THE EFFECT OF ALTERED ENTEROHEPATIC CIRCULATION ON BILE ACID KINETICS AND BILIARY LIPID COMPOSITION

by

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A B S T R A C T

Aim

The aim of this thesis was to contribute to our understanding of the physiology of the enterohepatic circulation with particular reference to the enterohepatic cycling frequency of the bile acid pool and its role in the pathogenesis of gallstones.

Background

Cholesterol precipitation and gallstone formation occurs in bile supersaturated with cholesterol. It has subsequently been shown that the abnormal bile is secreted by the liver and that patients with gallstones have a small bile acid pool with a shortened biological half-life suggesting that the pool is being depleted more rapidly. However, there is no difference between faecal bile acid loss in gallstone patients and controls. A similar faecal loss to normal with a smaller pool is inappropriately large and could be due to defective reabsorption or increased enterohepatic cycling of the smaller pool. The former seems unlikely and would also require a hepatic defect. There is some evidence for a relationship between pool size and cycling frequency but there are no studies in which cycling frequency has been controlled and its effect on bile acid pool size determined.

Method

A suitable model of controlled interruption of the enterohepatic circulation has been developed in the Rhesus monkey and has been used extensively in studies of bile acid metabolism. Effluent bile from a biliary fistula was allowed to accumulate in a reservoir. Cycling frequency was controlled by the regular return of the effluent bile into a duodenal fistula. Using isotope dilution techniques bile acid kinetics and biliary lipid composition were determined at each cycling frequency. Truncal vagotomy, a stimulus likely to alter enterohepatic cycling frequency, was investigated in man using duodenal bile sampling. Bile acid kinetics and biliary lipid composition were determined before and after vagotomy.

Results

1. An inverse relationship was demonstrated between cycling frequency and bile acid pool size.
2. Fractional turnover rate was directly related, and cholate synthetic rate inversely related to cycling frequency.
3. Alterations in enterohepatic cycling frequency did not affect biliary lipid composition.
4. Bile acid pool size increased after truncal vagotomy and calculated cycling frequency decreased.
5. Vagotomy did not affect biliary lipid composition.

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STATEMENT OF ORIGINALITY

When work for this thesis began in 1975, the abnormalities of biliary lipid composition (Admirand and Small, 1968) and bile acid kinetics (Vlahcevic et al., 1970) in cholesterol gallstone disease had been defined but the relationship between them was unclear.

For this reason I modified the chronic bile fistula monkey model (Dowling et al., 1968) which enabled a study of the direct effects of alterations in enterohepatic cycling frequency to be made.

It was found that increasing cycling frequency caused a reduction in bile acid pool size but did not alter biliary lipid composition. This is an original observation. It was also found that bile acid pool size increased after truncal vagotomy in man and that this was probably due to decreased enterohepatic cycling frequency.

All of the experimental procedures and physiological measurements in both the animal and clinical studies were carried out personally. Most of the analytical procedures were also carried out personally. After returning to England help with biliary lipid analyses was received from Professor I. A. D. Bouchier and the University of Dundee. Statistical advice was obtained from a member of the John Ellicott Computer Centre at The London Hospital.

GENERAL STATEMENT OF THE PROBLEM TO BE INVESTIGATED

Can the frequency of enterohepatic circulation be shown to have a direct effect on the size of the bile acid pool?

PATHOPHYSIOLOGY OF CHOLELITHIASIS

Introduction

The three biliary lipids, cholesterol, phospholipids, and bile acids, all undergo an enterohepatic circulation but to varying degrees. Only small quantities of cholesterol (Borgstrom, 1969, Grundy et al., 1969) and phospholipids (Saunders, 1970) undergo an enterohepatic circulation, but in health 95% or more of bile acids are reabsorbed (Lack and Weiner, 1967). Bile acids are detergent-like molecules which emulsify lipids, aiding their digestion, and maintain the products of digestion in micellar solution, which aids their absorption in the jejunum (Verzar, 1936, Hofmann and Borgstrom, 1964). The bile acids themselves are reabsorbed in the ileum. It was noted as long ago as 1878 that bile acids were poorly absorbed from the jejunum but rapidly absorbed from the ileum (von Tappeiner, 1878). Quantitative studies with isolated rat jejunal and ileal segments confirmed this observation and showed that the reabsorption was by an active transport mechanism (Baker and Searle, 1960, Lack and Weiner, 1961). The need for this efficient conservation of bile acids becomes evident when one considers

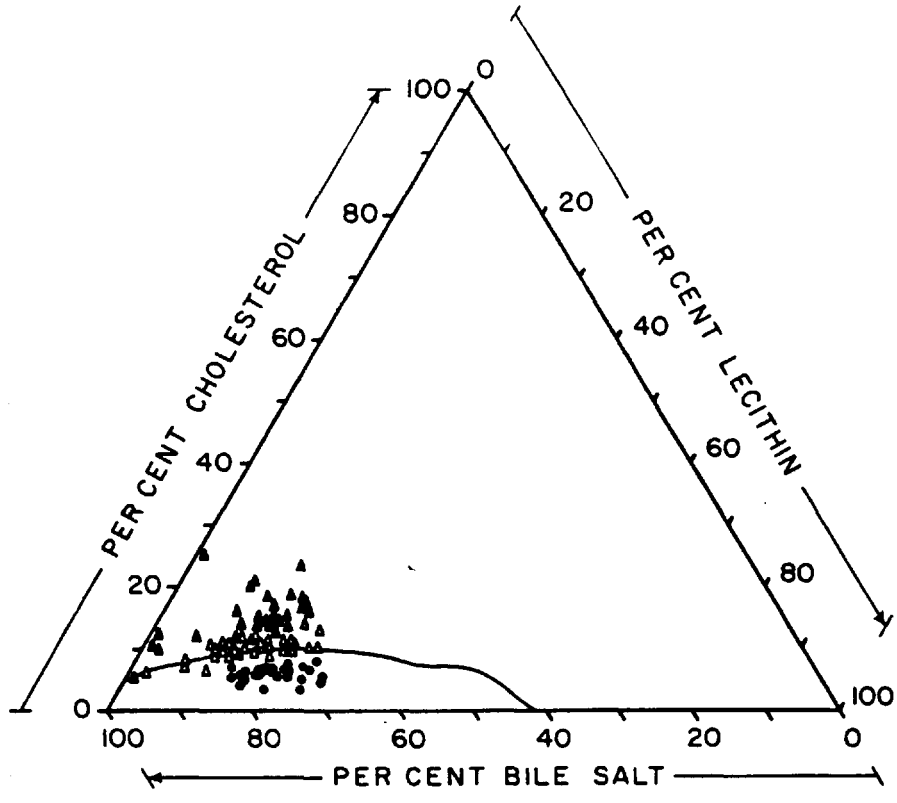
bile acid pool size, hepatic secretion of bile acids, and hepatic synthesis of bile acids. The normal bile acid pool in man is approximately 2 - 5 grams (Lindstedt, 1957, Danielsson et al., 1963, Vlahcevic et al., 1970) whereas daily hepatic secretion is of the order of 20 - 25 grams/day (Hofmann, 1965, Hofmann, 1967, Grundy and Metzger, 1972). Such a high hepatic secretion rate, with a relatively small bile acid pool, could only be maintained by an extremely high rate of hepatic biosynthesis or by highly efficient reabsorption of the small pool which is then recirculated between six and ten times each day. Bile acid kinetic studies have shown the hepatic daily synthesis is in fact very low, in the order of 500 mg per day (Grundy et al., 1965, Vlahcevic et al., 1970) and that with complete interruption of the enterohepatic circulation synthesis only increased fourfold (Shaffer et al., 1972). The efficient enterohepatic circulation of bile acids thus conserves these physiologically important compounds. The small amount lost in the faeces is balanced by hepatic biosynthesis to maintain a constant bile acid pool size. Hepatic biosynthesis of bile acids is regulated by feedback inhibition by the amount of bile acids returning to the liver in portal blood (Bergstrom and Danielsson, 1958, Shefer et al., 1970). An increased return reduces synthesis and a decreased return increases synthesis.

Physical Chemistry

Bile acids also have an important role in the hepatic secretion

of cholesterol. Hepatic bile is 90% water in which cholesterol is virtually insoluble but the daily hepatic secretion of cholesterol is approximately 1 - 1.5 grams/day (Wood, 1969, Grundy et al., 1970). The cholesterol is held in solution in mixed micelles of bile acids and phospholipids (Isaksson, 1952). Under normal circumstances the formation of cholesterol-bile acid-phospholipid micelles prevents the precipitation of cholesterol which has been recognised as a prerequisite for gallstone formation and growth for over a century (Budd, 1853, Thudicum, 1863). Until recently this precipitation of cholesterol was thought to be due to either stasis, biliary tract infection or deranged gallbladder function (Drury et al., 1924, Rains, 1964). This generally held view was supported by the fact that very few patients developed recurrent stones in the common bile duct following cholecystectomy and therefore the gallbladder was the cause, as well as the site, of the disease. Further support for a primary defect within the gallbladder came from analyses of gallbladder bile. Numerous studies on the composition of gallbladder bile showed wide variations in concentrations of bile acids, cholesterol, and phospholipids in normal human subjects. No consistent differences in the absolute composition of bile was found between these subjects and patients with cholesterol gallstones (Isaksson, 1952, Isaksson, 1954 a, Isaksson, 1954 b, Polanovski and Bourillon, 1952, Large et al., 1960, Tera, 1963, Burnett, 1965, and Dam et al., 1966). Some of these differences

were probably due to variations of the analytical method. Further wide variations occurred when results were related to unit volume since the volume depended upon the degree to which the bile had been concentrated at the time of sampling. Binary ratios of cholesterol:bile acid or cholesterol:phospholipid also failed to separate the biles of cholesterol gallstone subjects from controls (Dam et al., 1966). Physical chemical studies on ternary and quaternary model systems of cholesterol, phospholipids, and bile acids (Small et al., 1966 a, Small et al., 1966 b, Bourges et al., 1967 a, Bourges et al., 1967 b) led to the development of an in vitro model in which the physical chemical state of all possible combinations of cholesterol, phospholipids, bile acids and water was defined within the bounds of a tetrahedron. For any given concentration of water the relative composition of the remaining three components could be plotted on a triangular phase diagram. Using this model Admirand and Small defined the limits of cholesterol solubility in a one-phase micellar solution of cholesterol, phospholipids, and bile acids and the line of separation between it and two- or three-phase crystal, liquid, and liquid-crystal solutions (Fig. 1). When the relative composition of gallbladder biles from patients with gallstones and from normal controls were compared with the previously defined limit of cholesterol solubility it was found that all the biles from the controls fell within the



W. H. Admirand and D. M. Small

Fig. 1

The composition of gallbladder bile from normal subjects (closed circles) and patients with gallstones (triangles). The closed triangles indicate the presence of cholesterol microcrystals and the open triangles represent biles without microcrystals. The solid line is the limit of cholesterol solubility. (Admirand and Small, 1968)

micellar phase whereas those from subjects with cholesterol gallstones were either very close to the limits of solubility or, in most cases, beyond the limits of solubility (Admirand and Small, 1968). Further studies on American Indians, amongst whom cholesterol gallstones are very prevalent (Comess et al., 1967, Thistle and Schoenfield, 1970, Sampliner et al., 1970), showed that the bile secreted by the liver was supersaturated with respect to cholesterol suggesting that the primary defect lay in the liver and not in the gallbladder as previously thought (Small and Rapo, 1970). These findings in American Indians were confirmed by other workers (Vlahcevic, 1972, Grundy et al., 1972, Metzger et al., 1973, Adler et al., 1974). Further studies confirmed the abnormality in hepatic bile composition (Vlahcevic, Bell and Swell, 1970, Shaffer et al., 1972 a, Shaffer et al., 1972 b, Simmons et al., 1972, Almond et al., 1973).

The results of these studies strongly suggested that cholesterol gallstone disease results from the secretion of hepatic bile which is supersaturated with respect to cholesterol. The rate of secretion of each of the biliary lipids determines the <u>absolute</u> composition of the secreted hepatic bile but the <u>relative</u> composition determines the solubility of the cholesterol.

Role of the Bile Acid Pool

In 1970 Vlahcevic and co-workers measured bile acid kinetics and bile acid pool size in patients with gallstones and in normal controls. The average pool size in the gallstone patients was 1.29 G compared with 2.38 G in the controls. They suggested that hepatic bile acid synthesis was reduced and that the reduced bile acid pool size might be the defect underlying the relative lack of bile acids in hepatic bile to maintain cholesterol in solution (Vlahcevic et al., 1970). To make this hypothesis it was necessary to assume that the reduced bile acid pool in the gallstone patients underwent the same number of enterohepatic cycles in each twenty-four hours as the normal controls, and that the hepatic bile acid secretion rate was therefore reduced. Subsequent studies have cast doubt on this assumption, though the evidence has been indirect in each case. Low-Beer and Pomare measured bile acid pool size in patients with coeliac disease, post-cholecystectomy subjects, and normal controls (Low-Beer and Pomare, 1973). Previous work had shown that gallbladder contraction is impaired (Low-Beer, et al., 1971) and entry of bile into the intestine is delayed (Di Magno, et al., 1972) in coeliac disease. Reduced enterohepatic cycling would seem likely in these circumstances. After cholecystectomy, they suggested that the bile acid might circulate continuously and at an increased frequency. They found that pool size was increased in patients with coeliac disease and decreased after cholecystectomy, suggesting an inverse relationship

between bile acid pool size and the frequency of enterohepatic circulation. In another study (Hepner, 1975) bile acid pool size was measured in normal subjects when receiving a low-fat, low-protein diet and when receiving a normal diet. Hepner suggested that the low-fat, low-protein diet would reduce gallbladder emptying and hence cycling frequency. Bile acid pool size increased during the low-fat, low-protein diet. Further indirect evidence to support the view that there is an inverse relationship between bile acid pool size and frequency of enterohepatic circulation was provided by Northfield and Hofmann who showed no significant difference in bile acid secretion of patients with gallstones and normal controls, as measured by a duodenal perfusion technique (Northfield and Hofmann, 1973, Northfield and Hofmann, 1975). Calculated cycling frequency, derived from their secretion data and bile acid pool size measurements, suggested an inverse relationship between pool size and enterohepatic cycling frequency.

Grundy and co-workers measured faecal bile acid excretion in American Indians and detected no significant difference between controls and patients with gallstones (Grundy et al., 1972). Calculated reabsorption was similar in both groups and was approximately 95%. If the reduced bile acid pool in the subjects with gallstones circulated at the same frequency as the normal controls then one would anticipate a reduced faecal bile acid loss. Faecal bile acid loss similar to the controls was therefore inappropriately

high.

There are two possible explanations for this excessive faecal bile acid loss. First, if the bile acid pool in gallstone patients was cycled through the enterohepatic circulation with the same frequency as in normal subjects the total amount of bile acids presented to the ileum in each twenty-four hours would be less. A similar faecal bile acid excretion in both groups would imply a defect in reabsorption by the intestine. Secondly, if the diminished bile acid pool in patients with gallstones was cycled through the enterohepatic circulation with an increased frequency, as compared with normal subjects, the total amount of bile acids presented to the ileum in each twenty-four hours might be the same as in normal subjects.

A defect in reabsorption seems unlikely and would need to be accompanied by a defect in the feedback regulation of hepatic synthesis. Reduced reabsorption acts as a partial interruption of the enterohepatic circulation which increases bile acid synthesis in monkeys (Dowling et al., 1970) and in gallstone patients following cholecystectomy (Shaffer et al., 1972). Hepatic bile acid synthesis in patients with gallstones is not significantly different from normal controls (Vlahcevic et al., 1970, Grundy et al., 1972). Increased enterohepatic cycling frequency, compensating for the reduced bile acid pool size in gallstone patients,

provides a simpler explanation for the observation that faecal bile acid excretion is similar to that of normal subjects. If this theory is true then the diminished bile acid pool, excessive faecal bile acid excretion, and the shortened bile acid biological half-life in patients with gallstones might all be due to altered enterohepatic cycling rather than any hepatic defect. Indirect evidence suggests that there might be a relationship between bile acid pool size and enterohepatic cycling (Low-Beer and Pomare, 1973, Hepner, 1975, Northfield and Hofmann, 1975). No reports have been found in the literature which directly link bile acid pool size and enterohepatic cycling frequency.

Aims of the Thesis and Choice of Experimental Technique

It seems in theory, therefore, that enterohepatic cycling frequency might be a major determinant of bile acid pool size. It is less certain whether cycling frequency by influencing bile acid pool size would have any influence on biliary lipid composition, as proposed by Vlahcevic and co-workers (Vlahcevic et al., 1970). It was the aim of this study to attempt to fill some of the gaps in our knowledge on the role of enterohepatic cycling in the kinetics of the bile acid pool.

