\circ\circ\circ}$ = $p < 0.001$

FIGURE 28: Hepatic bile output

FIGURE 29: Phospholipid concentrations

FIGURE 30: Cholesterol cohcentrations

FIGURE 31: Bile salt concentrations

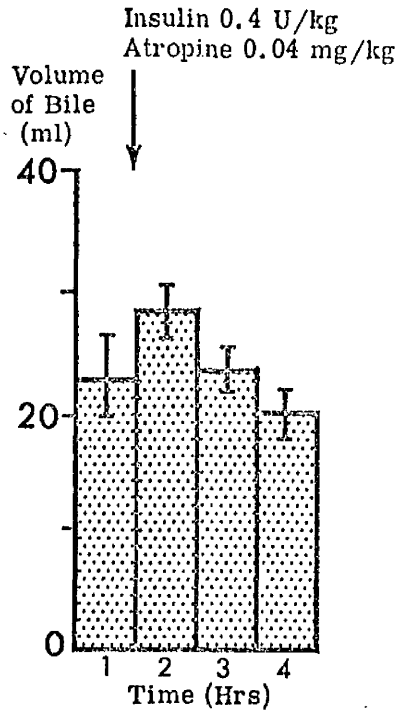


FIGURE 28.

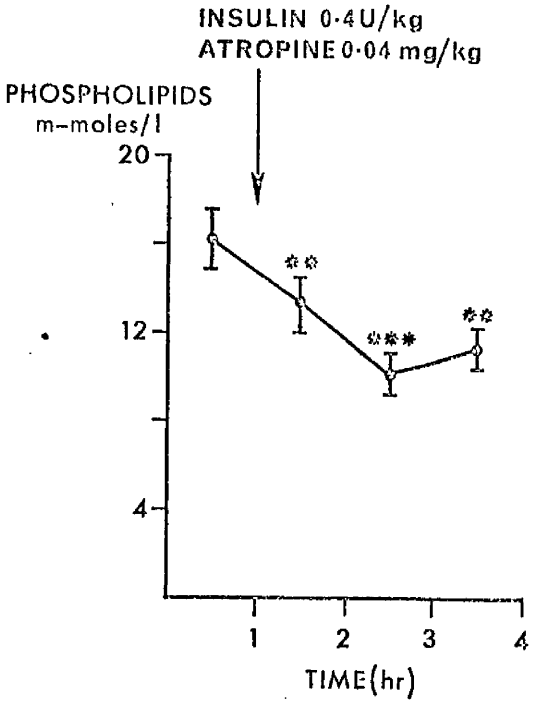


FIGURE 29.

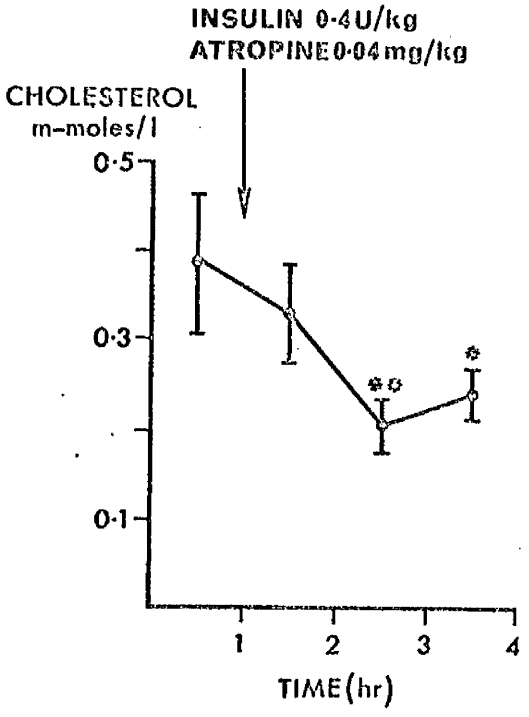


FIGURE 30.

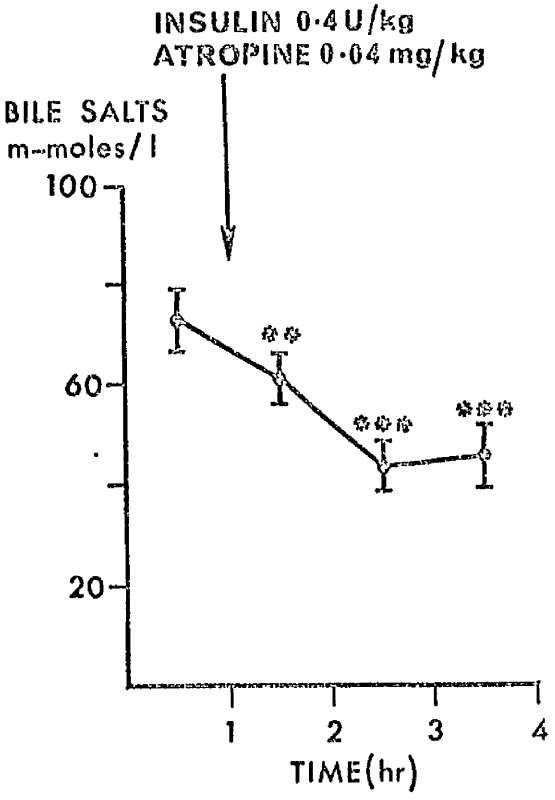


FIGURE 31.

THE EFFECT OF INSULIN AND ATROPINE TOGETHER ON THE OUTPUT OF PHOSPHOLIPIDS, CHOLESTEROL AND BILE SALTS

Values shown are the mean of 8 determinations on the six dogs under study. ^{o} = $p < 0.05$; ^{oo} = $p < 0.01$.

FIGURE 32: Phospholipid output over the four hour period

FIGURE 33: Cholesterol output over the four hour period

FIGURE 34: Bile salt output over the four hour period

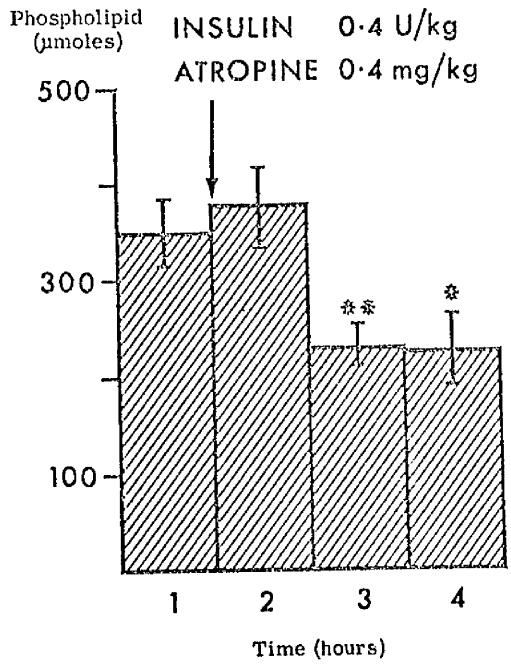


FIGURE 32.

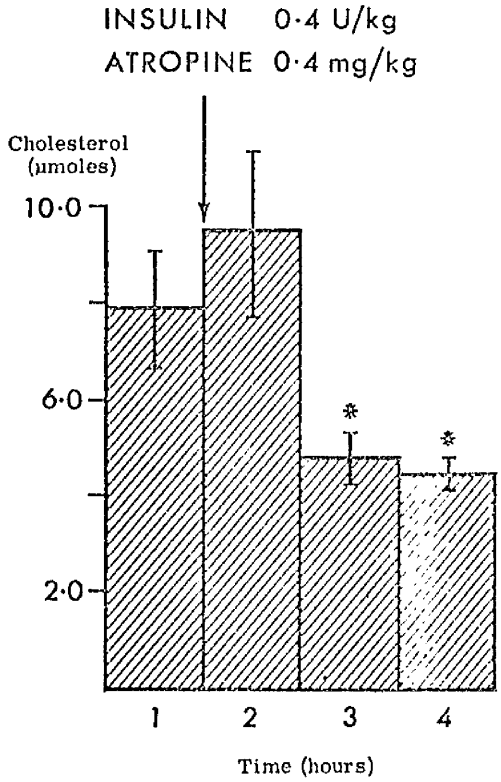


FIGURE 33.

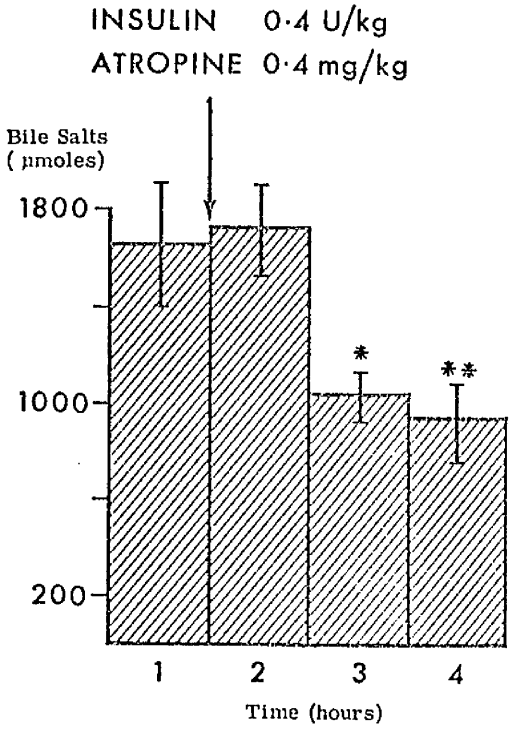


FIGURE 34.

THE EFFECT OF INSULIN FOLLOWED BY ATROPINE ONE HOUR LATER ON BILE FLOW AND CONCENTRATIONS OF PHOSPHOLIPIDS, CHOLESTEROL AND BILE SALTS

Values shown are the mean $\pm$ S.E. of 11 determinations in the six dogs under study. . = $p < 0.05$; .. = $p < 0.01$; ... = $p < 0.001$.

FIGURE 35: Hepatic bile output

FIGURE 36: Phospholipid concentrations

FIGURE 37: Cholesterol concentrations

FIGURE 38: Bile salt concentrations

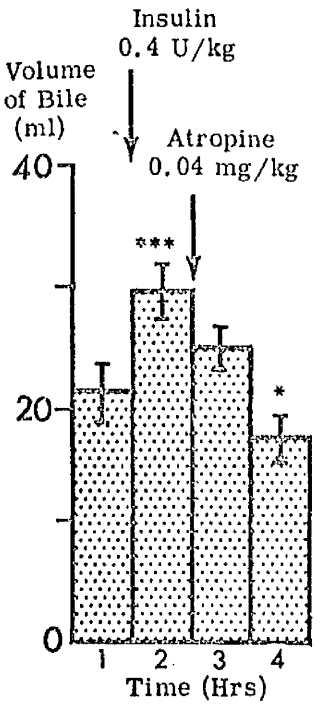


FIGURE 35.

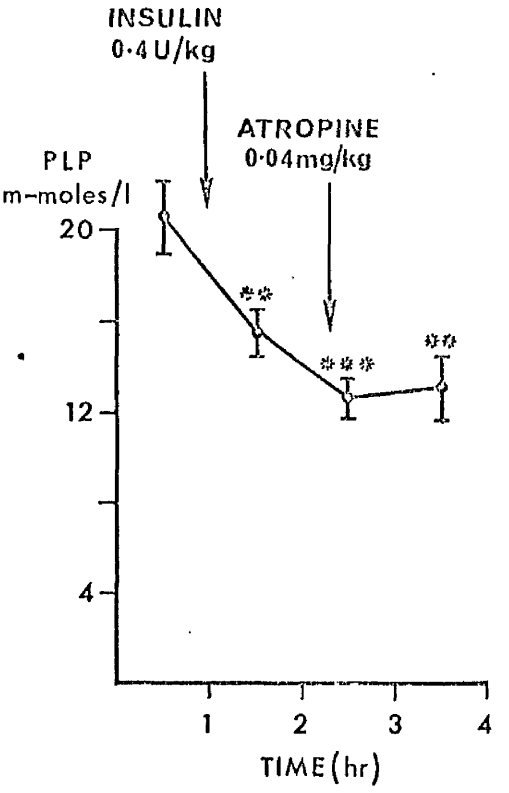


FIGURE 36.

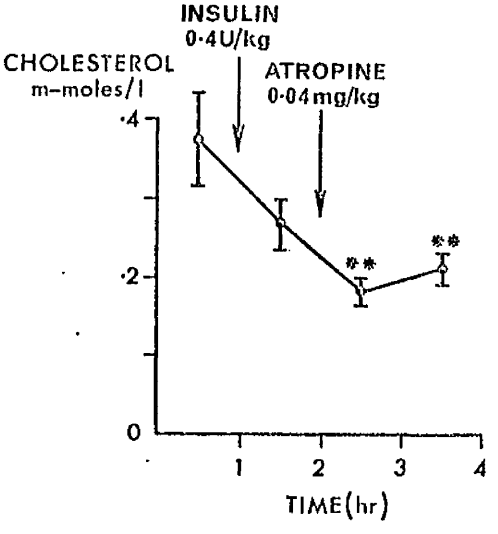


FIGURE 37.

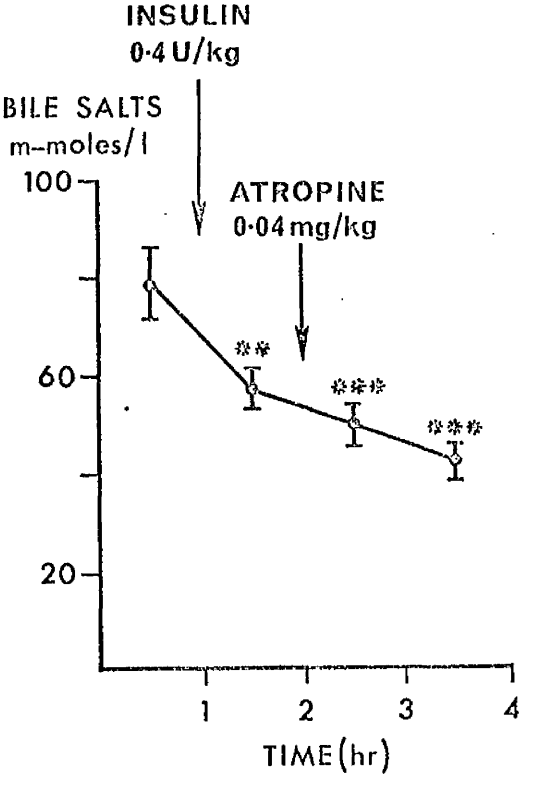


FIGURE 38.

THE EFFECT OF INSULIN FOLLOWED BY ATROPINE ONE HOUR LATER ON THE OUTPUT OF PHOSPHOLIPIDS, CHOLESTEROL AND BILE SALTS

Values shown are the mean $\pm$ S.E. of 11 determinations on the six dogs under study. . = $p<0.05$; .. = $p<0.01$; ... = $p<0.001$.