A considerable number of techniques have been developed for the study of biliary physiology in man and experimental

animals. Fasting gallbladder bile may be easily collected in man by intubation of the duodenum, under fluoroscopic control, with a double lumen Dreiling tube. The proximal lumen drains the stomach, minimising any dilution of duodenal samples by gastric contents, and the distal lumen, with a weighted tip to aid its passage through the pylorus, lies in the duodenum. Contraction of the gallbladder is stimulated by intravenous injection of cholecystokinin and bile is collected by siphonage. The technique is well validated and ideal for serial collections of gallbladder bile for biliary lipid composition and for isotope dilution studies of bile acid kinetics if used in conjunction with radioactive bile acid markers (Vlahcevic et al., 1970, Hofmann et al., 1970). Studies of hepatic secretion of biliary lipids can be performed using a triple- or quadruple-lumen tube, the tip of which lies in the upper jejunum (Go et al., 1970, Grundy and Metzger, 1972, Northfield and Hofmann, 1975). Non-absorbable markers are perfused at a steady rate, together with a liquid diet, via one of the more proximal ports and after mixing in the intestinal lumen, usually over a distance of 30 cms, samples are aspirated from the distal port and secretion rates calculated from dilution of the non-absorbable marker. Faecal bile acid excretion rates may be determined by gas-liquid chromatography (Grundy et al., 1965), but the technique is not widely used, mainly because more information can be obtained with fewer sources of error by either of the methods

already described. All of these techniques are suitable for measurement of various aspects of biliary physiology but in none of them is it possible to directly manipulate the enterohepatic cycling frequency. Of the methods described duodenal intubation and aspiration of gallbladder bile is the most useful for direct measurements of biliary lipid composition and bile acid pool size from which hepatic synthesis and fractional turnover may be calculated. With certain assumptions indirect estimates of cycling frequency may be made.

Direct access to the biliary tree in man is possible in patients who have undergone cholecystectomy and choledocholithotomy. Bile drainage via the T-tube gives one a limited control over enterohepatic cycling (Shaffer et al., 1972 b). The technique would not be suitable for this study for a number of reasons: firstly, it would be difficult to be certain that all hepatic bile was draining via the T-tube and that small quantities of bile were not entering the duodenum and maintaining some continuous enterohepatic circulation. Secondly, bile acid pool size and biliary lipid composition might not be stable in the post-operative period, and lastly, one would be examining patients who had had gallstones in whom there might be pre-existing abnormalities of hepatic secretion or biosynthesis. A balloon-occludable T-tube has been described (Baldwin, 1966) which seems to effectively divert all hepatic bile. It has been used

in acute studies lasting 8 - 12 hours (Soloway et al., 1975) but not for several days as required for these studies. It is doubted whether complete occlusion could be maintained throughout the study period.

More direct studies on biliary physiology are possible in the experimental animal. Acute studies of bile acid secretion may be performed in the bile-fistula rat but this model is unsuitable for long-term studies. In order to test the hypothesis that the frequency of the enterohepatic circulation of bile acids affects bile acid pool size one must be able to directly control the enterohepatic cycling frequency. As explained above, this cannot be done in man but a model of controlled interruption of the enterohepatic circulation has been perfected in the Rhesus monkey (Dowling et al., 1968 , Dowling et al., 1970 and Dowling et al., 1971). In this model Rhesus monkeys are prepared with biliary and duodenal fistulae. Effluent bile from the biliary fistula activates a stream-splitting pump which returns 95% of the bile to the animal via the duodenal fistula and diverts 5% of the bile to a collecting vial for subsequent analysis. The model has been used extensively in studies of bile acid metabolism by Dowling and co-workers and several other groups (Campbell et al., 1971, Small et al., 1972, Redinger et al., 1973, Deveney et al., 1974, Strasberg et al., 1974, Beaudoin et al., 1975) and would seem to be most suitable for this study. An additional advantage of the Rhesus monkey is the similarity

of its biliary lipid composition to that of man.

In order that complete control over the enterohepatic circulation could be achieved, it was realised that certain modifications would need to be made to the model described by Dowling. Firstly, at the time of construction of the biliary and duodenal fistula it would be necessary to remove, or in some way exclude, the gallbladder from the circulation of bile to prevent sequestration of part of the circulating pool within it. Secondly, it would be necessary, for most of the experiments, to develop different techniques for collection of the efferent bile from the biliary fistula in a reservoir and delay its return via the duodenal fistula in such a manner that cycling frequency would be controlled. Collection of bile in the reservoir would simulate filling of the gallbladder and return of the bile would simulate gallbladder emptying. Cycling frequency would be determined by the length of time for which bile was withheld. Once established at a particular frequency it would be possible to label the bile acid pool with radioactive bile acid and perform the bile acid kinetic studies. When studies were complete at a particular cycling frequency, the animals would be established at a new frequency and the kinetic studies repeated. Bile samples would also be analysed to determine the biliary lipid composition at each cycling frequency.

Additional Studies on Truncal Vagotomy

To supplement the experiments described above, in which cycling frequency could be controlled directly, it was proposed to perform clinical studies in patients. Cycling frequency would not be controlled directly but the patients would be subject to a stimulus, truncal vagotomy, which might be anticipated to have some influence on cycling frequency. It was realised that the efficiency of intestinal absorption would have to be assumed when indirectly calculating the cycling frequency from the bile acid kinetic data. It seemed unlikely that vagotomy would sufficiently alter absorption so as to invalidate the study.

For many years there has been controversy as to whether there is an association between truncal vagotomy and the subsequent development of cholesterol gallstones. Much of the evidence, reviewed below, is anecdotal and the case still remains unproven. It seems reasonable to hypothesise that if vagotomy does predispose subjects to cholesterol gallstone disease, then the abnormalities of biliary lipid composition (Admirand and Small, 1968) and bile acid pool size (Vlahcevic et al., 1970) should be detectable after vagotomy. Alternatively, the association between vagotomy and cholesterol gallstones might be fortuituous. If this were so then any changes observed in bile acid pool size, and much more speculatively in biliary lipid composition, might be due to alterations in enterohepatic cycling frequency.

It was therefore hoped that additional studies on bile acid kinetics before and after truncal vagotomy would extend the evidence for the original hypothesis, and also provide further evidence for or against an association between vagotomy and the subsequent development of cholesterol gallstones.

The current surgical treatment of chronic duodenal ulcer disease is truncal vagotomy (Dragstedt and Owens, 1943, Dragstedt and Camp, 1948) or its subsequent modifications, selective vagotomy (Burge, 1964) and proximal gastric vagotomy (Johnston and Wilkinson, 1969, Amdrup and Jensen, 1970). Although a relationship between gastric surgery in general and the subsequent development of gallstones has been reported (Majoor and Suren, 1947, Griffiths and Holmes, 1964), truncal vagotomy in particular has been implicated as an aetiological factor in cholesterol gallstone disease following surgery. Cholecystograms performed one to ten years after truncal vagotomy in 91 patients demonstrated gallstones in 21 (22.8%) (Clave and Gaspar, 1969). Fifty-five of these patients had normal oral cholecystograms prior to vagotomy. Although there was no control group there was a significantly increased incidence when compared with the prospective epidemiological study in Framingham (Friedman et al., 1966). In another retrospective study 17 (15.5%) of 110 patients had developed gallstones within ten years (Nobles, 1966). Four patients with normal pre-operative cholecystograms developed gallstones within

three years of truncal vagotomy (Nielsen, 1964). Acute cholecystitis has also been described shortly after vagotomy in man (Schein and Gliedman, 1978) and similar findings have been demonstrated in experimental animals (Schein et al., 1969). Oral cholecystographic studies by Johnson and Boyden demonstrated dilatation of the gallbladder after truncal vagotomy (Johnson and Boyden, 1952) and this finding has been supported by several other groups (Cox et al., 1958, Schein et al., 1969, Schein et al., 1970, Fagerberg et al., 1970, Inberg and Vuorio, 1969, and Rudick and Hutchison, 1965. Vagotomy has also been shown to decrease the pressure required to overcome the resistance of the cystic duct and the sphincter of Oddi (Beneventano et al., 1969). Conversely, no change in gallbladder volume, as calculated from oral cholecystograms, was reported by three other groups (Clave and Gaspar, 1969, Glanville and Duthie, 1964, Williams and Irvine, 1966), and the value of cholecystographic studies has been questioned (Kramhoft et al., 1972).

Overall, the clinical studies suggest an association between truncal vagotomy and the subsequent development of gallstones but the evidence is insufficient to reach a definite conclusion.

Because the human biliary tree is relatively inaccessible a considerable amount of experimental work has been carried out in animals. Amdrup and Griffith performed oral cholecystograms in dogs before and after truncal vagotomy and

failed to demonstrate dilatation of the gallbladder (Amdrup and Griffith, 1970). Barnett and Hilburn demonstrated that dissolution of human gallstones placed in the gallbladders of dogs was retarded by vagotomy (Barnett and Hilburn, 1966) but two other groups found no difference between vagotomised and control groups (Loeb and Nicoloff, 1968, Amdrup and Griffith, 1970).

Studies of biliary lipid composition might be expected to clarify the situation. Fletcher and Clark found that the cholic acid concentration was significantly decreased in dog bile following truncal vagotomy (Fletcher and Clark, 1969), suggesting an increased likelihood of gallstone formation. However, when their results are plotted on the triangular coordinates of Admirand and Small the differences are no longer significant. In a subsequent study from the same laboratory the concentration of cholate remained constant but cholesterol concentration increased significantly (Cowie and Clark, 1972). This finding was confirmed by two other groups (Fagerberg et al., 1970, and Machleder, 1971). Studies of biliary lipid composition in man pre-operatively and one year post-operatively failed to demonstrate a significant change in the relative composition although there was an increase in the absolute concentration of all three lipids (Smith et al., 1973). White and co-workers measured bile acid pool size in dogs before and after vagotomy and found that pool size had doubled by one week after truncal

vagotomy and was unchanged when bile acid kinetic studies were repeated after five months (White et al., 1974).

These experimental studies have failed to consistently demonstrate the abnormalities of biliary lipid composition associated with cholesterol gallstone disease.

Choice of Experimental Technique

To test the extension of the original hypothesis that changes in bile acid pool size after vagotomy are due to alterations in the enterohepatic cycling frequency, and to determine whether vagotomy produces the changes in bile acid kinetics and biliary lipid composition of cholesterol gallstone disease, it is proposed to perform a series of experiments in patients presenting for elective surgery for chronic duodenal ulcer. It would be necessary to exclude patients with co-existing gallstones by oral cholecystography. The available techniques have been discussed already. After labelling the bile acid pool with radioactive bile acid serial samples of fasting gallbladder bile would be collected using the duodenal intubation technique (Vlahcevic et al., 1970), and bile acid pool size and kinetics determined. Samples would also be analysed to determine biliary lipid composition. After recovery from truncal vagotomy bile acid kinetic and biliary lipid composition studies would be repeated.

It is as well to note that most of the experimental work on the relationship of vagotomy to gallstone disease has looked at biliary lipid composition - little attention being paid to bile acid pool size.

Summary

In summary, therefore, this thesis in part sets out, using chronic bile-fistula Rhesus monkeys, to test the hypothesis that changes in enterohepatic cycling frequency can cause alterations in bile acid pool size, such that an increase in cycling frequency results in a decrease in pool size and vice versa.

In addition the influence of changes in enterohepatic cycling on biliary lipid composition will be determined. Further data will be collected on bile acid kinetics and biliary lipid composition before and after truncal vagotomy in patients with chronic duodenal ulcer disease.

CRITICAL REVIEW

BILE ACIDS AND THE ENTEROHEPATIC CIRCULATION

Introduction

Bile acids are present in all invertebrates (Sobotka, 1937) and were first described in man during the nineteenth century (Thenard, 1807, Hoppe-Seyler, 1863, Socolov, 1875, Hoppe-Seyler, 1877). They aid the absorption of dietary fat, the excretion of cholesterol by the liver, and undergo an enterohepatic circulation. The first and second parts of this review discuss in detail the physiology of the intact and the interrupted enterohepatic circulation. In subsequent sections the various factors known to influence bile acid pool size, biliary lipid composition, and biliary lipid secretion, are reviewed and their relationships to enterohepatic cycling are discussed. The experimental animal model used in this work is a modification of the chronic bile fistula monkey described by Dowling and co-workers (Dowling et al., 1968). Its use in studies of biliary lipid metabolism is reviewed.

1. THE NORMAL ENTEROHEPATIC CIRCULATION OF BILE ACIDS

In man the bile acid pool is 6 - 12 mM in health (Lindstedt, 1957, Danielsson et al., 1963, Vlahcevic et al., 1970, Vlahcevic et al., 1971) and comprises a mixture of primary bile acids (cholic acid and chenodeoxycholic acid) synthesised in the liver, and secondary bile acids (deoxycholic acid and lithocholic acid) produced from the two primary bile acids by bacterial degradation in the ileum and colon. Bile acids are secreted into bile and then stored and concentrated in the gallbladder. The stimulus of feeding causes the gallbladder to contract and the bile acids enter the intestinal lumen to fulfil their role in the absorption of dietary fat. The bile acids are reabsorbed in the ileum and enter the portal blood to be returned to the liver so completing their enterohepatic circulation.

The bile acid pool circulates 2 - 3 times during each meal and therefore undergoes 6 - 10 enterohepatic circulations each day (Borgstrom et al., 1957, Hofmann and Hoffman, 1974). Highly efficient reabsorption of bile acids in the terminal ileum reduces faecal loss to approximately 1 mM/day (Grundy et al., 1972). Hepatic biosynthesis has been shown to be controlled homeostatically by the quantity of bile acid returning to the liver via the portal circulation (Shefer et al., 1969).

(a) Hepatic Phase

The liver synthesises bile acids, conjugates free bile acids (both those newly synthesised and any deconjugated bile acids returning from the intestine), and secretes the conjugated bile acids into bile.

i) Hepatic Biosynthesis (Fig. 2)

Bile acids are synthesised by catabolism of cholesterol, which is also synthesised in the liver. Cholesterol is the obligatory precursor for bile acid synthesis. Cholesterol undergoes 7α-hydroxylation to form 7α-hydroxycholesterol which is then transformed to 7α-hydroxycholest-4-en-3-one. This unsaturated ketone is the last intermediate compound common to both primary bile acids. It may be transformed into 5β-cholestane-3α,7α-diol and hence to chenodeoxycholic acid or undergo 12α-hydroxylation to 7α, 12α-hydroxy-cholest-4-en-3-one and conversion to cholic acid (Staple, 1969, Elliott and Hyde, 1971, Mosbach, 1972, Danielsson, 1973). The rate of bile acid synthesis by the liver appears to be controlled by the size, cycling frequency, and composition of the bile acid pool (Shefer et al., 1969, Shefer et al., 1970). It seems likely that the hepatic bile acid flux influences the concentration of enzymes that are rate-limiting for bile acid synthesis though the exact mechanism is not known. Cholesterol 7α-hydroxylase, which catalyses the first step in bile acid synthesis, appears to be

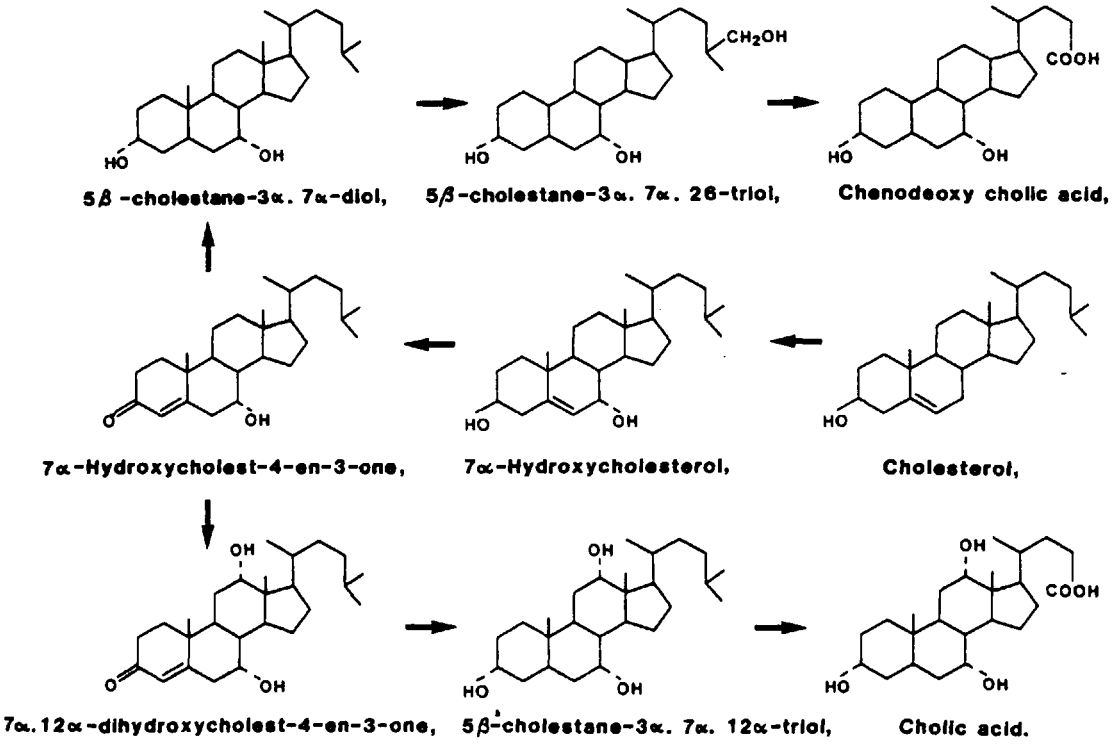


Fig. 2

The metabolism of cholesterol to cholic acid and chenodeoxycholic acid.

rate-limiting in animals (Shefer et al., 1970, Mosbach et al., 1971) and is probably rate-limiting in man (Einarsson, 1973). Since cholesterol is the precursor of bile acids its rate of production could control bile acid biosynthesis. 3-hydroxy-3-methylglutaryl CoA reductase is known to be the rate limiting enzyme for cholesterol biosynthesis and might, therefore, indirectly regulate bile acid synthesis.

ii) Conjugation of free bile acids

In health all bile acids secreted into bile are conjugated with the amino acids, taurine or glycine in the hepatocyte. The ratio between glycine and taurine conjugates is normally 2:1 (Dam, 1966), though this can be reversed by feeding a diet rich in taurine (Sjovall, 1960). The ratio found in man can be explained by the relative excess of glycine available from metabolism and the relative shortage of taurine in a normal diet.

iii) Bile Acid Secretion

Bile acids returning to the liver in portal blood, together with small amounts of newly synthesised bile acid are secreted into bile by an active transport mechanism. The rate of secretion of bile is related to the secretion rate of bile acids. Schiff first demonstrated this choleretic effect of bile acids over 100 years ago by re-feeding bile to bile-fistula dogs (Schiff, 1870). Infusion of bile acids intravenously increases bile acid

secretion until the limit of the active transport system is reached. Any further increases in the rate of infusion do not further increase bile acid secretion (O'Maille et al., 1965, Alpert et al., 1969, Hardison and Apter, 1972). At very low bile acid secretion rates a watery bile is secreted, and extrapolation of secretion curves has shown there to be a "bile-salt independent fraction" of bile flow (Wheeler et al., 1968, Boyer and Klatskin, 1970). At low bile acid secretion rates there is a linear relationship to the secretion rates of cholesterol and phospholipids which is not maintained at high secretion rates (Hardison and Apter, 1972). Cholesterol and phospholipid secretion rates both plateau at the same bile acid secretion rate supporting the concept that micelle formation is an important mechanism of biliary cholesterol excretion. It has been noted by many observers that, although the absolute secretion rates of cholesterol and phospholipid are highest at high bile acid secretion rate, the relative concentrations are highest at low bile acid secretion rates (Dowling et al., 1971, Hardison and Apter, 1972, Wheeler and King, 1972, Soloway and Schoenfield, 1975). Thus bile acid secretion rate is an important determinant of the cholesterol saturation of bile.