FIGURE 39: Phospholipid output over the four hour period

FIGURE 40: Cholesterol output over the four hour period

FIGURE 41: Bile salt output over the four hour period

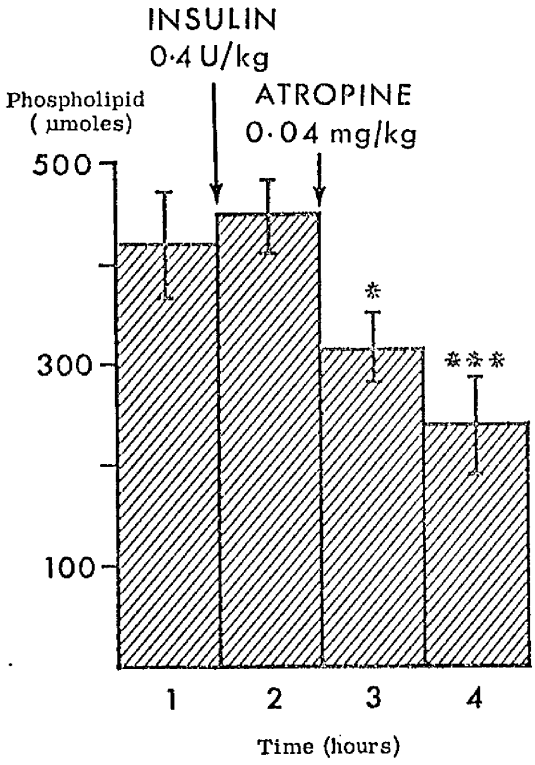


FIGURE 39.

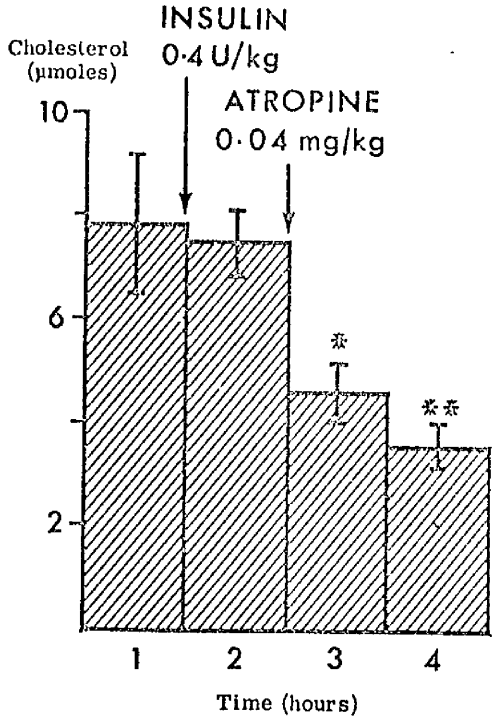


FIGURE 40.

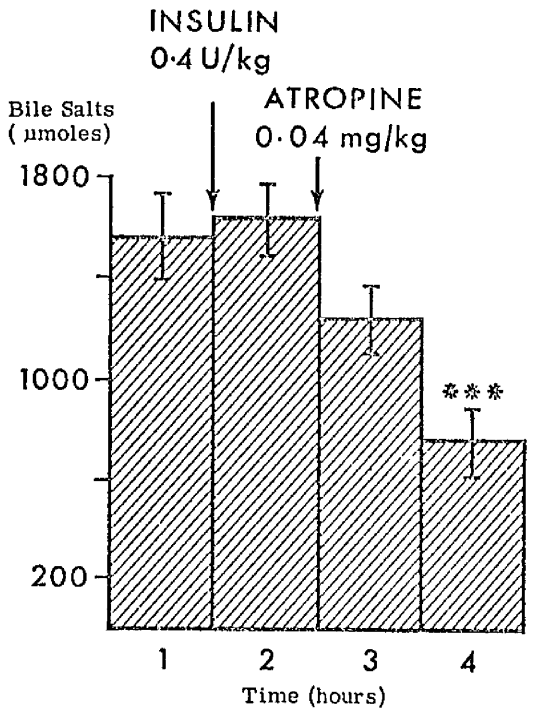


FIGURE 41.

TABLE 12.

THE EFFECT OF INSULIN AND ATROPINE ON THE RELATIVE COMPOSITION OF HEPATIC BILE

	INSULIN 0.4 units/kg.			INSULIN 0.4 units/kg. followed ONE HOUR LATER BY ATROPINE 0.04 mg/kg.			INSULIN 0.4 units/kg. AND ATROPINE 0.04 mg/kg. SIMULTANEOUSLY		
HOUR	CHOLESTEROL %	PHOSPHOLIPIDS %	BILE SALTS %	CHOLESTEROL %	PHOSPHOLIPIDS %	BILE SALTS %	CHOLESTEROL %	PHOSPHOLIPIDS %	BILE SALTS %
BASAL	0.48 ± 0.04	18.74 ± 0.64	80.76 ± 0.66	0.36 ± 0.04	21.26 ± 1.45	78.38 ± 1.44	0.44 ± 0.08	18.19 ± 0.92	81.32 ± 0.98
1	0.46 ± 0.04	18.51 ± 0.76	81.03 ± 0.77	0.36 ± 0.02	21.67 ± 1.57	78.01 ± 1.55	0.44 ± 0.05	17.98 ± 1.22	81.58 ± 1.25
2	0.37 ± 0.05 **	15.99 ± 0.51 ***	83.65 ± 0.53 ***	0.31 ± 0.03	20.85 ± 1.88	78.84 ± 1.90	0.40 ± 0.06	18.74 ± 1.36	80.85 ± 1.39
3	0.41 ± 0.04	17.65 ± 0.81	82.10 ± 0.79	0.39 ± 0.03	23.52 ± 1.46	76.09 ± 1.46	0.46 ± 0.09	20.17 ± 1.57	79.25 ± 1.40

* = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

All values represent the means ± S.E.

CONCLUSIONS

If information is to be obtained on the composition of bile that will lead to an understanding of gallstone formation, then sensitive and accurate methods of analysis are essential. The studies here presented, started with a careful assessment of procedures for the determination of cholesterol, phospholipids, and bile salts. During the early stages of these investigations a new colorimetric method for the determination of cholesterol was developed. This should also be a useful method for the determination of those 3β-hydroxysterols that do not react in the Liebermann-Burchard reaction.

Studies of the chemical composition of bile have often been made on specimens which have been stored in the frozen state. These specimens are thawed prior to analysis yet little attention has been paid to the effect of freezing and thawing on cholesterol solubilisation. In pursuing this possibility, it was found that considerable amounts of cholesterol were brought out of solution in gallbladder bile from patients with gallstones, by freezing and thawing the samples. It seems possible that failure to observe this in the past may account for some of the discrepancies in the literature.

It is still uncertain whether vagotomy effects changes in bile composition such as would predispose the patient to the later development of gallstones. In a preliminary study it was shown that bile obtained by duodenal intubation was representative of gallbladder bile. This method was used to obtain bile from a group of duodenal

ulcer patients before and after vagotomy and drainage. There was no significant alteration in the relative composition of bile in the 8 to 10 day period after operation. However this does not exclude the possibility of later changes in bile composition which might predispose to gallstone formation.

The above method of obtaining bile samples should also be of value in future studies, for example in studying the effect of diet on the composition of bile. At present it is being used to obtain information on normal individuals.

We have shown that gallstones can occur in the presence of hepatic bile which is saturated or even undersaturated with cholesterol. It would therefore appear that supersaturation of hepatic bile is not the sole factor responsible for the precipitation of cholesterol. Some other factors may be responsible for the precipitation of cholesterol from the mixed micelles of cholesterol, bile salts and phospholipid.

Because of the difficulty of accurately measuring the volume of bile secreted in patients, animal models were used in these studies. For this purpose, dogs with chronic biliary fistulae were prepared. This preparation assures total diversion of bile flow, and it has been shown that sampling only 10 per cent for the purpose of chemical analysis produces no significant change in the composition of bile. There is therefore no significant diversion of the enterohepatic circulation of bile salts.