(b) Intestinal Phase

In their passage through the small intestine the bile acids play an important role in the absorption of dietary fat. During the intestinal phase bacterial activity converts some of the primary bile acids to secondary bile acids. Bile acids are largely reabsorbed in the terminal ileum by an active transport mechanism and return to the liver via the portal circulation.

i) Bile acids and absorption of dietary fat.

Dietary fat is hydrolysed in the intestinal lumen by pancreatic lipase. Verzar and McDougall demonstrated that the fatty acids released by this hydrolysis were soluble in bile salt solutions at the pH of intestinal contents (Verzar and McDougall, 1936). Monoglycerides and diglycerides were also demonstrated after hydrolysis of triglycerides by pancreatic lipase in vitro (Artom and Reale, 1936). For many years it was thought that fat absorption took place from a triglyceride emulsion (Fraser, 1958). It is now known that bile acids emulsify lipids because of their detergent properties (Hofmann, 1965) and, that above a critical concentration, bile acid molecules, like all other detergents, aggregate to form micelles (Rains & Crawford, 1953, Rains, 1955, Garrett, 1961). In an elegant study, Hofmann and Borgstrom demonstrated that, with prolonged centrifugation, intestinal contents could be

separated into an infranatant micellar phase and a supernatant oil phase (Hofmann and Borgstrom, 1964). Most of the fatty acids and monoglycerides were in the micellar phase, the oily phase consisting mainly of diglycerides and triglycerides. It is known that dietary fat is mainly absorbed in the form of fatty acids and monoglycerides (Reiser, 1955). The results of the study by Hofmann and Borgstrom strongly suggested that emulsification of fats was a prelude to formation of micelles from which absorption takes place at the microvillus of the brush border of the intestinal epithelial cell. Adsorption of fatty acids to the cell is probably reversible (Mishkin et al., 1972) but becomes irreversible by synthesis of triglyceride (Hofmann, 1978). The bile acid micelle is thus freed to transport further fatty acid and monoglyceride molecules from the oil/water interface of the intestinal emulsion to the microvillus. This micellar solubilisation of lipolytic products increases the rate of absorption of dietary fat by a factor of 100 (Hofmann, 1976). Although fat can be absorbed throughout the small intestine this highly efficient mechanism normally completes the process within the proximal half of the jejunum (Borgstrom et al., 1957).

iii) Bacterial Degradation of Bile Acids

Bacterial action on bile acids is largely concerned with two processes: deconjugation and 7α-hydroxylation. The initial reaction is splitting of the taurine or glycine moiety from the bile acid mainly by obligate anerobes

(Norman and Grubb, 1955, Lewis and Gorbach, 1972). 7α-dehydroxylation of the free bile acids converts cholic to deoxycholic acid and chenodeoxycholic to lithocholic acid. In vitro studies suggested that deconjugation must take place before 7α -dehydroxylation can occur (Norman, 1970), but subsequent in vitro studies with radio-labelled glychocholic acid have shown that, in man at least, this is not always necessary (Hepner et al., 1972 a, Hepner et al., 1972 b). Deconjugation mainly occurs in areas of stasis and in health this seems to be restricted to the large bowel and perhaps the terminal ileum. Studies on patients with an ileostomy by Gorbach and co-workers have shown that deconjugation of the primary bile acid, cholic acid, occurs but that dehydroxylation to the secondary bile acid, deoxycholic acid, does not occur (Gorbach et al., 1967). It is therefore likely that dehydroxylation and reabsorption of deoxycholic acid occur in the colon. Most of the bile acids appearing in the faeces are in the secondary forms of deoxycholic and lithocholic acid. The latter is extremely insoluble, is also toxic, and very little undergoes an enterohepatic circulation.

iii) Reabsorption of Bile Acids

In the latter part of the last century von Tappeiner observed that bile acids, unlike other water soluble materials, were poorly absorbed in the jejunum but well absorbed in the ileum (von Tappeiner, 1878). Classic experiments by two

groups working independently on isolated loops of small intestine conclusively demonstrated that the ileum was the major site of bile acid reabsorption (Baker and Searle, 1960, Lack and Weiner, 1961). In addition, the study by Lack and Weiner also demonstrated that an active transport mechanism was involved. Further studies showed that ileectomy, but not jejunectomy, completely abolished the enterohepatic circulation of bile acids in the dog (Playoust, Lack and Weiner, 1965). Subsequent studies in normal human subjects (Borgstrom et al., 1963) and in patients with extensive ileal resection (Austad et al., 1967) confirmed the site of bile acid absorption and the interruption to the enterohepatic circulation of bile acids by the resection. Passive absorption can occur in both the large and small intestine. It probably accounts for very little of the small intestinal reabsorption but for most of the large intestinal reabsorption (Tyor et al., 1971).

The active reabsorption of bile acids in the ileum is extremely efficient. More than 95% of the circulating pool is reabsorbed during each enterohepatic cycle conserving these physiologically important compounds for cholesterol excretion and fat absorption.

(c) Portal Circulation

Absorbed bile acids are rapidly returned to the liver in the portal blood. The large volume of portal blood flow

makes other potential routes of return, such as lymph, quantitatively insignificant. Serum bile acids in the systemic circulation are normally very low because the liver efficiently clears returning bile acids from the portal blood. However, with modern radioimmunoassay techniques La Russo and co-workers have shown rises in serum bile acids after meals in normal subjects indicating that hepatic clearance is not complete (La Russo et al., 1974).

2. THE INTERRUPTED ENTEROHEPATIC CIRCULATION

The study by Playoust and co-workers, which demonstrated a complete interruption of the enterohepatic circulation by ileal resection in dogs (Playoust et al., 1965), stimulated several other groups to study bile acid metabolism in patients with ileal disease or resection. In 1965, Hofmann and Grundy published an abstract describing a patient with extensive ileal and colonic resection in whom they had studied bile acid kinetics. Intraluminal bile acid concentrations were low. The fractional turnover rate and daily faecal excretion of 14-C Cholic acid were both increased with a corresponding decrease in the half-life of the cholic acid pool (Hofmann and Grundy, 1965). Their findings were confirmed in similar studies on patients with ileal resection (Hardison and Rosenberg, 1966, Stanley and Nemchausky, 1966), and similar findings

were also described in ileal disease, rather than ileal resection (Austad, Lack and Tyor, 1967). It was realised that the interruption of the enterohepatic circulation was contributing significantly to their diarrhoea. Because of the failure of bile acid reabsorption in the small intestine much larger amounts of bile acids were entering the large intestine and preventing normal water reabsorption by their irritant effect on the colonic mucosa. Although the intraluminal concentration of bile acids was reduced in the upper small intestine (Hofmann and Grundy, 1965) resulting in impaired fat absorption and steatorrhoea, treatment with oral bile acids was not effective. Jejunal absorption of fat could be improved, reducing the steatorrhoea, but the increased bile acid load could not be reabsorbed and the diarrhoea was therefore exacerbated. Treatment with a bile acid-binding resin (Cholestyramine) further depletes the circulating bile acid pool but prevents the bile acids exerting their cathartic effect in the colon (Hofmann and Poley, 1969, Hofmann and Poley, 1972).

The effects of interruption of the enterohepatic circulation on biliary physiology were quantified in a series of important papers by Dowling and co-workers. Their studies on Rhesus monkeys with an exteriorised enterohepatic circulation which could be partially or completely interrupted demonstrated that a partial interruption of up to 20% of the enterohepatic circulation was completely compensated by increased hepatic biosynthesis so that daily bile acid secretion remained

constant. Interruptions of more than 20% exceeded the capacity of the liver to increase synthesis and daily bile acid secretion fell incrementally for each increase in the percentage interruption (Dowling et al., 1968, Dowling et al., 1970). With complete acute diversion of all effluent bile, i.e. 100% interruption, bile acid secretion fell over 3 - 6 hours to a low point representing basal hepatic synthesis. About twelve hours after complete interruption synthesis started to increase reaching a maximum 2 - 4 days later. Resection of the distal 1/3 of the small intestine considerably reduced the enterohepatic circulation and daily bile acid secretion rate but not to the same extent as occurred with complete interruption (Dowling et al., 1970). In a further study they investigated the secretion rates of all three biliary lipids (Dowling et al., 1971). As with bile acids, 5% or 10% interruptions of the enterohepatic circulation produced no changes in daily cholesterol or phospholipid secretion, but when greater interruptions occurred there was an incremental decrease in their secretion rates parallel to the decrease in bile acid secretion rate. With large interruptions the decrease in bile acid secretion rate was greater than that of the other two lipids and there was, therefore, a relative increase in cholesterol leading to a supersaturated bile. After 24 hours the compensatory increase in hepatic synthesis of bile acid returned the relative composition to near normal levels.

These experiments demonstrate the ability of the liver to compensate for interruption of the enterohepatic circulation by increasing biosynthesis 4 - 5 fold. Extensive ileal resection did not produce as complete an interruption to the enterohepatic circulation as biliary diversion because some bile salt reabsorption was occurring in the residual intestine.

3. BILE ACID POOL SIZE

(a) Cholesterol Gallstone Disease

An association between reduced bile acid pool size and cholesterol gallstone disease was first reported by Vlahcevic and co-workers in an important study comparing gallstone patients and normals using the isotope dilution technique (Vlahcevic et al., 1970). The patients with gallstones had a bile acid pool which was 46% smaller than the control group. This difference was highly significant ($P < 0.001$), and was confirmed in further studies (Swell et al., 1971, Bell et al., 1972). These findings stimulated research into the physiology and pathophysiology of the bile acid pool. Studies on American Indians, who had been shown to be a high-risk group for cholesterol gallstone disease (Sampliner et al., 1970, Thistle and Schoenfield, 1970), confirmed the finding that patients with gallstones had a reduced bile acid pool size (Vlahcevic et al., 1972). However their

studies also identified a sub-group amongst the normal controls, whose bile was supersaturated with cholesterol and whose bile acid pool size was reduced. These findings, present in both males (Bell et al., 1972) and females (Vlahcevic et al., 1972) suggested that reduction of bile acid pool size might be responsible for the development of bile supersaturated with cholesterol, so-called "lithogenic bile" (Metzger et al., 1972), and the subsequent development of gallstones.

(b) Effect of Cholecystectomy

Few patients develop truly recurrent common bile duct stones after cholecystectomy. A natural development from the findings described above was to investigate bile acid pool size before and after cholecystectomy, but there have been conflicting results. Hepner and co-workers reported normal bile acid pool size after cholecystectomy, implying an increase from the small pool of gallstone patients (Hepner et al., 1974) while others have found that the small pool persists (Almond et al., 1973). In animal studies on dogs (Crook et al., 1971) and hamsters (Bergman and van der Linden, 1974) it was found that cholecystectomy further reduced the bile acid pool size. A reduction in pool size was also found in human subjects with cholesterol gallstones (Pomare and Heaton, 1973 a, Shaffer and Small, 1977) and with pigment gallstones (Shaffer and Small, 1977). The variations between these

studies are probably due to methodological differences. Almond and co-workers investigated the same patients before and after cholecystectomy, but they compared cholecystokinin-stimulated gallbladder bile collected pre-operatively with hepatic bile post-operatively. Hepner and co-workers and Pomare and Heaton both compared post-cholecystectomy subjects with normal controls. Again cholecystokinin-stimulated gallbladder bile was collected from the controls and hepatic bile from the post-cholecystectomy subjects. In the animal studies, bile acid pool size was measured by the same washout technique before and after cholecystectomy, and in the studies by Shaffer and Small pool size was measured by the same perfusion technique before and after cholecystectomy. In a study on gallstone patients with functioning gallbladders Pomare and Heaton demonstrated a reduction in bile acid pool size similar to, but less marked, than found after cholecystectomy (Pomare and Heaton, 1973 b). Overall, it seems likely that the small bile acid pool in gallstone patients remains small and may well further decrease in size.

(c) Effect of Obesity

Many patients with cholesterol gallstone disease are obese and it might be postulated that the differences observed between patients and controls are due to obesity. Two studies have found that obese subjects without gallstones

have large bile acid pools (Bennion and Grundy, 1975, Shaffer and Small, 1977). Pool size in obese subjects with gallstones is reduced by 25% when compared with normal controls but the difference is greater (31%) when compared with obese controls. The increased bile acid pool size had no beneficial effect on the lithogenicity of bile in these obese subjects since cholesterol secretion was greatly increased. This will be discussed fully in the sections on biliary lipid composition and biliary lipid secretion below.

(d) Effect of Diet

Alterations in diet can alter bile acid pool size. Redinger and co-workers investigated the effects of diet on pool size in the Rhesus monkey (Redinger et al., 1973). They found pool size increased when triglycerides were added to the diet and decreased during a fat-free diet and when fasting. Secretion of all three biliary lipids increased during the diet and it seems likely that the expansion of pool size was in response to a need for increased lipid metabolism. Conversely, when fasting, decreased lipid metabolism would result in decreased lipid secretion and bile acid pool size. In their study on obese subjects, Bennion and Grundy found bile acid pool size to be much larger than in normal controls. These obese subjects were given a 1,000 calorie weight-reducing

diet and after 4 weeks, while still on the diet, bile acid pool studies were repeated. Pool size had decreased by 42% to normal levels, reflecting decreased biliary lipid secretion (Bennion and Grundy, 1975). Hepner studied normal subjects on a normal and a low-fat, low-protein diet, which might decrease the frequency of gallbladder contraction. Pool size increased and daily fractional turnover decreased during the diet. The data suggest stimulation of hepatic biosynthesis by reduced bile acid return from the ileum, and that a considerable enterohepatic circulation occurred in the absence of dietary stimulation of gallbladder contraction. In an interesting study on gallstone patients, Pomare and co-workers measured bile acid pool size during a normal diet and one rich in fibre (Pomare et al., 1976). Total pool size did not change but there was a reduction in secondary bile acids and an increase in primary bile acids in gallbladder bile. It seems likely that bran reduces colonic reabsorption of deoxycholic acid which stimulates compensatory hepatic biosynthesis.

(e) Effect of Bile Acid Feeding

Considerable interest has been shown in the effects of bile acid feeding, particularly in patients with gallstones. In 1973, Danziger and co-workers published results of feeding chenodeoxycholic acid to patients with radiolucent stones

in functioning gallbladders (Danziger et al., 1973). As might be expected total bile acid pool size increased and chenodeoxycholic became the predominant bile acid. Cholic and deoxycholic pools decreased markedly by negative feedback inhibition of cholic acid synthesis. In 5 out of 6 patients gallstones decreased in size or dissolved. Two further studies (La Russo et al., 1975, Northfield et al., 1975) confirmed that chenodeoxycholic accounted for 90% of the bile acid pool in all cases but failed to demonstrate increase in total bile acid pool in just under half of their cases.

Cholic acid feeding increased cholic and deoxycholic acid pools in all cases, and increased total bile acid pool size in half of a group of patients with gallstones (La Russo et al., 1975). Ingestion of deoxycholic acid by normal subjects suppressed synthesis of both cholic and chenodeoxycholic acids but did not expand the total pool (La Russo, 1977).

(f) Effect of Vagotomy

Taurocholate metabolism was investigated in 8 patients before, and after truncal vagotomy with pyloroplasty (5 patients) or antrectomy (3 patients) using a doubly-labelled sodium 35S-Tauro-7-3H-Cholate (Arnesjo and Stahl, 1974). The taurocholate pool size and half-life decreased

but synthesis remained unaltered. They suggested that these changes were due to increased bacterial degradation since they had shown 7α-hydroxylation to be increased by comparing the fractional turnover rates of taurine and cholic acid. However, there was no change in the cholic acid:deoxycholic acid ratio, which would be expected in the presence of increased bacterial degradation. Neither total bile acid pool size or faecal excretion were measured.