Comparison of the relative composition of gallbladder and hepatic

bile in the dog has shown that the former contained significantly more bile salts and significantly less phospholipids than the latter. There was no significant difference in the relative amounts of cholesterol. When these data were plotted on triangular co-ordinates the relative composition of both gallbladder and hepatic bile lay well within the micellar zone of cholesterol solubility. This may be the reason why cholesterol gallstones are rarely found in dogs.

Measurement of the bile salt excretion and pool size in the dog showed that the mean pool size was 6.60 m-moles $\pm$ S.E. 0.7 with the gallbladder intact. After cholecystectomy it was 4.6 m-moles $\pm$ S.E. 0.3. From these data, and measurement of the bile salt output, it was calculated that the pool circulates about 6 times per day. The data also established that the pool size was large compared to man or monkey. This might be due to differences in the eating habits of the different species.

Since the bile salt pool is held mainly in the gallbladder, removal of the latter significantly reduces the space of the extra-hepatic biliary tree. This was used in the dog to examine a report that patients with gallstones had a significantly lower pool size than normals. In these animals a significant reduction in the pool size occurred as a result of cholecystectomy, and it is concluded that in patients, the presence of stones in the gallbladder may effectively reduce the pool size, suggesting that a reduced pool size may be a result, and not a cause of the disease.

The final part of this thesis elaborated on the clinical

observations made in Part I, which reported a study of bile composition before and after vagotomy. Dogs with chronic biliary fistulae were studied to assess the effects of vagal stimulation by insulin hypoglycaemia. The data showed that vagal stimulation by insulin hypoglycaemia produced a water choleresis. This could be blocked by atropine, either when given together with insulin, or one hour after insulin. The findings suggest that insulin choleresis is mediated by the vagus. In both situations, the observation was made for the first time, that atropine inhibited the secretion of bile salts, phospholipids and cholesterol.

REFERENCES

ADLERSBERG, D. and SOBOTKA, H. (1958) In Cholesterol p. 417. Ed. by Cook, R.P. New York Acad. Press.

ADMIRAND, W.H. and SMALL, D.M. (1968) J. clin. Invest., 47: 1043.

AMER, M.S. (1969) Endocrinology 84: 1277.

ANDERSON, F. and BOUCHIER, I.A.D. (1969) Nature (Lond.) 221: 372.

ANDREWS, E., SCHOENHEIMER, R. and HRDINA, L. (1932) Arch. Surg. 25: 796.

ASATOOR, A.M. and KING, E.J. (1954) Biochem. J., 56: xliv.

BALDWIN, J.N. HEER, F.W., ALBO, R., PELOSO, O., RUBY, L. and SILEN, W. (1966) Amer. J. Surg., 3: 66.

BARNETT, W.O. and HILBUN, G.R. (1966) Surgery 60: 840.

BARTLETT, G.R. (1959) J. biol. Chem., 234: 466.

BEHER, W.T. and ANTHONY, W.L. (1954) J. Nutr., 52: 519.

BOUCHIER, I.A.D. (1968) Brit. J. Hosp. Med., 1: 33.

BOUCHIER, I.A.D. (1970) Gut, 11: 799.

BOUCHIER, I.A.D. (1971) Lancet, 1: 711.

BOUCHIER, I.A.D. and FRESTON, J.W. (1968) Lancet 1: 340.

BOUCHIER, I.A.D. and COOPERBAND, S.R. (1967) Clin. chim. Acta, 15: 291.

BROOKS, F.P. (1969) Amer. J. dig. Dis., 14: 343.

BROWN, H.H., ZLATKIS, A., ZAK, B. and BOYLE, A.J. (1954) Analyt. Chem., 26: 397.

BURNETT, W. (1965) In The Biliary System, North Atlantic Treaty Organisation Advanced Study Institute. A Symposium of the Institute, Newcastle-upon-Tyne. (1963) pp. 601, 618. Ed. by Taylor, W. Oxford.

CALDWELL, F.T.Jr. and LEVITSKY, K. (1967) Ann. Surg., 166: 753.

CAMPBELL, B.A. and BURTON, A.C. (1949) Surg. Gynec. Obstet., 88: 731.

CHAPA, J.S. and ENGEL, G.C. (1959) Arch. Surg., 78: 307.

COMESS, L.J., BENNETT, P.H. and BURCH, T.A. (1967) New Engl. J. Med., 277: 894.

COOK, R.P. (1958) In Cholesterol, p. 155. Ed. by Cook, R.P. New York Academic Press.

CRAWFORD, N. (1955) J. med. Lab. Technol., 13: 304.

DISCHE, Z. (1947) Fed. Proc., 6: 278.

DISCHE, Z. (1955) In Methods of Biochemical Analysis, Vol. 2, p. 327. Ed. by Glick, D. New York/London Interscience.

DISCHE, Z. (1962) In Methods in Carbohydrate Chemistry, Vol. I, p. 488. Ed. by Whistler, R.L. and Wolfrum, M.L. New York Acad. Press Inc.

DISCHE, Z. and DANILCHENKO, A. (1967) Analyt. Biochem., 21: 119.

DOUGLAS, M.C., WATTS, M.M., JABLONSKI, P. and OWEN, J.A. (1969) Gut, 10: 79.

DOWLING, R.H., MACK, E. and SMALL, D.M. (1969) Clin. Res., 17: 301.

DOWLING, R.H., MACK, E. and SMALL, D.M. (1970) J. clin. Invest., 49: 232.

FEICHTMEIR, T.V. and BERGERMAN, J. (1953) Amer. J. clin. Path., 23: 599.

FLETCHER, D.M. and CLARK, C.G. (1968) Brit. J. Surg., 55: 895.

FLETCHER, D.M. and CLARK, C.G. (1969) Brit. J. Surg., 56: 103.

FLETCHER, D.M. and CORRY, R.L. (1968) Gut, 9: 129.

FREY, C., THORPE, C. and ABRAMS, G. (1968) Amer. J. Surg., 115: 75.

FRITZ, M.E. and BROOKS, F.P. (1963) Amer. J. Physiol., 204: 825.

GANSHIRT, H., KOSS, F.W. and MORIANZ, K. (1960) Arzneimittel Forsch., 10: 943.

GEIST, R.E. and JONES, R.S. (1971) Gastroenterology, 60: 566.

GEIST, R.E., JONES, R.S. and HALL, A.D. (1970) Clin. Res., 18: 129.

GREGORY, R.A. and TRACY, H.J. (1964) Gut, 5: 103.

GRIFFITHS, J.M.T. and HOLMES, G. (1964) Lancet, 2: 780.

GROSS, D.B.M. (1929) J. Path. & Bact., 32: 503.

HEATON, K.W. and READ, A.E. (1969) Brit. med. J., 3: 494.

HOFFMAN, A.F. and SMALL, D.M. (1967) Ann. Rev. Med., 18: 333.

HOLLANDER, F. (1946) Gastroenterology, 7: 607.

HORWITZ, A. and KIRSON, S.M. (1965) Amer. J. Surg., 109: 760.

HURLOCK, B. and TALALAY, P. (1957) J. biol. Chem., 227: 37.

INGELFINGER, F.J. (1968) Gastroenterology, 55: 102.

ISAKSSON, B. (1951) Acta Soc. med. Upsal., 56: 177.

ISAKSSON, B. (1953-1954) Acta Soc. med. Upsal., 59: 277.

JONES, D.D. (1968) Clin. chim. Acta., 19: 57.

JONES, R.S. and BROOKS, F.P. (1967) Amer. J. Physiol., 213: 1406.

JONNARD, R. and SCALERA, F. (1956) Clin. Chem., 2: 254.

JUNIPER, K. and BURSON, E.N. (1957) Gastroenterology, 32: 175.

KILIANI, O.G.T. (1945) In New and Nonofficial Remedies, p. 319. Chicago. American Medical Association.

KRAUSE, U. (1963) Acta Chir. Scand. Suppl., 310.

LEFFLER, H.H. (1959) Amer. J. clin. Path., 31: 310.

LEVINE, J.B. and ZAK, B. (1964) Clin. chim. Acta, 10: 381.

LIEBER, M.M. (1952) Ann. Surg., 135: 394.

LOEB, M. and NICOLOFF, D.M. (1968) Surgery, 63: 806.

LUNDMAN, T., ORINIUS, E. and THORSEN, G. (1964) Acta chir. scand., 127: 130.

McALLISTER, R.A. (1971) Analyt. Biochem., 43: 647.

McALLISTER, R.A., CROOK, J.N., SMITH, D.C. and MacKAY, C. (1972) Biochem. Med. In the Press.

MAJOOR, C.L.H. and SUREN, T.J.J. (1947) Brit. med. J., 2: 8.

MAKI, T., SATO, T., YAMAGUCHI, I. and SAITO, Y. (1964) Tohoku J. exp. Med., 84: 37.

MARTINEK, R.G. (1966) J. Amer. med. Technol., 28: 123.

MUELLING, R.J. and COPELAND, B.E. (1967) Amer. J. clin. Path., 47: 654.

NAKAYAMA, F. (1966) Clin. chim. Acta, 14: 171.

NAKAYAMA, F. (1969) J. Lab. clin. Med., 73: 623.

NAKAYAMA, F. and JOHNSTON, C.G. (1960) Proc. Soc. exp. Biol. Med., 104: 73.

NAKAYAMA, F. and MIYAKE, H. (1966) J. Lab. clin. Med., 67: 78.

NAKAYAMA, F. and VAN DER LINDEN (1970) Acta chir. scand., 136: 605.

NEIDERHISER, D.H., ROTH, H.P. and WEBSTER, L.T. (1966) J. Lab. clin. Med., 68: 90.

NILSSON, S. (1970) Acta chir. scand. Suppl., 405.

NILSSON, S. and SCHERSTEN, T. (1969) Gastroenterology, 57: 525.

NORMAN, A. (1965) In The Biliary System, North Atlantic Treaty Organisation Advanced Study Institute. A Symposium of the Institute, Newcastle-upon-Tyne. (1963) p. 165. Ed. by Taylor, W. Oxford.

OKEY, R. (1930) J. biol. Chem., 88: 367.

PHILLIPS, G.B. (1960) Biochim. biophys. Acta, 41: 361.

POWELL, K.C., MILLER, L.C. and BROOKS, F.P. (1965) Proc. Soc. exp. Biol. Med., 118: 481.

RAINS, A.J.H. (1962) Brit. med. J., 685: 5306.

RAINS, A.J.H. (1964a) Gallstones, Causes and Treatment, p. 13. London Heinemann Medical Books.

RAINS, A.J.H. (1964b) Gallstones, Causes and Treatment, p. 11. London Heinemann Medical Books.

RAINS, A.J.H. (1964c) Gallstones, Causes and Treatment, p. 88. London Heinemann Medical Books.

RAPPAPORT, F. and KLAPHOLZ, R. (1933) Biochem. Ztschr., 258: 467.

SARLES, H., CROTTE, C., GEROLANI, A., MULLE, A., DOMINGO, N. and MAUTON, J. (1971) Scand. J. Gastroent., 6: 189.

SCHERSTEN, T., NILSSON, S. and CAHLIN, E. (1970) Scand. J. Gastroent., 5: 473.

SMALL, D.M. (1968) New Engl. J. Med., 279: 588.

SMALL, D.M. (1970) Advanc. intern. Med., 16: 243.

SMALL, D.M., BOURGES, M. and DERVICHIAN, D.G. (1966) Nature (Lond.), 211: 816.

SMALL, D.M. and RAPO, S. (1970) New Engl. J. Med., 283: 53.

SPERRY, W.M. and WEBB, M. (1950) J. biol. Chem., 187: 97.

SPITZER, H.L., KYRIAKIDES, E.C. and BALINT, J.A. (1964) Nature (Lond.), 204: 288.

TANTURI, C.A., IVY, A.C. (1938) Amer. J. Physiol., 121: 270.

TERA, H. (1960) Acta chir. scand. Suppl., 256.

TERA, H. (1963) Ann. Surg., 157: 468.

THISTLE, J.L. and SCHOENFIELD, L.J. (1971) New Engl. J. Med., 284: 177.

THUREBORN, E. (1962) Acta chir. scand. Suppl., 303.

THUREBORN, E. (1965) Arch. Surg., 91: 952.

TOMPKINS, R.K., KRAFT, A.R. and ZOLLINGER, R.M. (1970) Surg. Forum, 21: 396.

TOMPKINS, R.K., BURKE LINDA, G., ZOLLINGER, R.M. and CORNWELL, D.G. (1970) Ann. Surg., 172: 936.

TONKS, D.B. (1967) Clin. Biochem., 1: 12.

TURUNEN, M. and ANTILA, L. (1964) Acta chir. scand., 127: 134.

VAHOUNY, G.V., BORJA, C.R., MAYER, R.M. and TREADWELL, C.R. (1960) Analyt. Biochem., 1: 371.

VAHOUNY, G.V., MAYER, R.M., ROE, J.H. and TREADWELL, C.R. (1960) Arch. Biochem. Biophys., 86: 210.

VAN DER LINDEN, W., and NORMAN, A. (1967) Acta chir. scand., 133: 307.

VARLEY, H. (1964) Practical Clinical Biochemistry 3rd. ed., p. 241. London. Wm. Heinemann.

VLAHCEVIC, Z.R., COOPER BELL, C. and SWELL, L. (1970) Gastroenterology, 59: 62.

VLAHCEVIC, Z.R., COOPER BELL, C., BUHAC, I., FARRAR, J.T. and SWELL, L. (1970) Gastroenterology, 59: 165.

VLAHCEVIC, Z.R., COOPER BELL, C., JUTTIJUDATA, P. and SWELL, L. (1971) Dig. Dis., 16: 797.

WEBSTER, W.W., NICHOLS, C.W. and CHAIKOFF, I.L. (1959) Arch. Biochem. Biophys., 82: 195.

WINDAUS, A. (1909) Ber. Deut. Chem. Ges., 42: 238.

WOMACK, N.A., ZEPPA, R. and IRVINE, G.L. (1963) Ann. Surg., 157: 670.

YOUNG, D.S. and MEARS, T.W. (1968) Clin. Chem., 14: 929.

ZAK, B., LUZ, D.A. and FISHER, M. (1957) Amer. J. Med. Technol., 23: 283.

ZAK, B. and ZLATKIS, A. (1953) Abstr., p. 61. 12th. Meeting Amer. Chem. Soc.

DETERMINATION OF 3 β-HYDROXYSTEROLS BY THE CYSTEINE-SULPHURIC ACID REACTION

by

R.A. McALLISTER

J.N. CROOK

D.C. SMITH

C. MACKAY

University Department of Surgery,

Western Infirmary, Glasgow, W.1.

Cholesterol is the major constituent of human gallstones[1,2]. This substance is totally insoluble in water and is maintained in aqueous solution in bile by the formation of mixed micelles with bile salts and phospholipids. The relative amounts of these three substances present in bile have been shown to be important in determining its physicochemical characteristics[3], and their estimation is a necessary adjunct in the study of gallstone disease. Fletcher and Clark[4] have reported that gallstones are more commonly found in patients who have previously undergone gastric surgery, and at present we are studying this problem in patients and in dogs with chronic biliary fistulae.