In a study on dogs with cannulated gallbladders White and co-workers measured pool size using the isotope dilution technique (White et al., 1974). Both the measured cholic acid pool size and the calculated total bile acid pool size expanded within one week of operation, and remained so when re-investigated after 6 weeks and 5 months. Similar findings have recently been reported in post-vagotomy patients whose pool size (8.3 mM) was significantly greater than in matched controls (5.8 mM) (Duane and Stempel, 1977). The changes in bile acid pool size found in cholesterol gallstone disease have not been reproduced by vagotomy in these studies, which therefore raise doubts regarding an association between vagotomy and the subsequent development of gallstones.

(g) Effect of Age

The effects of ageing on bile acid pool size have been studied by a group working in Chile, where there is a high prevalence

of cholesterol gallstone disease (Valdiviseo et al., 1978). They compared bile acid pool size in young (mean age 20 years) and elderly (mean age 59 years) asymptomatic Chilean women with normal weights and recent normal cholecystograms. No significant differences were found between the two groups.

(h) Regulation of Bile Acid Pool Size

Bile acid pool size is dependent upon hepatic biosynthesis and intestinal reabsorption of bile acids. A decrease in either will tend to reduce bile acid pool size. Normally, any reduction in reabsorption decreases the quantity of bile acid returning to the liver which results in an increase in synthesis to maintain pool size constant (Shefer et al., 1969). By this mechanism the flux of bile acids through the liver regulates bile acid synthesis (Bergstrom and Danielsson, 1958, Low-Beer et al., 1972, Mosbach et al., 1972). Alterations in enterohepatic cycling frequency will affect faecal excretion and hepatic flux of bile acids and might, therefore, influence pool size. Each of these factors will now be considered in turn.

i) Hepatic Biosynthesis

Decreased daily synthesis, unresponsive to the inevitably reduced hepatic bile acid flux, will result in a reduction of pool size. Vlahcevic and co-workers suggested that this was the mechanism resulting in a new steady state

at a smaller pool size in their patients with gallstones (Vlahcevic et al., 1970, Swell et al., 1971). However, other investigators have found no difference in synthetic rate in patients and normal controls (Northfield and Hofmann, 1975). Studies on subjects without gallstones have shown little variation in synthetic rates despite a wide range in pool sizes (Low-Beer et al., 1973, Mok et al., 1977). Salen and co-workers demonstrated a marked reduction of cholesterol 7α-hydroxylase, the enzyme regulating bile acid synthesis, in gallstone patients when compared with controls (Salen et al., 1975). Although bile acid synthesis was not measured they suggested that the reduced enzyme levels might indicate reduced synthesis.

If the feedback regulation of synthesis was activated at a lower than normal hepatic flux then faecal loss might not be fully compensated by synthesis. Pool size would contract until a new steady state is reached with faecal losses again equal to synthesis. Synthetic rate would be similar but the ability to expand pool size by increasing synthetic rate would be absent. This appears to be the predominant mechanism in American Indian women with gallstones (Grundy et al., 1975).

ii) Intestinal Reabsorption

Small bile acid pool sizes in patients with ileal disease or resection are readily explained by decreased intestinal reabsorption. Decreased reabsorption reduces hepatic bile

acid flux stimulating synthesis. Faecal losses exceed synthesis and pool size contracts (Hofmann, 1972). However, lesser degrees of loss can be compensated by increases in synthesis. This has been demonstrated in the Rhesus monkey (Dowling et al., 1970) and in man (Garbutt and Kenney, 1972). In the latter study normal subjects were fed the bile acid-binding resin cholestyramine, which effectively created a partial interruption of the entero-hepatic circulation. Increased synthesis compensated for increased loss and pool size was unchanged. Grundy and co-workers estimated bile acid reabsorption in American Indian women with gallstones and in a control group of Indian men without gallstones (Grundy et al., 1972). Although reabsorption tended to be slightly lower in the women (mean 94%) than the men (mean 97%) the differences were not significant. In another study, normal subjects with a wide range of pool sizes were found to have no significant differences in intestinal absorption (Mok et al., 1977).

The evidence suggests that intestinal reabsorption in gallstone disease is normal. If a defect in absorption did exist it would be of small magnitude and insufficient, by itself, to reduce bile acid pool size.

iii) Cycling Frequency

Cycling frequency would influence pool size in two ways. An increased frequency would increase hepatic flux suppressing

synthesis (Shefer et al., 1969). Secondly, since a small fraction of the pool is lost in the faeces during each enterohepatic cycle, increasing cycling frequency could increase daily faecal excretion. Both these mechanisms would tend to reduce pool size. In 1973, Low-Beer and Pomare suggested that the differences they observed in the bile acid pool sizes of patients with coeliac disease, post-cholecystectomy subjects, and normal controls might be due to altered enterohepatic cycling (Low-Beer and Pomare, 1973). Support for this view was provided by a study demonstrating relatively constant bile acid secretion for a variety of pool sizes (Northfield and Hofmann, 1975). Recently, Mok and co-workers examined pool size in normal subjects (Mok et al., 1977). Their data supported the hypothesis of Low-Beer and Pomare that cycling frequency could influence pool size, but failed to repeat the findings of Northfield and Hofmann that secretion rates were constant. At low pool sizes secretion rates were also low, indicating a defect of hepatic synthesis. Hepatic secretion increased with increasing pool size but this was partly compensated by reduction in cycling frequency, particularly with very large pool sizes.

Regulation of bile acid pool size is a complex process not yet fully elucidated. Reduced hepatic synthesis appears to be an important factor, particularly in subjects with a small pool. Intestinal reabsorption does not seem to be an

important factor. Finally, cycling frequency does appear to influence pool size, probably by its effects on faecal excretion and hepatic flux of bile acids. What determines cycling frequency is, as yet, unknown. The frequency of gallbladder emptying and intestinal transit time might be expected to be the principle factors.

4. BILIARY LIPID COMPOSITION

The three major lipid constituents of bile are cholesterol, phospholipids and bile acids. Bile is 90% water, in which cholesterol is almost insoluble, and physical chemical studies have shown that the detergent-like bile acids form mixed micelles with cholesterol and phospholipid (Small et al., 1966 a, Small et al., 1966 b).

(a) Cholesterol Gallstone Disease

Since most gallstones are predominantly cholesterol, with small quantities of bile pigments, calcium salts, and other substances (Holt and Tarnoky, 1953, Sutor and Wooley, 1969, Sutor and Wooley, 1971, Trotman et al., 1974, Sutor and Wooley, 1976), it seemed likely that patients with gallstones might have a disturbance of biliary lipid composition. However, many studies had failed to separate gallstone patients from controls (Isaksson, 1952, Polonovski and Bourrillon, 1952, Isaksson, 1954 a, Isaksson, 1954 b,

Large et al., 1960, Tera, 1963, Burnett, 1965, Dam et al., 1966). A major advance in our understanding of cholesterol gallstone formation was made by Admirand and Small, who first separated cholesterol gallstone patients from controls on the basis of the relative biliary lipid composition (Admirand and Small, 1968). Supersaturation of bile with cholesterol is the cornerstone of our present understanding of gallstone formation. Admirand and Small's findings have been confirmed in many subsequent studies (Vlahcevic et al., 1970, Dam et al., 1971, Dam and Hegardt, 1971, Bell et al., 1973, Holzbach et al., 1973, Adler et al., 1974). Alternative lines of separation between the stable micellar solution of bile and the unstable cholesterol-supersaturated bile were drawn by two groups (Dam and Hegardt, 1971, Holzbach et al., 1973), but the differences are not great.

Attention was focussed on the role of the gallbladder in gallstone formation, hitherto unquestioned, by studies published almost simultaneously (Small and Rapo, 1970, Vlahcevic, Bell and Swell, 1970). The relative composition of biliary lipids in hepatic and gallbladder bile was investigated. Both hepatic and gallbladder bile were found to be abnormal, suggesting that gallstones were due to a disorder of the liver, not the gallbladder. This radical hypothesis raised questions regarding the role of the gallbladder. What event in the gallbladder disrupts the delicate balance of the supersaturated bile? Why, and how, does the

precipitated cholesterol aggregate to form stones instead of remaining as a finely dispersed suspension? It seems likely that bile pigments or mucus, both of which have been found to exist at the centre of gallstones (Rains et al., 1960, Bills and Lewis, 1975, Been et al., 1979) may be the cause. Further studies, reviewed in following sections, have shown that this conceptually simple view of the pathogenesis of gallstones is not entirely valid. It is now realised that there is considerable variation in biliary lipid composition such that, at times, normal subjects may secrete super-saturated bile and gallstone patients may secrete a normal unsaturated bile (Smallwood et al., 1972, Northfield and Hofmann, 1975).

(b) Effect of Cholecystectomy

A natural sequel to the demonstration of abnormal bile in gallstone patients was the investigation of biliary lipid composition after cholecystectomy. In 1972 groups working in Great Britain (Simmons et al., 1972) and in America (Shaffer et al., 1972 b) reported their studies which both found a marked improvement in biliary lipid composition. Simmons and co-workers compared cholecystectomy patients to normal controls and gallstone patients. Bile from the gallstone patients was supersaturated with cholesterol but bile from the cholecystectomy patients was very similar in composition to the controls, and lay well within the micellar zone. Shaffer and co-workers investigated bile collected at operation and by post-operative T-tube drainage in gallstone patients and a control group undergoing bile duct surgery for stricture. They also found a return to normal composition post-operatively in the cholecystectomy patients and similar findings were reported by others (Kernahan and Danziger, 1975). Conflicting results were reported by Almond and co-workers investigating a group of patients before and after operation (Almond et al., 1973). They found no improvement in composition or bile acid pool size. There were methodological differences in bile collection before and after operation which might have affected their results. However, similar results were reported in a study which compared cholecystectomy subjects with normal controls (McDougall et al., 1975),

and in an uncontrolled study of cholecystectomy subjects (Kimball et al., 1976). Further support for these findings was provided by Adler and co-workers who examined the same group of Indian women before and after cholecystectomy, with no variations in methodology, and found consistently abnormal bile. The differences between these studies might be due to the comparison of dissimilar biles. Intermittent secretion of lithogenic bile was demonstrated in post-cholecystectomy patients (MacKay, 1972, Smallwood et al., 1972, Heller and Bouchier, 1973) and in healthy man (Nakayama and van der Linden, 1970, Holzbach et al., 1973). Another study showed considerable diurnal variation in composition due to feeding and fasting (Metzger et al., 1973). Sequestration of much of the bile acid pool in the gallbladder after an overnight fast reduced the bile acid available to form micelles with cholesterol. Cholesterol secretion was not greatly reduced and lithogenicity therefore increased. The influence of such variations in bile acid output had been clearly demonstrated by Schersten and co-workers who showed that bile was in the micellar zone at high bile acid secretion rates but in the unstable super-saturated zone at low secretion rates (Schersten et al., 1971). Shaffer and Small repeated their studies on a group of gallstone patients before and after cholecystectomy using the perfusion technique (Grundy and Metzger, 1972) previously employed in the studies by Adler and co-workers (Shaffer and

Small, 1977). They again found an improvement in composition, and were able to demonstrate that this was due to an increase in bile acid secretion resulting from the more continuous circulation of the small bile acid pool in the absence of the gallbladder. This suggestion was supported in studies on patients with gallstones who were re-investigated after the development of a non-functioning gallbladder and again after cholecystectomy (Antsaklis et al., 1975, Redinger, 1976). During gall-bladder function lithogenic bile was secreted. With loss of function the biliary lipid composition improved and a stable micellar solution was secreted. There was little further change in composition when the gallbladder was removed.

These studies indicate that, although the secretion of an abnormal bile is a pre-requisite for gallstone for-mation, the gallbladder plays an active role influencing biliary lipid composition by virtue of the effect on the frequency of circulation of the bile acid pool and by providing conditions in which precipitation and stone growth can occur.

(c) Racial and Species Differences

There are marked differences in biliary lipid composition

between species and there are considerable racial differences in humans. There is a high correlation between the degree of cholesterol saturation of bile and the propensity for a particular race or species to form gallstones (Small, 1972).

Animals with very low concentrations of cholesterol relative to bile acids and phospholipids such as the ox, dog, pig and chicken, do not form gallstones (Kruse and Dam, 1963, Nakayama, 1969, Cowie and Clark, 1972). Rhesus monkeys, squirrel monkeys, and Syrian hamsters have higher concentrations of cholesterol in their bile, but are still well within the micellar zone (Prange et al., 1962, Dowling et al., 1971, Osuga and Portman, 1972) but stone formation can occur with a high cholesterol diet. In the baboon, bile is saturated with cholesterol and stones form spontaneously (McSherry et al., 1971).

Similarly, by studying biliary lipid composition in different races, it is possible to predict their likelihood of cholesterol gallstone disease. In a cholecystographic study Sampliner and co-workers drew attention to the high prevalence of gallstones among the American Indians of the Southwest (Sampliner, 1970). Studies of biliary lipid composition have shown a high incidence of bile supersaturated with cholesterol among the normal population (Thistle and Schoenfield, 1970, Bell et al., 1972, Vlahcevic et al., 1972,

Bell et al., 1973). A moderately high incidence of gallstone disease is found in Denmark and Sweden and again there is close correlation with the higher than normal incidence of supersaturated bile (Dam et al., 1966, Nakayama and van der Linden, 1970). Conversely, in countries such as Japan, where biliary cholesterol is low the incidence of gallstones is also low. When cholesterol saturation of bile is very low, as in the Masai tribe in Africa, gallstones are virtually unknown (Biss et al., 1971).

(d) Effect of Obesity

It is well known that patients with gallstones are frequently obese, often grossly so. Is this due to coincidental association between two common conditions, or are obese subjects at a greater risk of developing gallstones than the rest of the population?

Osler first reported an increased incidence of gallstones in obese patients (Osler, 1897). More recently, in the epidemiological study in Framingham, Massachusetts, a positive correlation was reported between obesity and gallstone prevalence (Friedman et al., 1966). Over the 10 years of the study obese patients had about twice the risk of developing gallstones than their thinner counterparts. The suggestion that the metabolic defect of obese patients forming gallstones might be different from that of non-

obese patients was first made by Grundy and co-workers (Grundy et al., 1972). In studies on American Indian women with gallstones they found that, although bile acid secretion rate was less than in Indian men or Caucasian women without gallstones, cholesterol secretion was significantly increased and contributed to the defective biliary lipid composition. They suggested that this increased cholesterol secretion was related, in part, to obesity. In a further study on Caucasian women with and without gallstones they again found increased cholesterol secretion in the obese gallstone patients (Grundy et al., 1974). Another group studying obese patients without gallstones also demonstrated markedly increased cholesterol secretion (Mabee et al., 1976). Studies by Nestel and co-workers had demonstrated an increased daily production of cholesterol in the obese (Nestel et al., 1973). Since biliary secretion is the main route of cholesterol excretion in man it might be expected that biliary cholesterol secretion would be increased in obesity. In an extensive study of obese subjects Bennion and Grundy showed that obesity was associated with supersaturation of bile with cholesterol (Bennion and Grundy, 1975). The relative composition of bile improved after weight loss, though it remained more saturated than matched non-obese controls. During weight loss, cholesterol supersaturation was at its highest because bile acid and phospholipid secretion decreased while cholesterol was mobilised from adipose tissue and

secreted. Their findings were confirmed in another study of obese and non-obese subjects, with and without gallstones (Shaffer and Small, 1977).

These studies indicate that two different secretory abnormalities account for abnormal bile in obese and non-obese subjects with gallstones. In non-obese subjects secretion of the solubilising lipids, bile acids and phospholipids, is reduced while in grossly obese subjects cholesterol secretion is increased. Moderately obese subjects may have a mixed defect. Loss of weight by obese subjects improves biliary lipid composition, though saturation increases during weight loss. It therefore seems likely that, although the risk of gallstone formation may be transiently increased during weight loss, a maintained weight reduction should decrease the chances of developing gallstones.

(e) Effect of Diet

In an epidemiological study of railroad workers in India, Malhotra found that the incidence of gallstones was seven times more frequent in North India than in South India (Malhotra, 1968). In North India the diet is considerably higher in fat content than in the South. In a retrospective dietary survey among patients with gallstones and well-matched controls, Sarles and co-workers demonstrated a

significantly higher daily calorie intake among the patients (Sarles et al., 1969). They also studied post-operative patients with indwelling T-tubes and demonstrated that the bile was more lithogenic during a high calorie diet (Sarles et al., 1971). However, calorie intake alone is not the only dietary factor. Alterations in the composition of the diet with no change in total calories can influence biliary lipid composition. Bile lithogenicity increased when normal subjects changed from cholesterol-free to high cholesterol eucaloric diets (Den Bensten et al., 1973), and a low cholesterol diet enhanced the improvement of biliary lipids by chenodeoxycholic acid in patients with gallstones (Maudgal et al., 1978). It is also known that dietary manipulation can induce gallstone formation in susceptible animals such as the hamster (Prange et al., 1962, Dam, 1971), the guinea pig (Schoenfield and Sjovall, 1966), the baboon (Glenn and McSherry, 1970, McSherry et al., 1971) and the squirrel monkey (Osuga and Portman, 1971).

The benefits of a dietary fibre for large bowel function are now unquestioned (Painter and Burkitt, 1971). In an interesting study of normal subjects, Pomare and co-workers investigated biliary lipid composition during normal and high-fibre diets (Pomare et al., 1976). Bile was significantly less saturated with cholesterol during the high-fibre diet. It seemed likely that deoxycholic acid reabsorption from the colon was decreased since the deoxycholic acid pool was smaller. There was a compensatory

increase in chenodeoxycholic acid synthesis which has been shown to suppress cholesterol synthesis (La Russo et al., 1975, Adler et al., 1975). Their findings were confirmed in a similar study (McDougall et al., 1977).

(f) Effect of Bile Acid Feeding

In 1972, a group from the Mayo Clinic reported that cholesterol gallstones could be successfully dissolved by oral treatment with chenodeoxycholic acid (Danziger et al., 1972). Investigation of biliary lipid composition of patients receiving chenodeoxycholic acid showed that bile became unsaturated with cholesterol and, not surprisingly, that chenodeoxycholic became the predominant bile acid (Danziger et al., 1973, La Russo et al., 1975, Northfield et al., 1975). On cessation of treatment with chenodeoxycholic acid in gallstone patients bile promptly returned to a supersaturated state (Thistle et al., 1974). Prevention of recurrence might be achieved by a low dose maintenance therapy or by the use of a high fibre diet. Although the improvement in lipid composition by the diet is unlikely to cause stone dissolution it might well be sufficient to prevent recurrence (Pomare, 1976). Cholic acid, the other primary bile acid, was not as effective as chenodeoxycholic acid since it did not decrease cholesterol synthesis (La Russo et al., 1975). Feeding the secondary bile acid, deoxycholic acid, produced no significant alteration

in the degree of cholesterol saturation of bile (La Russo et al., 1977).

The mechanism by which chenodeoxycholic acid seems to specifically inhibit hepatic cholesterol synthesis and secretion is unknown. Studies of cholesterol metabolism have been carried out during chenodeoxycholic acid treatment in both man (Hoffman et al., 1974) and animals (Webster et al., 1975). There was no significant change in the exchangeable cholesterol pool, nor any change in serum lipids.

(g) Effect of Drugs

The effects of a number of different drugs on biliary lipid composition have been studied. Since gallbladder disease is more frequent in women than in men particular interest has been shown in synthetic steroid hormones. Studies have also investigated clofibrate which lowers serum cholesterol, phenobarbitone, a known inducer of hepatic enzymes, and cholestyramine, a bile acid-binding resin.

i) Oestrogens

Use of oral contraceptives and post-menopausal oestrogen therapy have both been associated with an increased incidence of cholesterol gallstones in an epidemiological study (Boston Collaborative Drug Surveillance Programme, 1973, 1974). Incidence of gallbladder disease was doubled in

a study of patients with coronary artery disease taking oestrogens when compared with those taking a placebo (Coronary Drug Project Research Group, 1977). Three patients studied by Pertsemlidis and co-workers had increased bile lithogenicity while taking an oestrogen-progestin combination (Pertsemlidis et al., 1974). More extensive studies have now been performed. In a study of 22 women before, during, and after oral contraceptive use, Bennion and co-workers also found a marked increase in cholesterol saturation of bile during treatment (Bennion et al., 1976). In another study both biliary lipid composition and bile acid pool size were measured (Di Padova et al., 1978). A similar increase in lithogenicity was demonstrated, though bile acid pool size remained unchanged. Saturation returned to normal levels on cessation of treatment. The biochemical changes of gallstone disease predate the radiological appearance by months or years (Small, 1970) and in addition there is often a delay of several years from radiological appearance of stones to the first symptoms (Sampliner et al., 1970). These delays mean that the full effect of the oral contraceptive pill and post-menopausal oestrogens on the incidence of gallstones will be manifest in the years to come (Small, 1976).

ii) Clofibrate

Clofibrate has been used extensively to decrease plasma cholesterol and triglycerides in patients with ischaemic heart disease or hyperlipidaemia (Oliver, 1968, Berkowitz,

1971). In hyperlipidaemia clofibrate increases faecal cholesterol excretion and decreases faecal bile acid excretion (Grundy et al., 1972). Thistle and Schoenfield observed a marked increase in cholesterol saturation of bile during combined clofibrate and cholic acid treatment such that bile was consistently supersaturated (Thistle and Schoenfield, 1971). In studies on post-cholecystectomy patients with indwelling fine bore biliary catheters, Pertsemlidis and co-workers examined the effects of clofibrate alone on biliary lipid composition (Pertsemlidis et al., 1974). Cholesterol concentration increased and bile acid concentration decreased in 6 out of 7 patients studied. These changes made several of the biles supersaturated with cholesterol and suggested that patients receiving clofibrate might be at a greater risk of developing gallstones than the normal population. This was borne out in a prospective epidemiological study amongst patients with ischaemic heart disease which showed an increased incidence of gallstone disease amongst those taking clofibrate when compared with a placebo group (Coronary Drug Project Research Group, 1977).

iii) Phenobarbitone

Phenobarbitone, a well known enzyme inducer, has been shown to increase the activity of 7α-hydroxylase in the rat thereby increasing bile acid synthesis (Shefer et al., 1972). The effects of phenobarbitone were further characterised in studies on the Rhesus monkey (Redinger and Small, 1973).

Phenobarbitone caused considerable increases in bile flow, both of the bile acid-dependent and bile acid-independent fractions. Cholesterol secretion was unaffected and there was, therefore, a decrease in the cholesterol saturation of bile. Phenobarbitone increased hepatic bile acid biosynthesis both in animals with an intact and an interrupted enterohepatic circulation suggesting that phenobarbitone could, in some way, "over-ride" the regulation of synthesis by feedback inhibition.

iv) Cholestyramine

Cholestyramine is a basic anion-exchange resin which is capable of sequestering bile acids. It has been used in the treatment of diarrhoea due to bile acid malabsorption following ileal resection (Hofmann and Poley, 1972). Biliary lipid studies in gallstone patients receiving cholestyramine showed that, while there was a marked increase in cholic acid concentration and marked decrease in concentrations of chenodeoxycholic and deoxycholic acids, there was no change in the overall biliary lipid composition of cholesterol, phospholipids and bile acids.

(h) Effect of Vagotomy

Truncal vagotomy has frequently been implicated as an aetiological factor in cholesterol gallstone disease, though most of the evidence has been retrospective and

anecdotal. It might be expected that, if vagotomy does predispose to gallstones, the changes in biliary lipid composition known to occur in gallstone disease would be present. However, the evidence to date is inconclusive. Several studies have been performed in dogs, whose bile is very undersaturated with cholesterol. When human gallstones are placed in the gallbladder of a dog, they dissolve. Truncal vagotomy has been found to slow the rate of dissolution, suggesting an adverse effect (Barnett and Hilbun, 1966). Fletcher and Clark found a decrease in bile acid concentration in dogs after vagotomy though the bile remained within the limits of solubility defined by Admirand and Small (Fletcher and Clark, 1969). In a subsequent study by the same group bile lithogenicity increased beyond the limits of solubility (Cowie and Clark, 1972). However, in this study bile acid concentration was unaltered and the changes seen were due to markedly increased cholesterol concentration. Phospholipid concentration was significantly reduced. These rather divergent results are better understood if one considers secretion rates rather than absolute concentration. From their data it is possible to calculate daily secretion rates. It becomes apparent that the observed changes in concentration are due to low secretion rates after vagotomy. Bile acid and phospholipid secretion are both reduced by more than 90% whereas cholesterol secretion is only reduced by 65%, thus increasing lithogenicity. This is in accord with

other studies of bile acid secretion (Dowling et al., 1970, Hardison and Apter, 1972). Other studies in dogs do not clarify the issue. Amdrup and Griffith and Inberg and co-workers both found no change in biliary lipid composition after vagotomy (Amdrup and Griffith, 1970, Inberg et al., 1971). Machleder found an increase in cholesterol concentration and a slight decrease in taurocholate concentration (Machleder, 1971). White and co-workers found that bile acid concentration decreased six weeks after vagotomy with no significant changes in cholesterol or phospholipid concentration (White et al., 1974). Bile acid concentration was still decreased five months post-operatively though the difference was less. In another study there were no significant changes in composition but 4 out of 8 dogs developed gallstones (Wilbur et al., 1975). Both these studies used chronic cannulation techniques and it is possible that infection could have influenced the results. However, in the study by Wilbur and co-workers bile cultures were negative during the study period 3 - 6 weeks after operation. Chronic inflammation was present on microscopy at 12 weeks.

There have been two recent studies in man. A group of 10 patients have been studied before, and 10 days and 1 year after, vagotomy (Smith et al., 1972, Smith et al., 1973). Cholecystokinin-stimulated gallbladder bile was collected by duodenal intubation on each occasion. There were no significant changes at 10 days post-operatively, but after

1 year the concentration of all three biliary lipids had increased. Cholesterol concentration increased disproportionately so that, although still within the micellar zone, the degree of saturation was closer to the limits of solubility. In another study comparing bile acid pool size and biliary lipid composition in post-vagotomy patients and controls exactly opposite changes were noted (Duane and Stempel, 1977). The bile acid pool expanded, bile acid concentration increased, and cholesterol concentration decreased making stone formation less likely.

Cholesterol gallstone disease is associated with well-defined changes in biliary lipid composition. The wide variety of changes in composition produced by vagotomy in dogs makes it impossible to draw any definite conclusions. The studies in man give no further evidence to support the view that vagotomy is an aetiological factor in gallstone disease.

(i) Effect of Age

The effect of ageing on biliary lipid composition has recently been studied in Chile, a country with a high prevalence of cholesterol gallstone disease (Valdiviseo et al., 1978). Twelve young and twelve elderly asymptomatic women with normal weights and recent normal oral cholecystograms were studied. Cholesterol saturation of

bile increased to make stone formation more likely. Whereas only 1 of the young group had supersaturated bile, 5 of the elderly group had supersaturated bile and a further 5 were close to the limits of solubility. An additional group of 4 elderly obese patients was studied, all of whom were found to have supersaturated bile.

It therefore appears that the known increase in incidence of gallstone disease with increasing age is due to a gradual increase in the cholesterol saturation of bile.

5. BILIARY LIPID SECRETION

When Vlahcevic and co-workers demonstrated a reduced bile acid pool size in gallstone patients they postulated that the reduction in pool size might be the causative factor in the production of abnormal bile found in these patients (Vlahcevic et al., 1970, Swell et al., 1971). Fundamental to this hypothesis was the assumption that the reduced bile acid pool in the gallstone patients circulated at the same frequency as in normals. If this were so bile acid secretion rate would necessarily be reduced. Phospholipid secretion, which depends on bile acid secretion (Nilsson and Schersten, 1969, Wheeler and King, 1972, Hardison and Apter, 1972), would also be reduced, cholesterol saturation would increase, and precipitation and stone formation would be more likely.

Studies of biliary lipid secretion have cast doubt on this assumption. In the Rhesus monkey partial interruptions of the enterohepatic circulation (20% or less) do not result in a reduction of bile acid pool size or of bile acid secretion rate (Dowling et al., 1970). It is therefore possible for increased hepatic biosynthesis to compensate for increased losses and maintain pool size and secretion rate. Greater interruptions (30% or more) exceed the liver's capacity for increased biosynthesis and both pool size and secretion rate fall. At these low secretion rates cholesterol saturation increases due to a disproportionate fall in bile acid secretion. The development of duodenal perfusion techniques (Grundy and Metzger, 1972) enabled accurate measurements of biliary lipid secretion to be made. Subsequent studies have shown there to be various abnormalities of lipid secretion. American Indian women have a mixed defect, with increased cholesterol secretion and decreased bile acid secretion (Grundy et al., 1972), whereas obese Caucasian women have increased cholesterol secretion but normal bile acid secretion (Grundy et al., 1974). Grossly obese subjects have markedly increased cholesterol secretion in the presence of normal or slightly raised bile acid secretion (Bennion and Grundy, 1975). Each of these secretory abnormalities results in a lithogenic bile.

In 1972, Shaffer and co-workers demonstrated an improvement

in biliary lipid composition by cholecystectomy and suggested that this was due to either an expansion of the bile acid pool or more rapid enterohepatic cycling (Shaffer et al., 1972). Support for an increase in cycling frequency after cholecystectomy was provided in two subsequent studies. Malagaleda and co-workers showed that bile acid secretion was continuous after cholecystectomy, and that there was an increased daily output (Malagaleda et al., 1973). In another study, Soloway and Schoenfield demonstrated significant increases in bile acid and phospholipid secretion in relation to meals (Soloway and Schoenfield, 1975). Their kinetic studies showed this to be due to increased re-circulation of bile acids rather than increased synthesis. The abnormalities of biliary lipid secretion in cholesterol gallstone disease have recently been further characterised in an elegant study by Shaffer and Small (1977). Bile acid pool size and secretion rate was measured before and after cholecystectomy in obese and non-obese patients, and in normal controls. The differences in biliary lipid secretion between obese and non-obese subjects, previously reported by Bennion and Grundy, were confirmed. Cholecystectomy caused a slight decrease in bile acid pool size and a marked increase in bile acid and phospholipid secretion rates. This could only be due to an increased frequency of enterohepatic circulation, and this was confirmed by their calculations which showed that cycling frequency had doubled. Of particular interest

were their preoperative studies on non-obese gallstone patients, a group essentially similar to the one originally studied by Vlahcevic in 1970. Both bile acid pool size and bile acid secretion rate were reduced confirming Vlahcevic's original hypothesis. Why hepatic bile acid biosynthesis is suppressed is unknown. Their study also confirmed that a different secretory defect is present in obesity. Bile acid pool size and secretion rate are normal but cholesterol secretion is increased. In either case cholesterol saturation of bile is increased and gallstone formation is more likely. The presence of these different mechanisms explain some of the differences observed by various investigators. Other differences have resulted from the comparison of dissimilar bile samples.

In health bile acid secretion is the principle determinant of biliary lipid composition. At high bile acid secretion rates bile becomes undersaturated with cholesterol but at low secretion rates cholesterol saturation increases. In cholesterol gallstone disease an undetermined hepatic defect suppresses bile acid biosynthesis. Pool size and secretion rate are both reduced. Removal of the gallbladder causes the small bile acid pool to re-circulate more frequently resulting in an increased bile acid secretion rate which improves biliary lipid composition. The reduced pool size and the underlying hepatic defect are unaltered.

6. ROLE OF THE GALLBLADDER

The separation of patients with gallstones from normals on the basis of the relative composition of gallbladder bile (Admirand and Small, 1968) and the subsequent finding that hepatic bile was also abnormal (Small and Rapo, 1970) focussed attention on the role of the liver in cholesterol cholelithiasis. Since then the predominant opinion expressed in the literature has been that cholesterol cholelithiasis results from either a genetic or metabolic defect within the hepatocyte or to a failure of the hepatocyte to respond to alterations in the enterohepatic circulation of bile (Swell et al., 1972).

However, the rare occurrence of truly recurrent stones after cholecystectomy and the improvement in relative biliary lipid composition after cholecystectomy (Shaffer et al., 1972, Simmons et al., 1972, Nahrwold et al., 1976, Shaffer et al., 1977) or loss of gallbladder function (Redinger, 1976) are difficult to reconcile with this view. These findings, discussed in the sections on the bile acid pool size and biliary lipid composition above, suggest that gallbladder motor function might predispose to cholesterol stone formation by altering the dynamics of the enterohepatic circulation. It is known that during fasting some of the secreted bile acids still enter the duodenum (O'Brien et al., 1974, Shaffer et al., 1974). The gallbladder must therefore

interact with the sphincter of Oddi to partition the total hepatic bile flow between the gallbladder and the duodenum.

The concept that hepatic bile is normally partitioned between the gallbladder and the duodenum, even while gallbladder filling occurs has important consequences when one considers the effect of an alteration in gallbladder motility. If gallbladder tone was reduced one would expect a greater proportion of hepatic bile to enter the gallbladder during fasting. Less bile would enter the duodenum and a smaller proportion of the bile acid pool would circulate during fasting. During feeding the larger volume of bile acids in the gallbladder would increase hepatic return and inhibit synthesis. It has been suggested that this brief but high rate of return can suppress synthesis for a period of several hours (Small et al., 1972). If this is so, then the synthesis of bile acids would remain decreased, or increase more slowly during the subsequent fasting period. As a result the <u>effective</u> bile acid pool would be smaller for significant periods of time when synthesis was depressed and much of the total pool was sequestered in the gallbladder. At this time bile acid secretion rate would be low and lithogenic bile formation would be more likely (Hardison and Apter, 1972). However, the <u>total</u> pool as measured by isotope dilution, might be expected to be larger than normal because of the increased

amount of bile acids stored in the gallbladder. Such a situation may well occur after truncal vagotomy in which gallbladder dilation occurs as a result of denervation (Johnson and Boyden, 1952, Inberg and Vuorio, 1969, Fagenberg et al., 1970) but still responds normally to cholecystokinin (Liedberg, 1969).

Most Caucasian patients with gallstones have a reduced bile acid pool but synthesis does not increase to restore the pool to normal. It has recently been shown that gallbladder emptying in response to meals is increased in patients with gallstones (Maudgal et al., 1978). In this situation there might again be prolonged suppression of hepatic synthesis. If this occurred repeatedly, daily synthesis would be decreased and pool size would contract until a new steady state had been reached.