Since little if any of the cholesterol in human and canine bile is present in an esterified form[5,6] we precipitate the sterol directly as the digitonide from isopropanol extracts of the bile samples, and determine the cholesterol content of the precipitates by the method of Sperry and Webb[7]. Due, however, to the relatively low levels of cholesterol in the normal hepatic bile of the dog[8] the above procedure was found to be too insensitive for the determination in some of our experiments, where cholesterol levels of the order of 0.2 to 0.5 m. mole/l. were encountered.

We describe here a method that has been developed for the determination of as little as 10 µg. of cholesterol in extracts of bile and tissue homogenates. Our procedure is based upon the colorimetric determination of the hexose moities in the side chain of cholesterol digitonide by means of the cysteine-sulphuric acid reaction[9], and has

afforded a method that is about ten times more sensitive than the Sperry-Webb procedure[7], and gives also a sixfold gain in sensitivity over methods[10,11] using the anthrone reaction.

MATERIALS AND METHODS

Cholesterol with an analytical specification according to Young and Mears[12] was obtained from the British Drug Houses. Sulphuric acid, glacial acetic acid, diethyl ether, methanol, digitonin, hydrochloric acid, acetone, ethanol and isopropanol were all 'Analar' grade (British Drug Houses). L-Cysteine hydrochloride was reagent grade.

Preparation of Standard Solutions

159 mg. digitonin was dissolved in glacial acetic acid and made to 100 ml. with this solvent. Prior to use this was diluted 1 in 2 and 1 in 5 with glacial acetic acid to give solutions containing 79.5 and 31.8 µg. of the saponin in 0.1 ml. On the basis of the formation of a 1:1 molecular complex of cholesterol and digitonin[13], these amounts are equivalent to 25 and 10 µg. of the sterol respectively. For use as an internal standard in the method 1 ml. of cholesterol standard (30 mg. cholesterol in 100 ml. of isopropanol) was used throughout.

All samples of bile were rapidly frozen after collection and stored at -25°C until analysed. Bile from the gallbladder was centrifuged prior to analysis.

Procedure for Cholesterol Determination

An amount of the sample, usually 0.1 to 0.5 ml. and containing

approximately 0.04 to 0.20 mg. cholesterol, was added to 4.5 ml. of isopropanol in tapered centrifuge tubes. After mixing, the samples were allowed to stand for 10 minutes at room temperature and then centrifuged. Three millilitres of the supernatants were removed and transferred to similar tubes. At the same time, 1 ml. of cholesterol standard, containing 0.3 mg. of the sterol in isopropanol, was added in duplicate to centrifuge tubes containing 2 ml. of isopropanol. To each was then added 1.5 ml. of digitonin (1% w/v digitonin in methanol). The contents of the tubes were then mixed on a vortex mixer, and 1.5 ml. of water added to each. The contents of the tubes were again mixed and allowed to stand overnight in the dark.

The tubes were then centrifuged, and the supernatants discarded. To minimise contamination of the necks of the tubes we remove most of the supernatants by mild suction through a fine glass capillary. The remainder is then removed by drainage over filter paper.

Purification of Cholesterol Digitonides

This was carried out by the method of Vahouny, et.al.[10]. Two ml. of acetone:ether (1:2) were added to the precipitates which were then suspended with a fine glass rod. The tubes were then centrifuged for 10 minutes at 3000 r.p.m. The supernatants were removed and 2 ml. of boiling acetone:water (1:1) added. After again mixing with a glass rod, the tubes were again centrifuged. The supernatants were removed, and the washing process with boiling acetone-water repeated. After again centrifuging, the supernatants were removed and the tubes drained over filter paper.

The digitonides were then dissolved in glacial acetic acid, 1 ml. of the latter being used for the standards, and 0.5 to 0.2 ml. for hepatic bile samples. Solution was effected by placing the tubes in near boiling water for a few minutes. After cooling, 0.1 ml. of each was transferred to glass-stoppered test tubes. A blank consisting of 0.1 ml. of glacial acetic acid was also set up, as were standards consisting of 0.1 ml. of digitonin standards in glacial acetic acid equivalent to 10 and 25 µg. of cholesterol respectively.

To each was then added 0.4 ml. of water, followed by 0.5 ml. of 1.5N HCl, and the tubes placed in crushed ice for about 10 minutes. Five ml. of 6:1 (v/v) H_2SO_4 was then added to each, the tubes shaken after 1 minute, then placed in tap water for 2 minutes. The tubes were then heated for exactly 3 minutes in a boiling water bath, then cooled in tap water. 0.1 ml. of cysteine reagent (freshly prepared 3% w/v cysteine hydrochloride in deionised water) was then added to each, the tubes stoppered and vigorously shaken then allowed to stand at room temperature for 18 hours. The extinctions of the yellow coloured products were then determined at 414 nm. with the blank in the reference cell.

Recovery Values for Sterols Added to Rat Liver Extracts

One gm. of rat liver was homogenised in 25 ml. of acetone:ethanol (1:1) and the tube and its contents heated to ebullition. After cooling the extract was made to 100 ml. with the solvent mixture, the proteins removed by filtration, and 5 ml. aliquots of the filtrate taken

to dryness. To other 5 ml. aliquots were added 100 µg. cholesterol, and to a similar series, 50 µg. of cholesterol and 50 µg. of its saturated analogue 5α-cholestan-3 β-ol. These were taken to dryness as before, and all residues redissolved in 3 ml. of isopropanol.

The sterol digitonides were precipitated and purified as described previously, then dissolved in the appropriate amount of glacial acetic acid. Free cholesterol was then determined by the method of Sperry and Webb[7], and the total β-hydroxysterols by the proposed method.

Recovery values for added sterols are shown in Table 1. Each figure represents the mean of 4 determinations, with standard deviations.

In further experiments, 4 ml. of bile from a patient with a T-tube in the common bile duct was extracted with 90 ml. of isopropanol at room temperature for 30 minutes, and the extract filtered. Ten 3 ml. aliquots were taken and the sterols precipitated with digitonin as described previously. The cholesterol content of the digitonides was determined by the Sperry and Webb method[7], and gave a mean value of 2.71 m.mole/l. with a standard deviation of 0.08. A further ten 3 ml. aliquots were taken and the 3β-hydroxysterols determined by the cysteine-sulphuric acid reaction. These gave a mean value of 2.72 m.mole/l., with a standard deviation of 0.09.

RESULTS AND DISCUSSION

When standard solutions containing up to 30 µg. of cholesterol were put through the method, there was a linear relationship between the extinctions at 414 nm. and concentration, and close agreement as shown in Figure 1 with the results obtained with equivalent amounts of free digitonin. This also demonstrates the efficacy of the washing procedure used to free digitonin from the cholesterol digitonides[10]. Vahouny and his co-workers[10] have shown that the precipitation of sterol digitonides can be effected in 15 minutes by the use of aluminium chloride as a gathering agent, and it would appear that this technique could be applied in our method, thereby obviating the overnight precipitation step.

In Figure 2 are shown the absorption curves of the products of the reaction with cholesterol digitonide and digitonin. Both gave similar absorption maxima at 410-420 nm. In earlier experiments, we used a cysteine-sulphuric acid reaction[14], in which HCl was absent, and the H_2SO_4 content slightly less, but with otherwise similar reaction conditions, and found that digitonin and cholesterol digitonide gave, in the primary reaction, absorption maxima at 410-412 nm. In this case, measurements were made one hour after the addition of cysteine, since as Dische[14] has pointed out, there is a shift in the maxima after this time, particularly in the presence of galactose. In the above procedure, pentoses do not react[14], and from our results, which are in agreement with those obtained with the free hexoses by Dische[14] and the data presented in Figure 2, we assume that the colour reaction with digitonin

and cholesterol digitonide is due to the hexoses (2 molecules glucose plus 2 molecules galactose) which together with xylose (1 molecule) comprise the glycosidic side chain at C_3 in the saponin.