This new steady state would, in addition to the smaller than normal pool, have a greater degree of interruption of the enterohepatic circulation during fasting even though gallbladder tone was normal. Cholecystectomy would be expected to improve bile composition in either of the situations just described since a more constant circulation of bile acids would prevent prolonged suppression of synthesis after meals.

It therefore seems possible that factors affecting the motility of the gallbladder and sphincter of Oddi could

have an influence on biliary lipid composition.

(a) Biliary Motility

Gallbladder muscle tone reflects the compliance of the smooth and fibroelastic tissue of the gallbladder wall and is modulated by autonomic and hormonal mechanisms. Dynamic studies of the pressure volume relationship of the baboon's gallbladder have demonstrated that gallbladder tone remains constant over a 2 - 4 hour period despite large fluctuations in volume (Schoetz et al., 1978 a). This property of slow readjustment of muscle tone would allow filling during the period of increased bile secretion after gallbladder emptying, whereas a rapid readjustment of tone would present an increased resistance to flow and tend to continue the circulation of the bile acid pool by diverting more bile into the duodenum.

The role of the autonomic nervous system in modulating gallbladder tone have been studied by nerve stimulation and pharmacological methods. The parasympathetic nervous system maintains tone (Pallin et al., 1964) and vagotomy decreases resting gallbladder pressure (Liedberg, 1969). Administration of atropine after vagotomy further reduces resting pressure indicating that intramural cholinergic activity is also involved in the maintenance of resting

tone. Vagal stimulation potentiates the effect cholecystokinin (Pallin et al., 1964) but vagotomy does not decrease its effect (Liedberg, 1969). Acetylcholine and pilocarpine both increase gallbladder tone and this effect is blocked by atropine (Schoetz et al., 1978 b).

Cholecystokinin is the principle hormonal influence on the gallbladder and graded dose-response curves have been described both in vivo and in vitro muscle strip preparations (Ryan and Cohen, 1976 a, Ryan and Cohen, 1976 b). In addition there are interactions with other gastrointestinal hormones, gastrin, secretin and glucagon. Gastrin increases gallbladder pressure in the dog (Vagne and Grossman, 1968) but no independant effect has been demonstrated in man (Mack and Todd, 1968).

(b) Water Reabsorption

In vitro studies in the mouse gallbladder have suggested that there is an increase in water reabsorption by the gallbladder (Lee, 1978). This was associated with epithelial cell proliferation and increased glycoprotein content in the gallbladder wall. There is theoretical and experimental evidence that the initial formation of gallstones takes place near the gallbladder wall (Amberg, 1967, Cussler et al., 1970). When bile is concentrated within the gallbladder water is extracted from bile at the

mucosal surface. The bile thus becomes more concentrated near the mucosa than in the centre of the gallbladder and the concentrations of the constituents of bile rise exponentially from the centre to the mucosa (Cussler et al., 1970). An increase in the rate of absorption will increase the concentration gradient and facilitate stratification, and will also concentrate the layer of mucus which lies next to the mucous membrane. These alterations in absorptive function may contribute to gallstone formation.

(c) Nucleation

Supersaturated bile occurs intermittently in normal subjects without gallstones (Smallwood et al., 1972) and in susceptible subjects such as the American Indian (Thistle and Schenfield, 1970) before gallstones form. Since these metastable biles do not contain gallstones it seems likely that some additional factor may be necessary to initiate the precipitation of cholesterol and nucleation of a gallstone. Studies by Holan and co-workers have shown a significant difference in the nucleation time of normal and abnormal biles (Holan et al., 1979). The mean nucleation time in abnormal bile was 2.9 days compared with 15 days in the controls. The nucleation time of 2.9 days in the abnormal biles is compatible with normal gallbladder function since it is known that gallbladder

emptying is incomplete and stratification of bile can also occur. Precipitation is most likely to occur in this retained stagnant bile.

7. THE CHRONIC BILE FISTULA MONKEY

The development of the chronic bile fistula Rhesus monkey model by Dowling and co-workers has been an important contribution to the study of biliary physiology (Dowling et al., 1968). Previously studies of the enterohepatic circulation had been limited by the difficulties of access, particularly for chronic studies, in man. Studies had been limited to analysis of faeces or samples obtained by duodenal drainage. The access to the enterohepatic circulation provided by their model allowed researchers to collect bile samples with ease and manipulate the enterohepatic circulation at will.

The success of the model was due, in part, to the system, simultaneously developed, whereby the effluent bile was automatically returned to the monkey to maintain the enterohepatic circulation. A stream-splitting pump, activated by bile flow, returned effluent bile to the monkey and a predetermined fraction was diverted to a collection tube for subsequent analysis. Initially, the system was optically activated, but the subsequent development of miniature ultrasonic transducers, which trigger the pump more reliably, has enabled the system to be improved (Berger, 1974).

In subsequent studies, Dowling and co-workers characterised the normal biliary lipid composition and bile acid pool

size in the monkey, and made extensive studies on the effects of acute and chronic interruption, and ileal resection, on the enterohepatic circulation (Dowling et al., 1970, Dowling et al., 1971). They conclusively demonstrated that increased hepatic bile acid biosynthesis could compensate for partial interruptions of the enterohepatic circulation. More extensive interruptions (in excess of 20%), or ileal resection, resulted in a decrease in bile acid secretion and pool size, and an increase in faecal fat excretion.

A disadvantage of animals frequently used in biliary physiology studies, such as the rat and the dog, is the marked undersaturation of bile with cholesterol. At the other extreme are animals which spontaneously form gallstones, such as the baboon (McSherry et al., 1971), or in whom gallstones may be produced by changes in diet, such as the hamster (Dam, 1971). For purely physiological studies these extremes are probably best avoided. Rhesus monkeys do not normally form stones and their biliary lipid composition is reasonably close to that of man, though less saturated with cholesterol (Campbell et al., 1971, Dowling et al., 1971).

Strasberg and co-workers defined the effects of increased biliary tract pressures on biliary lipid secretion (Strasberg et al., 1971). A pressure gradient of 2 cm of water existed between the bile duct and the exterior.

Back pressures of up to 12 cm water had no effect on bile flow or composition. Subsequent increases in pressure resulted in decreased bile flow and decreased secretion rates of all three lipids. When back pressure exceeded 30 cm of water bile flow ceased altogether. Redinger and Small examined the effects of phenobarbitone, a known hepatic enzyme inducer, in monkeys with intact and interrupted enterohepatic circulations (Redinger and Small, 1973). Phenobarbitone increased bile flow in all animals. Bile acid and phospholipid secretion increased but cholesterol secretion was unaltered. Effects of various diets and fasting were studied by the same group (Redinger et al., 1973). A high fat diet resulted in enhanced secretion of all biliary lipids with no significant change in composition. Diets supplemented with medium-chain triglycerides resulted in decreased cholesterol saturation while fasting increased cholesterol saturation and lithogenicity. Further studies during fasting (Strasberg et al., 1974) confirmed the close relationship between bile flow and bile acid secretion previously demonstrated in the fasted dog (Wheeler and Ramos, 1960) and the fed monkey (Dowling et al., 1968).

The bile fistula monkey has also been used by the same group to examine the complex interactions between bile acid and cholesterol secretion in acute (Strasberg and Ilson, 1975), and chronic (Strasberg et al., 1976) interruption of the enterohepatic circulation. Their acute studies

suggested that the observed correlations between bile acid secretion rate and cholesterol synthetic rate (which is equal to cholesterol secretion rate) might be due to an inverse relationship between bile acid and cholesterol synthetic rates. The chronic studies confirmed the relationships between bile acid secretion, bile acid synthesis and cholesterol synthesis rates, but large variations in cholesterol synthesis indicate that these are not the only factors concerned.

Alternative physiological models in primates have been described but neither give the investigator the same degree of control over the enterohepatic circulation. O'Brien and co-workers have described a model in which a cholecystocystopexy is performed so that gallbladder bile can be sampled by percutaneous needle puncture (O'Brien et al., 1974). The model has proved useful in studies of gallbladder function with normal enterohepatic circulation. Baboons were used in their studies and it is doubtful whether the technique could be easily performed in the Rhesus monkey which has a smaller, and often partially intrahepatic gallbladder. Wood and co-workers have described a model in Rhesus monkeys in which bile from the proximal common duct passes through a subcutaneous silicone tube before entering the distal common bile duct (Wood et al., 1977). This model obviates the need for a restraining chair, but only permits acute studies to be performed. Morbidity and mortality has been relatively high. Problems

with sediment obstructing the subcutaneous tube were noted.

Neither of these techniques offer the same degree of control over the enterohepatic circulation as the bile fistula monkey, which remains the most suitable model for prolonged studies of biliary physiology.

DETAILED STATEMENT OF THE PROBLEM TO BE INVESTIGATED

It appears from the above review of the literature that the size of the bile acid pool, by its influence on bile acid secretion and on biliary lipid composition, may be an important aetiological factor in cholesterol gallstone disease, particularly in the non-obese Caucasian. Most work has concentrated on biliary lipid composition and biliary lipid secretion and their relationships with bile acid pool size. Less attention has been paid to factors determining the size of the bile acid pool.

It is now possible to construct an animal model in which the enterohepatic circulation can be completely controlled.

This thesis sets out to study the physiology of the enterohepatic circulation with particular respect to the relationship between the frequency of the enterohepatic circulation and the size of tbe bile acid pool. In addition, the effects of alterations in enterohepatic cycling frequency on biliary lipid composition will be determined. Additional data will be collected in supplementary studies on truncal vagotomy in man.

In this work the chronic bile fistula Rhesus monkey has been used and an attempt has been made to answer the following questions:

(a) Can alterations in cycling frequency influence bile acid pool size?

(b) What effect do alterations in cycling frequency have on bile acid synthesis and daily fractional turnover rate?

(c) What effect do alterations in cycling frequency have on biliary lipid composition?

(d) Does truncal vagotomy alter bile acid pool size?

(e) Does truncal vagotomy alter biliary lipid composition?

(f) Could these changes be mediated by an alteration in hepatic cycling frequency?

MATERIALS AND METHODS

General Introduction

In the experimental part of this study chronic bile-fistula Rhesus monkeys, in whom the enterohepatic circulation was controlled, have been used. The animals were maintained in as near normal a physiological condition as was possible.

The first part of this section describes the preparation and care of the animal model and the operative and experimental procedures used.

The second part of this section is concerned with the clinical studies in patients and describes the methods used for bile collection and the operative procedure used.

The third, and final, part of this section describes the analytical techniques used in determining the biliary lipid composition and bile acid kinetics.

1. THE CHRONIC BILE-FISTULA RHESUS MONKEY

(a) The Experimental Animal

In these experiments female Rhesus monkeys, weighing 4 - 5 Kg were used. After completion of quarantine the monkeys were acclimatised to the restraining chair (Plas-Labs Inc., Lansing, Michigan, U.S.A.) in which the studies would be performed. The animals became fully acclimatised after one or two weeks.

i) The Restraining Chair

The restraining chair comprised a tubular alloy frame mounted upon a strong alloy base carried by lockable castors (Fig. 3). Extremely well-finished plastic components formed the restraining chair and tables. The upper shelf was a feeding tray. Two openings in this allowed the monkey to feed itself. At the back of this shelf was an adjustable head rest. The lower shelf supported a moulded fully adjustable seat on which the monkey sat. This shelf did not always prevent the monkey reaching the T-tubes and a plastic chest piece was therefore developed to prevent any access to the tubes. A foot-rest was suspended from the under side of the lower shelf. A sawdust-filled tray for collection of urine and faeces completed the assembly. During the studies precautions were taken for the safe disposal of the sawdust which

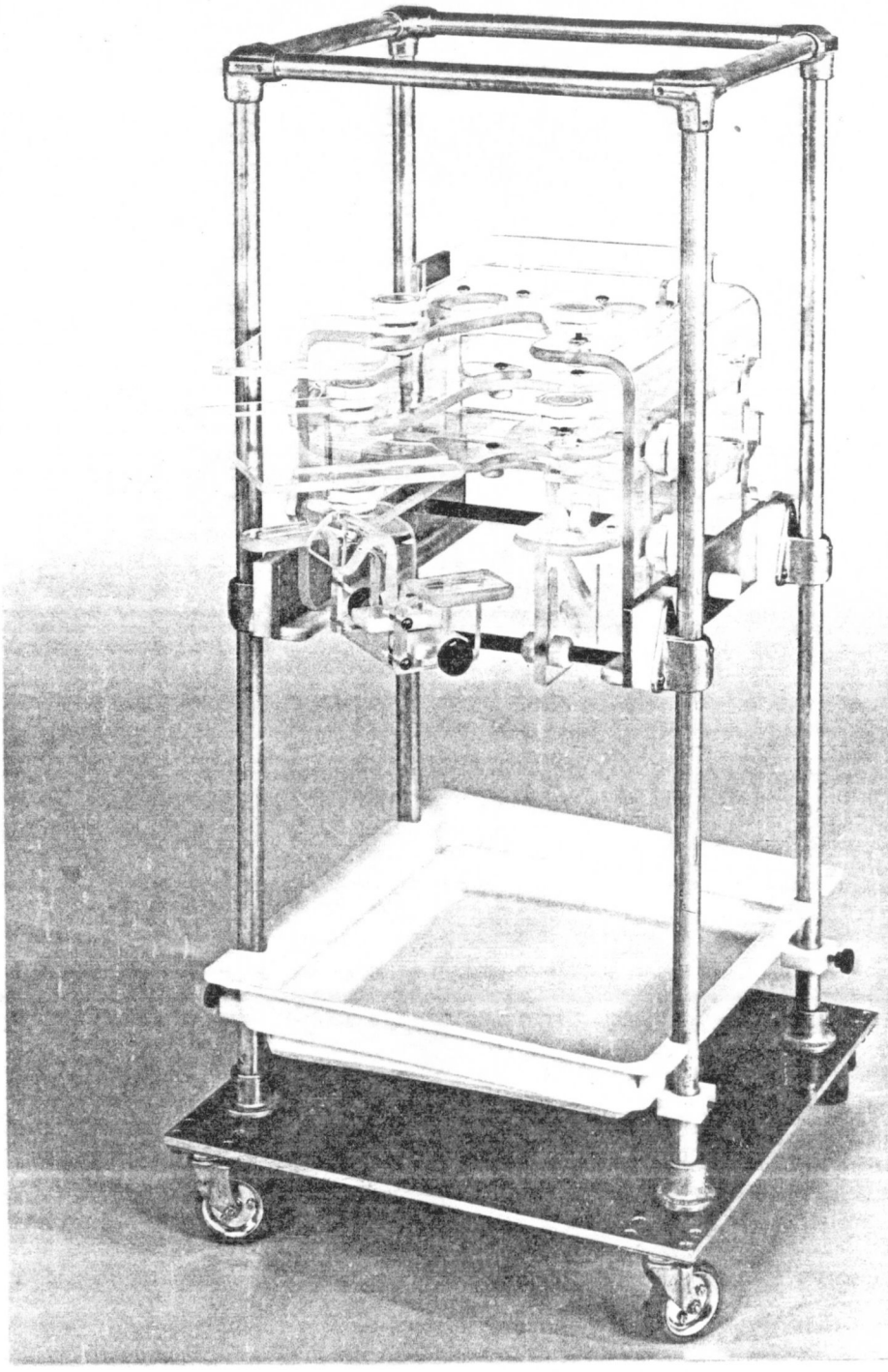


Fig. 3

The Restraining Chair

was contaminated by the radio-active faeces. The fully adjustable design of the restraining chairs made it possible to accommodate all variations in size and shape of the monkeys. None of the animals developed pressure sores.

(b) Animal Care

The monkeys were maintained on a diet of Purina monkey chow (Ralston, Inc., Chicago, U.S.A.) supplemented with fresh fruit and vegetables provided as two daily meals, the first at 9 a.m. and the second at 5 p.m. The monkeys were allowed water ad libitum from a feeding bottle suspended above the restraining chair. A normal diurnal rhythm was maintained with the laboratory illuminated for 12 hours and in darkness for twelve hours each day.

(c) Pre-Operative Preparation

The monkey was fasted from 9 p.m. on the night preceding operation. A premedication of ketamine (2 - 4 mg/Kg) was given 30 minutes prior to operation to enable the removal of the monkey from its restraining chair and transport to the operating theatre.

(d) Anaesthesia

Anaesthesia was induced with a 5% solution of sodium

thiopentone (0.5 ml/Kg body weight) given intravenously. A short skin crease incision was then made in the groin, usually the left, and the femoral artery and vein identified. The femoral vein was mobilised and then cannulated with sterile high grade silicone tubing, 2 mm. O.D. (Dow-Corning, Inc., Midland, Michigan, U.S.A.). The silicone tubing was approximately one metre in length and was connected to an infusion of sterile 0.9% saline outside the sterile field. Anaesthesia was maintained by intermittent additional intravenous boluses (0.5 - 1.0 ml) of the 5% solution of solium thiopentone, given by an assistant outside the sterile field.

(e) Operative Technique

The anaesthetised monkey was laid supine on the operating table and its position maintained by light ankle and wrist straps.