The difference between the total digitonin precipitable sterols as determined by the anthrone reaction, and that for cholesterol by the Liebermann-Burchard reaction[15] has been used as a measure of the saturated 3β hydroxysterol, cholestanol in plasma and tissues. In guinea pig bile, cholestanol was only tentatively identified by Schoenfield and Sjovall[16] using GLC, and working with canine hepatic bile, we find either duplicate or slightly higher results with our procedure than those obtained with the Liebermann-Burchard reaction, which in the absence of interfering substances, may be due to the small amounts of saturated sterols which have been stated to occur in hepatic and gallbladder bile[17]. This is the object of further study.

SUMMARY

A colorimetric method for the determination of 3β hydroxysterols in bile and tissue has been described. This is based upon the reaction of the purified sterol digitonides with cysteine and sulphuric acid. Spectrophotometric data has been presented, and evidence that the reaction depends upon the presence of hexoses in digitonin. The accuracy of the proposed method has been assessed and close agreement obtained with results for free cholesterol using the Liebermann-Burchard reaction. The sensitivity of the method is such that 10 μg. of cholesterol in bile or tissue extracts can be determined.

REFERENCES

1. Schersten, T., Nilsson, Sv. and Cahlim, E. Scand. J. Gastroent., 5: 473. (1970).

2. Nakayama, F. J. Lab. & Clin. Med., 73: 623. (1969).

3. Admirand, W.E. and Small, D.M. J. Clin. Invest., 47: 1043. (1968).

4. Fletcher, D.M. and Clark, C.G. Brit. J. Surg., 55: 895. (1968).

5. Isaksson, B. Acta Soc. Med. Upsaliensis Lix., 237. (1953-1954).

6. Norman, A. in "The Biliary System" (Taylor, W., ed.) p.165. Blackwell Scientific Publications, Oxford, 1965.

7. Sperry, A. and Webb, M. J. Biol. Chem., 187: 97. (1950).

8. Adlersberg, D. and Sobotka, H. in "Cholesterol" (Cook, R.P., ed.) p. 417. Academic Press, New York. (1958).

9. Dische, Z. and Damilchenko, A. Anol. Biochem., 21: 119. (1967).

10. Vahouny, G.V., Borja, C.R., Mayer, R.M. and Treadwell, C.R. Anal. Biochem., 1: 371. (1960).

11. Feichtmeir, T.V. and Bergerman, J. Amer. J. Clin. Path., 23: 599. (1953).

12. Young, D.S. and Mears, T.W. Clin. Chem., 14: 929. (1968).

13. Windaus, A. Ber., 42: 238. (1909).

14. Dische, Z. in "Methods of Biochemical Analysis" (Glick, D., ed.) vol. 2, p. 327. Interscience, New York/London, (1955).

15. Webster, W.W., Nichols, C.W. and Chaikoff, I.C. Arch. Biochem. Biophys., 82: 195. (1959).

16. Schoenfield, L.J. and Sjövall, J.S. Acta Chem. Scand., 20: 1297 (1966).

17. Cook, R.P. in "Cholesterol" (Cook, R.P., ed.) p. 155. Academic Press, New York (1958).

TABLE 1.

RECOVERY OF CHOLESTEROL AND 3β-CHOLESTANOL FROM RAT LIVER EXTRACTS

	3β Hydroxy Sterols (Cysteine-Sulphuric Acid Reaction)		Cholesterol (Sperry & Webb Method)	
	Total (µg./5 ml. extract)	Recovery (%)	Total (µg./5 ml. extract)	Recovery (%)
Free sterols	84.55 ± 0.69	-	86.21 ± 1.20	-
Free sterols + 100 µg. cholesterol	182.90 ± 0.93	98.45	184.71 ± 0.59	98.66
Free sterols + 50 µg. cholesterol + 50 µg. 3β cholestanol	181.83 ± 1.24	98.53	134.13 ± 0.77	99.46[a]

a = 3β-cholestanol does not give a colour in the Liebermann-Burchard reaction consequently the recovery value reported here refers only to added cholesterol.

FIGURE 1.

LINEARITY OF THE REACTION WITH CHOLESTEROL DIGITONIDE (o--o--o)

AND EQUIVALENT AMOUNTS OF DIGITONIN (x--x--x)

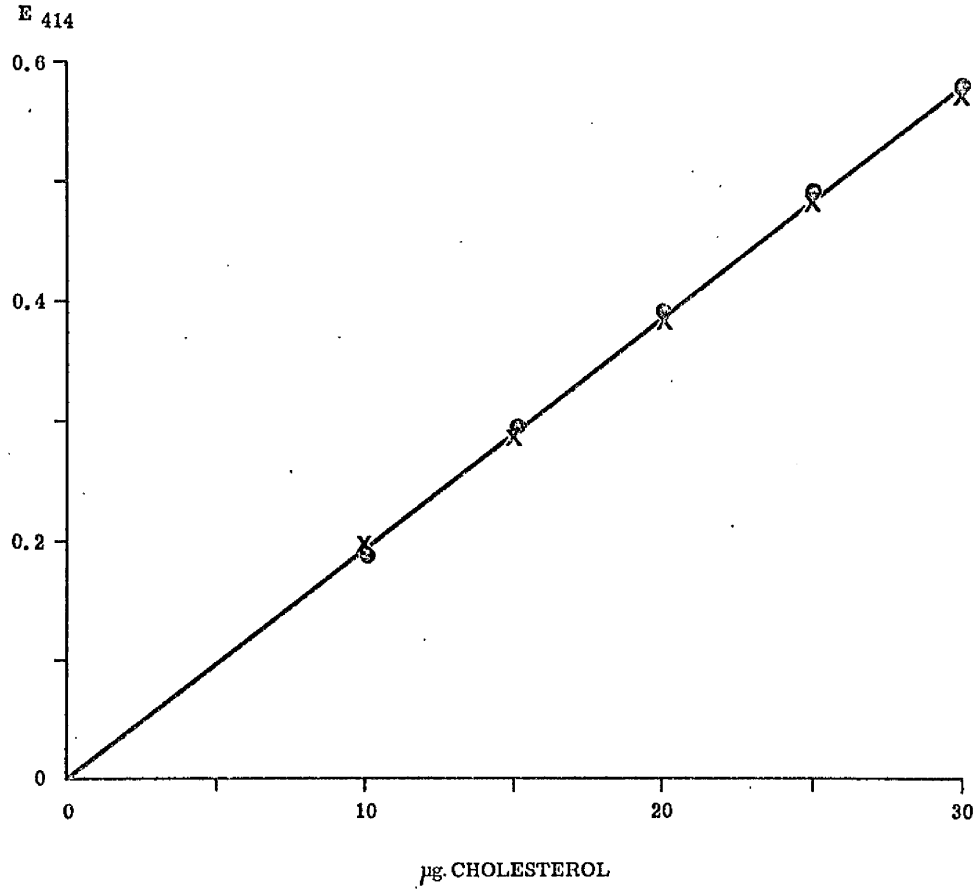
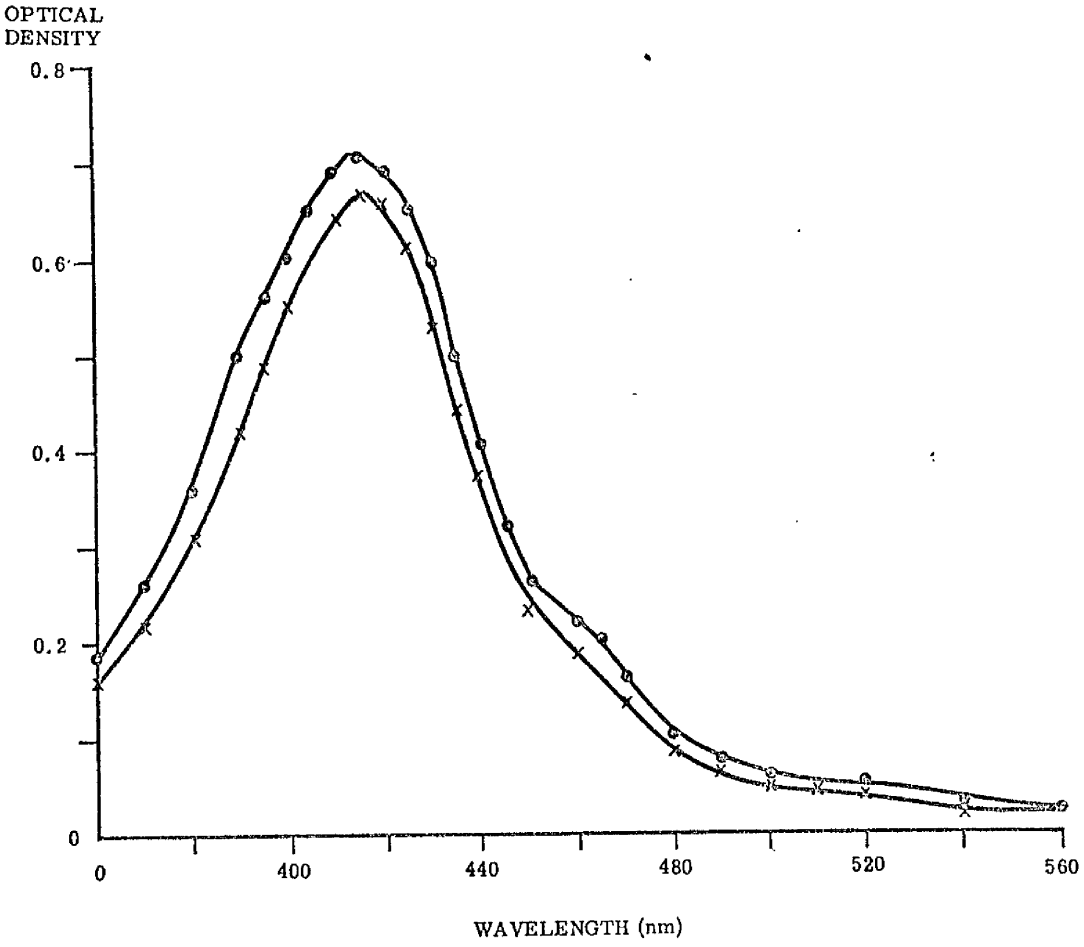