After preparation of the sterile field the abdomen was opened through an upper midline incision. The liver and gallbladder were carefully examined and confirmed to be normal. The common bile duct was then identified and its junction with the cystic duct exposed. The cystic duct was then isolated and doubly ligated with a non-absorbable suture. Bile was aspirated from the gallbladder until it was completely collapsed. The distal common bile duct was then carefully mobilised and divided between non-absorbable ligatures just

above the duodenum. A short vertical incision was then made in the common bile duct and a Silastic T-tube, 3 mm O.D., (Dow Corning, Inc., Midland, Michigan, U.S.A.) inserted. The choledochotomy was closed with interrupted absorbable sutures.

The second part of the duodenum was then exposed. A short stab incision was made at about its mid-point and a second Silastic T-tube, 3 mm O.D., inserted. Two purse string sutures were placed to close the incision. The omentum was then wrapped around both T-tubes which were then brought out through stab incisions in the anterior abdominal wall.

A stab incision was made in the most dependent part of the stomach and a 10 French gauge latex balloon catheter passed into the stomach as a gastrostomy tube to aid the early post-operative care. The stab incision was closed with two purse string sutures and the stomach was sutured to the abdominal wall through which the gastrostomy tube was passed.

Peritoneum and linea alba were then closed in a single layer with interrupted sutures and the skin was also closed with interrupted sutures.

(f) Post-Operative Care

The monkey was returned from the operating theatre and replaced in the restraining chair. A specially made chest piece was added to the chair assembly to prevent the monkey reaching and dislodging the T-tubes. The intravenous infusion was maintained and the gastrostomy and both T-tubes

allowed to drain freely. The monkey was started on water on the 2nd or 3rd post-operative day and usually was eating by the 5th day at which time the intravenous infusion was discontinued. The enterohepatic circulation was restored approximately one week post-operatively. The gastrostomy tube was removed ten days post-operatively.

To ensure complete recovery from the operative procedure four weeks elapsed before the studies were started.

2. STUDIES ON ENTEROHEPATIC CYCLING FREQUENCY

Three female Rhesus monkeys were prepared with biliary and duodenal fistulae as described above.

(a) Extracorporeal Bile Circuit for Controlled Cycling

Effluent bile from the T-tube in the common bile duct was collected in a glass beaker, which acted as a reservoir, attached to the restraining chair. Clear silicone tubing which reached to the bottom of the beaker was connected to a Holter pump (Extracorporeal Medical Specialties, Inc., King of Prussia, Pennsylvania, U.S.A.) which was in turn connected by clear silicone tubing to the duodenal T-tube. The electrical supply to the motor pump was interrupted by a time clock (Cole-Parmer, Chicago, Illinois, U.S.A.). The clock could be preset to activate the motor pump any number of times each day (Fig. 4). In practice the time clock was used at the higher cycling frequencies and the motor pump was activated by hand at the lower cycling frequencies.

EXPERIMENTAL MODEL FOR CONTROL OF FREQUENCY OF THE ENTEROHEPATIC CIRCULATION OF BILE

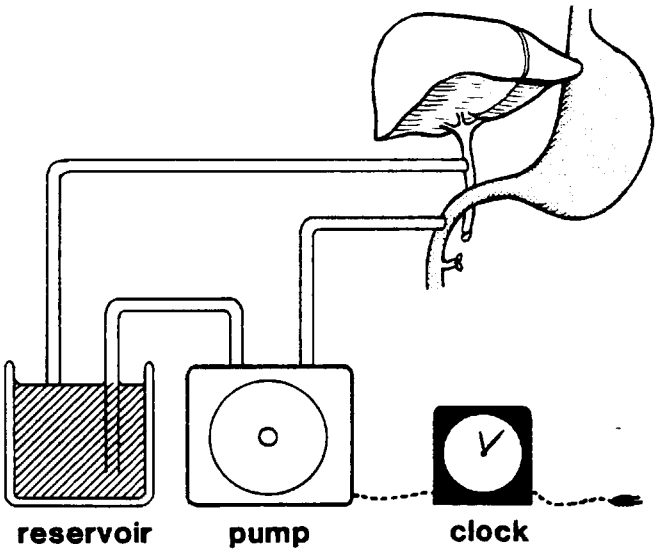


Fig. 4

i) The Holter Pump

The Holter pump is a compact and efficient self-priming motor pump. A wide range of flow rates can be achieved by the use of four different sizes of pump chamber and by a rheostat control which controlled the revolutions per minute. The flow rate was adjusted so that all the bile which had accumulated in the reservoir was returned within thirty minutes. The rate of flow was thus high at low cycling frequencies and low at high cycling frequencies, with the $t_{1/2}$ of the bile return period remaining constant.

ii) The Time Clock

The clock was electrically driven by 110 volt current. A series of levers around the face of the clock, when switched to their 'on' position, allowed the mains current to pass to another piece of apparatus, in this case the Holter pump. Each lever controlled a 10 minute time period and, therefore, the movement of three adjacent levers to the 'on' position allowed the Holter pump to run for 30 minutes and then switched it off.

iii) Tubing and Fittings

Clear silicone tubing (Dow Corning, Midland, Michigan, U.S.A.) was used throughout. A connecting tube with outside diameter (O.D.) of 4 mm and an internal diameter (I.D.) of 3 mm was sleeved over the end of the T-tube (3 mm O.D.). A short length of 5 mm O.D., 4 mm I.D. tubing coupled the connecting tube to the Holter pump avoiding the use of any plastic connectors which are prone to collect sediment.

(b) Preparation and Administration of Radio-Isotope

Radioactive 14-C Cholic acid obtained from Amersham Searle, Inc., (Arlington Heights, Illinois, U.S.A.) was purified by thin layer chromatography. Purity of the radioactive 14-C Cholic acid used exceeded 99%.

Ten microcuries (μc) of 14-C Cholic acid, together with 5 - 10 mg of carrier cholic acid as the sodium salt, was injected intravenously at 9 a.m. on the first day of a study to label the bile acid pool.

(c) Experimental Method

The monkey was stabilised at a particular cycling frequency for at least one week prior to a radio-isotope study.

In the first experiment bile was allowed to collect in the reservoir for twenty-four hours prior to return via the duodenal tube, thus giving a cycling frequency of one/day. Immediately prior to return of the bile at 9 a.m. each day bile samples were collected. The bile in the reservoir was agitated for 3 - 5 minutes, to ensure uniform suspension of any solid particles, and 1.5 ml of bile was taken for subsequent analysis. This volume represented 1 - 3% of the total daily bile output. Samples were collected in this manner each day for five days.

After an interval of two days a further tracer dose of 14-C

Cholic acid was given to the monkey and the study repeated, without any change in cycling frequency. It was anticipated that, particularly at the lower cycling frequencies, some residual radio-active cholic acid might remain in the bile from the previous study. An additional sample was therefore collected in the second and subsequent studies immediately prior to administration of the 14-C Cholic acid.

When two studies had been completed at a frequency of one cycle/day the enterohepatic cycling frequency was increased to two cycles/day. After an equilibration period of one week a further tracer dose of 14-C Cholic acid was given and the studies repeated. After further equilibration periods the studies were repeated at 4 and 8 cycles/day and with continuous recirculation of bile. For this last study the collection of bile was necessarily different.

(d) Extracorporeal Bile Circuit for Continuous Cycling

Effluent bile from the T-tube in the common bile duct passed through clear silicone tubing to a stream-splitting pump which returned 95% of the effluent bile to the monkey via clear silicone tubing and the duodenal T-tube. The remaining 5% was diverted to a reservoir (Fig. 5).

i) The Stream-Splitting Pump

The stream-splitting pump is a fraction collector designed to sample volumetrically small quantities of liquid, and

EXPERIMENTAL MODEL FOR CONTINUOUS ENTEROHEPATIC CIRCULATION OF BILE WITH COLLECTION OF A 5% FRACTION

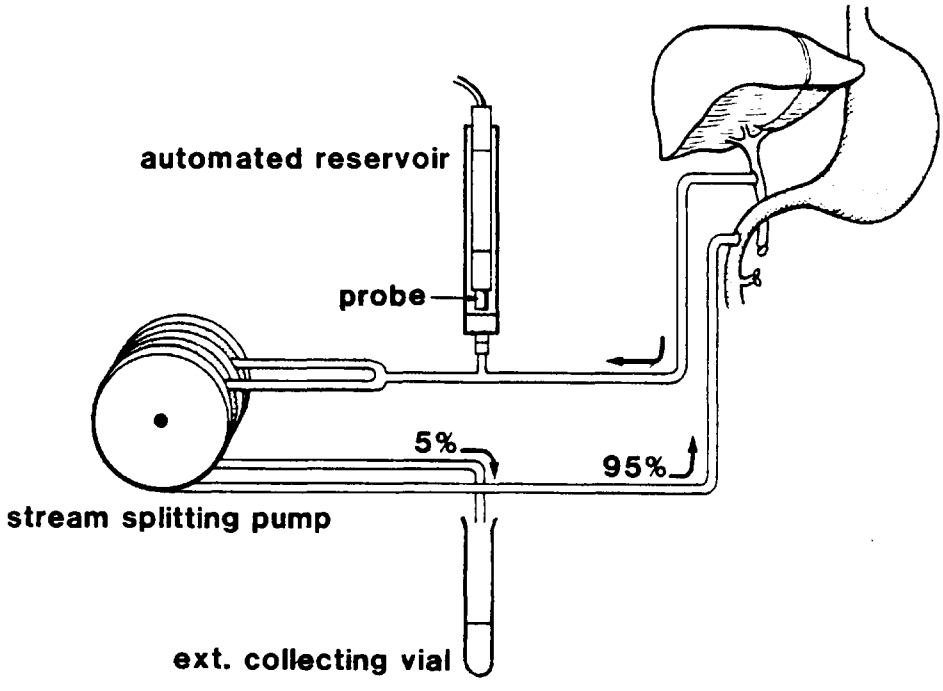


Fig. 5

was designed for studies on the enterohepatic circulation of bile (Berger, 1974).

The system comprises an ultrasonic probe, two concentric peristaltic pumps, and an electronic control unit, housed in a metal case (Fig. 6). Effluent bile from the monkey enters an open syringe from below. The ultrasonic probe lying in the syringe barrel acts as a sensor for a rise in the fluid level in the syringe, which via the electrical circuitry activates the pump. A time control determines the period for which the pump is activated, and thereby the volume required to trigger the pump. The range of volume required was 0.01 - 0.20 ml. Each time the bile level reached the probe the system was activated. A fraction control determined when the collection pump was activated in conjunction with the return pump. For all the experiments in these studies a 1/20 fraction was collected.

ii) Tubing and Fittings .

Clear silicone tubing (Dow Corning, Midland, Michigan, U.S.A.) was used throughout. To minimise the use of fittings to connect the various tubes together tube sizes were selected such that the outside diameter (O.D.) of one tube equalled the inside diameter of another. Thus the 3 mm O.D. T-tube was sleeved into a connecting tube with an internal diameter (I.D.) of 3 mm and an O.D. of 4 mm, which was the internal diameter of the tubing used to construct the pump chambers.

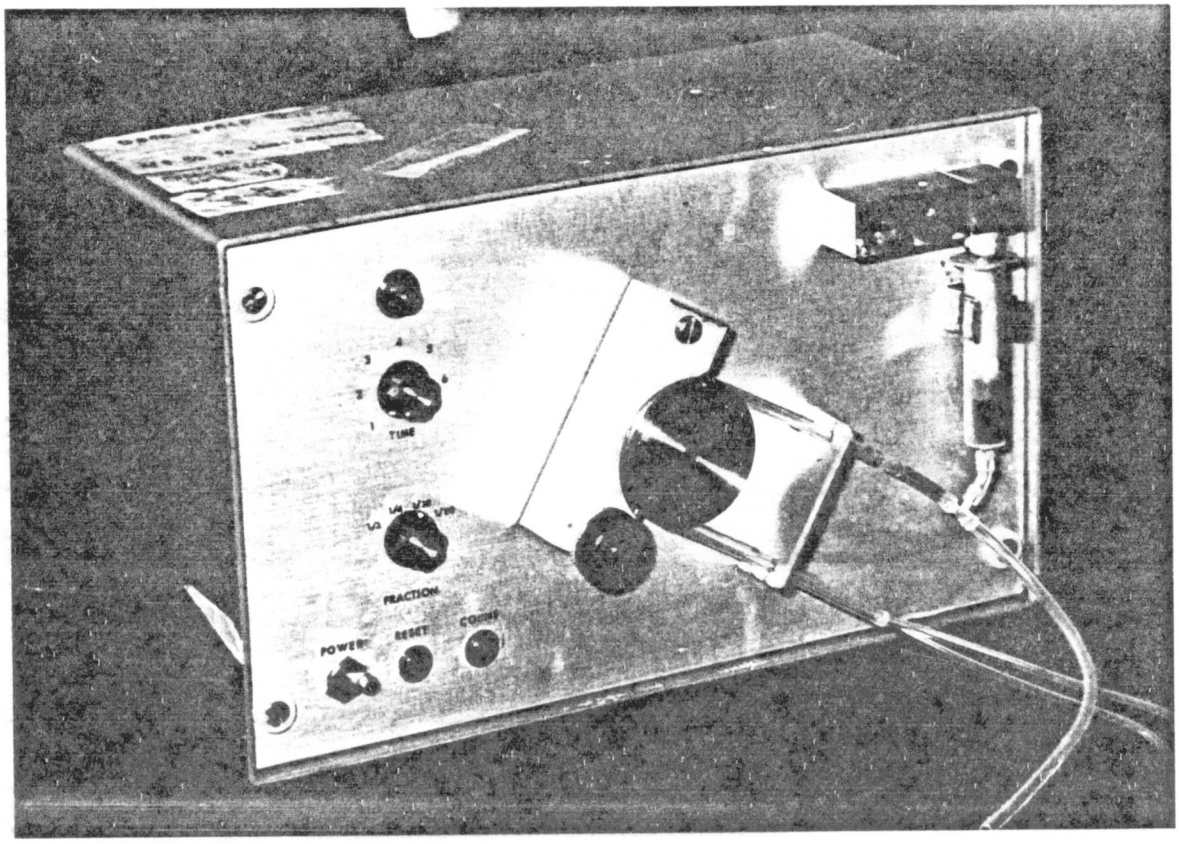


Fig. 6

The Stream-Splitting Pump

The pump chambers were made from 6 mm O.D., 4 mm I.D. silicone tubing. Collars of 6 mm I.D. tubing were glued to the tubes forming the pump chambers to prevent the chambers moving.

Two plastic connectors were used in the circuit. A 'T' connection coupled the syringe containing the ultrasonic probe to the system and a 'Y' connection divided the bile flow to the two peristaltic pumps. Unlike the silicone tubing, which remained clean throughout, the plastic fittings did collect a sediment and were therefore cleaned prior to each study.

(e) Preparation and Administration of Radio-Isotope

The techniques for purification and preparation of 14-C Cholic acid were identical to those used in the studies on enterohepatic cycling and are described in Section 2 b. As before 10 uc of 14-C Cholic acid, together with 5 - 10 mg of carrier cholic acid, was injected intravenously at 9 a.m. on the first day of a study to label the bile acid pool.

(f) Experimental Method

The monkeys were stabilised on continuous enterohepatic cycling for at least one week prior to a radio-isotope study.

After administration of the 14-C Cholic acid bile was allowed to accumulate in the collecting tube for 24 hours. The sample was thus a mean of the entire collection period.

At 9 a.m. each day the collecting tube was changed and the bile saved for subsequent analysis. Samples were collected in this manner each day for five days.

After an interval of at least one week, at which time no residual radio-active cholic acid remained, the study was repeated.

3. CLINICAL STUDIES ON THE EFFECT OF VAGOTOMY IN MAN

(a) Experimental Design

As in the animal studies the bile acid pool was labelled with 14-C Cholic acid. On the following 4 - 5 days the bile acid pool was sampled via a double-lumen duodenal tube, after stimulation of gallbladder contraction with cholecystokinin. A small sample of bile was retained for analysis, the remainder being returned to the duodenum. Truncal vagotomy was then performed and after an interval of three months the bile acid pool was again labelled with 14-C Cholic acid and serial bile samples collected.

(b) Ethical Approval

A detailed protocol of the experimental procedure was submitted to the local Ethical Committee and approved. As a radio-isotope was to be used in the study a protocol was submitted to the Isotope Advisory Panel at the Department of Health and Social Security, whose approval was also obtained.

(c) Selection of Patients

The patients included in the study were drawn from those attending the surgical out-patient department of the Norfolk and Norwich Hospital. All the patients had chronic duodenal ulcer. The diagnosis was confirmed in all cases by recent barium meal or endoscopy. The decision to submit the patients to operative treatment was based on the chronicity or severity of symptoms, or both, and was made by another surgeon unconnected with the study.

i) Informed Consent

The patients were initially approached by letter and invited to participate in the study sometime after being entered on the waiting list for admission. All patients were then personally interviewed by me and the nature of the experimental procedures described to them. Informed consent was received in all cases.

ii) Screening to Exclude Co-Existent Gallstone Disease

All patients were submitted to double-dose oral cholecystography. The studies were all performed when a radiologist was available so that the films could be reviewed immediately and tomography performed if necessary. Two patients were excluded at this stage when symptomless gallstones were demonstrated. All patients included in the study had cholecystograms free of abnormalities on supine or prone views with normal gallbladder contraction

after Lipiodol. Serum estimations of bilirubin, albumin, globulin, serum glutamine, oxaloacetic transaminase and serum pyruvate transaminase were performed to exclude any gross liver disease.