FIGURE 2

ABSORPTION CURVES OF THE REACTION WITH DIGITONIN (x--x--x)

AND CHOLESTEROL DIGITONIDE (o--o--o)



PRESENTATIONS TO THE SURGICAL RESEARCH SOCIETY

1. The Composition of Hepatic and Gallbladder Bile in Patients with Gallstones.

   By C. MacKay, J.N. Crook, D.C. Smith and R.A. McAllister (July, 1971).

2. The Effect of Cholecystectomy on the Size of the Bile Salt Pool in the Dog.

   By J.N. Crook, D.C. Smith, R.A. McAllister and C. MacKay (July, 1971).

3. Comparison of Gallbladder Bile in Gallstone Patients and in Duodenal Ulcer Patients Before and After Vagotomy.

   By D.C. Smith, J.N. Crook, R.A. McAllister and C. MacKay (January, 1972).

STUDIES ON THE STEROL AND LIPID COMPOSITION OF BILE

SUMMARY

The initial stage of these investigations was concerned with estimates of the precision of analytical methods for the determination of phospholipids, cholesterol and bile salts in human and canine bile samples. During the course of these studies a new method for the determination of cholesterol was developed. This was based on the determination of the hexoses present in the side chain at $C_{(3)}$ in cholesterol digitonide, by means of the cysteine-sulphuric acid reaction. The procedure was found to give an approximate tenfold gain in sensitivity over the Sperry and Webb method, and was about six times more sensitive than procedures using the anthrone reaction.

Since bile samples in these investigations were stored in the frozen state, the effect of freezing and thawing on cholesterol solubilisation in bile was studied. Data were presented to show that considerable amounts of cholesterol were brought out of solution in gallbladder bile from patients with gallstones as a result of freezing and thawing. It was suggested that this finding might explain some of the discrepancies found in the literature.

The first part of the thesis reported a study that was made of the supposed increase in the incidence of gallstone disease in patients who have undergone vagotomy. Initially it was shown that bile obtained by duodenal intubation following the injection of cholecystokinin was representative of gallbladder bile. This method was then used to obtain

bile samples from a group pre-operative duodenal ulcer patients and pre-operative gallstone patients, the latter group serving as controls. Samples of bile so obtained were analysed for cholesterol, phospholipids and bile salts. When the relative compositions were plotted on phase diagrams, bile from gallstone patients lay close the limits of cholesterol solubility, whereas bile from pre-operative duodenal ulcer patients lay within the micellar zone. The determinations were then repeated on the duodenal ulcer patients after vagotomy and drainage. No significant changes could be found in bile from these patients in the 0 to 10 day period after operation. This did not exclude the possibility of later changes in bile composition that might predispose to gallstone formation.

In the second part of this thesis, a study was made of the sterol and lipid composition of hepatic and gallbladder bile in patients with gallstones. When the relative compositions were plotted on phase diagrams, hepatic bile from these patients was found to be saturated with cholesterol, whereas gallbladder bile lay within the micellar zone. It was concluded that supersaturation of hepatic bile is not the sole factor responsible for the precipitation of cholesterol.

Part 3 described a model for the study of bile flow in the dog. This permitted total diversion of bile flow. No significant changes in bile composition were found to take place if ten per cent was sampled for chemical analysis. There was therefore no significant diversion of the enterohepatic circulation of bile salts.

When the relative compositions of canine gallbladder and hepatic

bile were compared, the former was found to contain significantly more bile salts and significantly less phospholipid than the latter. There was no significant difference in the relative amounts of cholesterol. When these data were plotted on phase diagrams, both gallbladder and hepatic bile lay well within the micellar zone. This might explain why cholesterol gallstones are rare in dogs.

The bile salt excretion and pool size were measured in dogs by a direct method. With the gallbladder intact, the pool size was 6.60 m-moles ± S.E. 0.7. After cholecystectomy it was 4.6 m-moles ± S.E. 0.3. From these values and measurement of the bile salt output it was calculated that in the cholecystectomised dog, the pool size circulated about 6 times per diem. The pool size was found to be large when compared with man and monkey. This might reflect differences in the eating habits of the different species.

Patients with gallstones are reported to have a reduced bile salt pool. This was studied in dogs by measuring the bile salt pool before and after cholecystectomy. There was a significant reduction in the pool size. Since removal of the gallbladder reduces the storage space of the extra-hepatic biliary tree, it was concluded that in patients the presence of stones in the gallbladder may effectively reduce the pool size. This suggested that a reduced bile salt pool in gallstone patients may be a result and not a cause of the disease.

The final part of the thesis elaborates on the clinical observations made in Part 1 in which studies were made on bile composition before and after vagotomy. Dogs with chronic biliary fistulae were used to study

the effect of vagal stimulation by insulin hypoglycaemia. Data was presented to show that vagal stimulation by this means produced a water choleresis. This could be blocked by atropine either when given together with insulin, or one hour after insulin. This suggested that insulin choleresis is mediated via the vagus. Atropine was also shown to inhibit the secretion of bile salts, phospholipids and cholesterol. This observation was made here for the first time.