(d) Preparation and Administration of Radio-Isotope

Radioactive 14-C Cholic acid obtained from Amersham Searle Inc., (Arlington Heights, Illinois, U.S.A.) was purified by thin layer chromatography. Purity of the radioactive 14-C Cholic acid used exceeded 99%.

Twenty microcuries of 14-C Cholic acid, together with 5 - 10 mg of carrier cholic acid as the sodium salt, was injected intravenously at 9 a.m. on the first day of study. The patient, who had been starved overnight was then given breakfast to stimulate gallbladder contraction and thorough mixing of the radioactive bile acid with the remainder of the bile acid pool.

(e) Experimental Method

During the five day study period the patient would eat and drink quite normally. The patient would be fasted from midnight each night until completion of the sample collection between 9.00 and 9.30 a.m. at which time they would receive breakfast.

The mouth and pharynx were anaesthetised with an amethocaine

lozenge and a Dreiling double-lumen tube (Fig. 7) was then passed with the patient sitting up. When the tip of the tube reached the stomach the patient was placed on his right side and the tube advanced 2 - 3 centimetres every few minutes. This usually ensured smooth passage of the tip of the tube through the pylorus. A fluoroscopic examination was then performed to confirm that the distal part of the tube lay in the duodenal loop (Fig. 8). Occasionally it was necessary to give metaclopramide (10 mg i.v.) or manipulate the tube under fluoroscopic control to pass the tip through the pylorus. Gallbladder contraction was then stimulated by an intravenous injection of Pancreozymin-Cholecystokinin. After a few minutes there was a brisk flow of concentrated bile which was collected by siphonage. The gastric tube was also dependent and draining by siphonage. It was also aspirated at intervals to minimise any contamination of the duodenal contents by gastric juice. After 5 - 10 minutes approximately 30 cc of bile had accumulated. A 1.5 ml sample of bile was taken for subsequent analysis and the remainder of the bile was returned via the duodenal tube and washed through with water. The Dreiling tube was then removed and the patient given breakfast. Samples were collected in a similar manner on four subsequent days. The patient was then prepared for operation on the next day.

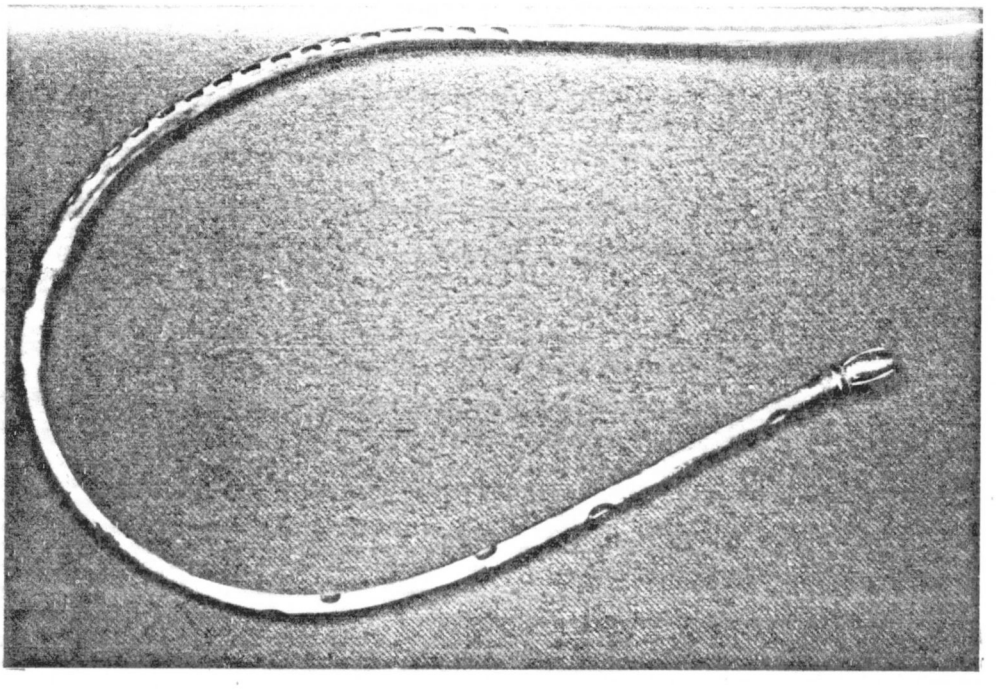


Fig. 7

The Dreiling Tube

A weighted tip aids the passage of the tube through the pylorus. The distal portion of the tube drains the duodenum and a second lumen, with multiple fenestrations, drains the stomach.

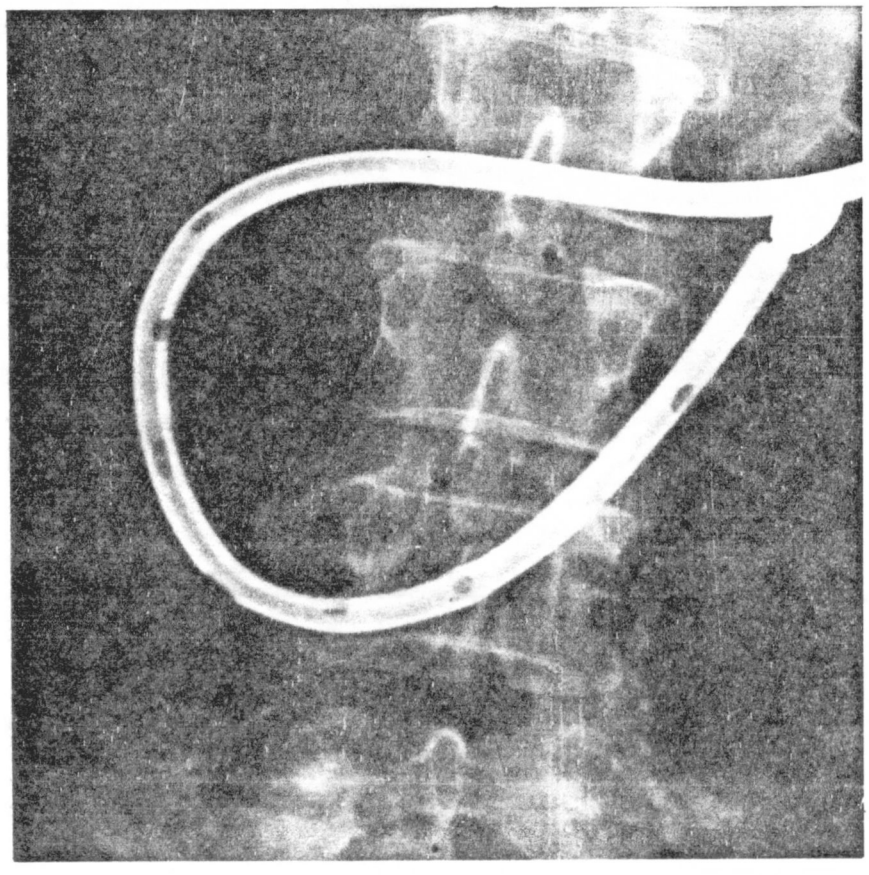


Fig. 8

The Dreiling Tube

A radiograph shows the tube positioned in the duodenum with its tip close to the ligament of Treitz.

(f) Anaesthesia

A premedication of Pethidine (100 mg) and Atropine (0.6 mg) was given one hour pre-operatively. Anaesthesia was induced with Sodium Thiopentone, titrated to its effect, and maintained with Nitrous Oxide, Halothane, and Oxygen.

(g) Operative Technique

The patient was placed supine on the operating table and the abdomen was opened through an upper midline incision. A careful laparotomy was performed with particular attention being paid to the liver and gallbladder.

The left triangular ligament of the liver was then divided and the left lobe of the liver retracted to the right. The body of the stomach was retracted downwards to expose the intra-abdominal oesophagus. The peritoneum was divided on its anterior surface and the oesophagus was dissected from its surroundings and encircled at first with a finger and then with a soft rubber tube. The anterior and posterior vagal trunks were then divided and segments were removed for confirmatory histology. A careful search was then made for any additional fibres and any found were divided.

The pylorus was then mobilised and divided longitudinally between stay sutures. The incision extended approximately

2 - 3 cm either side of the pyloric ring. The ends of the incision were then approximated and the incision closed transversely with a single layer of interrupted inverting sutures. A nasogastric tube was then placed in the body of the stomach and the abdomen closed in layers.

(h) Post-Operative Care

The patient was started on oral fluids on the second post-operative day and the nasogastric tube was usually removed on the third or fourth day. The patient was then started on a light diet and would be eating normally when discharged to home or convalescence 7 - 8 days post-operatively.

(i) Post-Operative Studies

After an interval of three months from the vagotomy bile acid kinetic studies were repeated. The patients were either readmitted or attended the hospital daily. As before the bile acid pool was labelled with 20 μc of 14-C Cholic acid at 9.00 a.m. on the first day of study and fasting gallbladder bile was sampled on the four subsequent days using the techniques already described in Sections (d) and (e).

5. LABORATORY AND ANALYTICAL METHODS

This section describes the methods used for quantitative analysis of bile acids, cholesterol and phospholipids and the methods used for determination of the specific activity of bile acids. The methods are briefly described below with full details appearing in Appendix 1.

(a) Handling and Storage of Bile Samples

In all the studies bile samples were divided before storage. 0.5 ml bile was added to 9.5 ml of a chloroform:methanol 2:1 solution which was then partitioned with 0.1 ml of water (Folch et al., 1951). Both this and the samples of whole bile were then stored frozen (-20°C) until analysed.

(b) Bile Acid Quantitative Analysis

Bile acids were analysed using Admirand's modification of Talalay's method (Talalay, 1960, Admirand and Small, 1968). A sample of bile was diluted with methanol and added to a reaction mixture containing hydrazine hydrate solution, beta nicatinamide adenine dinucleotide, glycine buffer and distilled water. The sample was then placed in the spectrophotometer and read against the blank to correct for background. A buffered solution of hydroxysteroid dehydrogenase was then added to the sample. After two

hours, when the enzymatic reaction was complete, the sample was again examined in the spectrophotometer at a wavelength of 340 mμ. The change in optical density from the previous reading was then determined. Comparison with a series of standards allowed the bile acid concentration to be determined.

(c) Cholesterol Quantitative Analysis

Cholesterol was analysed by the method described by Schoenheimer and Sperry (1934). A sample of bile was added to isopropyl alcohol, vortexed and then centrifuged for 10 minutes. The supernatant was removed and to this was added a 1% digitonin solution, which precipitated the cholesterol as its digitonide, and an equal volume of distilled water. The mixture was allowed to stand overnight at -4°C. The mixture was then centrifuged and the supernatant discarded. Glacial acetic acid, to dissolve the precipitated cholesterol digitonide, and the Lieberman-Burchard reagent (a mixture of glacial acetic acid and acetic anhydride) for the development of the colour reaction, were added to the precipitate of cholesterol and vortexed. After incubation for 20 minutes at 37°C the sample was placed in a spectrophotometer at a wavelength of 625 mμ and the optical density determined. Comparison with a series of standards allowed the concentration of cholesterol to be calculated.

(d) Phospholipid Quantitative Analysis

Phospholipid was analysed by the method of Fiske and Subbarow (1925). Bile was accurately diluted with distilled water and a small fraction of this dilute bile was placed in a Folin tube and incubated to dryness. 10 N sulphuric acid was added to oxidise the precipitate which was incubated at 160°C. Hydrogen peroxide was subsequently added and incubation continued until the sample was colourless. Ammonium molybdate and the Fiske-Subbarow reducing reagent (sodium bisulphite and amino-naphthol-sulphonic acid) were added to the sample and vortexed. Incubation in a boiling waterbath developed a blue colour. The sample was then placed in a spectrophotometer at a wavelength of 830 mμ and the optical density determined. Comparison with a series of standards allowed the concentration of phosphorus and thereby the concentration of phospholipid to be calculated.

(e) Determination of Bile Acid Specific Activity

Bile acid specific activity, expressed as disintegrations per minute per unit mass, is a measure of the dilution of the tracer dose of 14-C Cholic acid by non-radioactive bile acids. Almost all of the molecules of 14-C Cholic acid will have been conjugated with taurine or glycine during their enterohepatic circulation. These molecules must be deconjugated so that all the cholic acid, labelled and

unlabelled, is in its free state before the determination of the mass and the radioactivity of the sample.

i) Deconjugation and Extraction of Bile Acids.

A bile sample was added to sodium hydroxide in a screw-topped tube and autoclaved for three hours at two atmospheres to deconjugate taurine and glycine from cholic acid. Cholesterol was extracted with three ether washings which were then backwashed with alkaline water to return any small quantities of bile acid carried over by the washings to the sample. After acidification with hydrochloric acid the bile acids were extracted with ether and evaporated to dryness.

ii) Separation of Cholic Acid from Other Bile Acids

The dry sample of bile acid was taken up in methanol, pipetted onto an activated silica thin layer chromatographic plate, and separated with a moving phase with a mixture of iso-octane, ethyl acetate and glacial acetic acid. The cholic acid spot was identified with iodine vapour, scraped off the chromatographic plate and placed in a centrifuge tube. Aliquots of methanol were added to the tubes, centrifuged and the supernatants then pooled, and evaporated to dryness.

iii) Estimation of Specific Activity

The dry cholic acid was taken up in a measured volume of methanol and divided into two identical samples. One of

these samples was submitted to the enzymatic method of bile acid quantitative analysis described in Section 5 (b) above to determine cholic acid mass. The second sample was placed in a dry counting vial and allowed to evaporate to dryness. Aquasol liquid scintillation cocktail was then added to the vial and the counts per minute determined in a liquid scintillation spectrometer. Internal standards corrected for quenching and counting efficiency to convert counts per minute to disintegrations per minute, enabling the specific activity (disintegrations per minute per unit mass) to be calculated.

CRITICAL EVALUATION OF METHODS

This chapter is concerned with the validation of the experimental and laboratory methods used. The first part describes the methods used to assess the experimental animal model. The second part discusses the validation of the techniques used in the studies on human subjects and the final part evaluates the analytical techniques used.

1. ASSESSMENT OF THE EXPERIMENTAL ANIMAL MODEL

(a) Choice of Animal

The Rhesus monkey was chosen as the most suitable animal for this study. It is not possible to control enterohepatic cycling frequency in man, and the Rhesus monkey has similar, though slightly less saturated, bile to man. It is close to man on the evolutional scale and this may make extrapolation of findings to man more reliable.
The rat is unsuited for chronic studies with a biliary fistula and its bile is highly unsaturated with cholesterol. Although chronic bile fistulae can be constructed in dogs they are only suited to studies with continuous enterohepatic circulation. Furthermore their bile is more unsaturated with cholesterol than that of the Rhesus monkey. The baboon has the same advantages as the Rhesus monkey but its propensity for gallstone formation might confuse the results in a study of this nature. Furthermore, they are considerably larger than the Rhesus monkey and can be difficult to manage in restraining chairs. For these reasons the Rhesus monkey was chosen. Females were used throughout the study, obviating the influence of sex differences on the results. Females are also more suited to the restraining chair. In addition there was a considerable amount of data available from the studies of Dowling and co-workers (Dowling et al., 1968, Dowling et al., 1970).

(b) The Chronic Bile Fistula Monkey

In their studies on the Rhesus monkey Dowling and co-workers have described normal values for bile flow, bile acid secretion, and pool size as estimated by the "wash-out" technique (Dowling et al., 1968, Dowling et al., 1970). Each of these parameters were examined.

i) Bile Flow

Daily bile flow was measured in two monkeys during continuous enterohepatic circulation. The volume of the 5% fraction, diverted by the stream-splitting pump, was measured in graduated conical test tubes and the daily flow calculated. Mean daily bile flow in 34 animal days was 209 mls ($\pm$18 mls) which correlated well with the figure of 195 mls ($\pm$13 mls) reported by Dowling. Figures in brackets are 1 Standard Error of the Mean.

ii) Bile Acid Secretion Rate

Daily bile acid secretion rate was measured in two monkeys during continuous enterohepatic circulation. Bile acid concentration was determined on the 5% fraction samples collected over 24 hours by the stream-splitting pump. The samples were thus a mean of the bile secreted during the day and night. Secretion rates were calculated from the bile acid concentration and the daily bile volume. Mean daily bile acid secretion rate on 30 animal days was 9.13 mM/24 hours ($\pm$1.42) which correlated well with the

figure of 9.96 mM/24 hours ($\pm$0.89) reported by Dowling. Figures in brackets are 1 Standard Error of the Mean.

iii) Bile Acid Pool Size

Total bile acid pool size was measured by the "wash-out" technique described by Eriksson (Eriksson, 1957). The previously intact enterohepatic circulation is acutely interrupted. Bile acid secretion is initially unchanged as bile acids returning from the intestine maintain the pre-interruption secretion rate. After 1 - 3 hours, bile acid secretion rate falls precipitously to a plateau representing basal synthesis. The mass of bile acids secreted during this time, represented by the area under the secretion curve (Fig. 9), is the bile acid pool.

Pool sizes, determined by this method in two monkeys were 1.37 mM and 1.39 mM (mean 1.33 mM) which correlated well with the range of 0.88 - 1.60 mM (mean 1.27 mM) reported by Dowling and co-workers (Dowling et al., 1971).

(c) Stream-Splitting Pump

The accuracy of daily bile flow and bile acid secretion from samples collected from the stream splitting pump are obviously dependent on the size of the fraction collected. When set to collect a 5% fraction the ultrasonic impulse generated by bile flow would activate the return pump alone on 18 occasions. On the 19th impulse both the

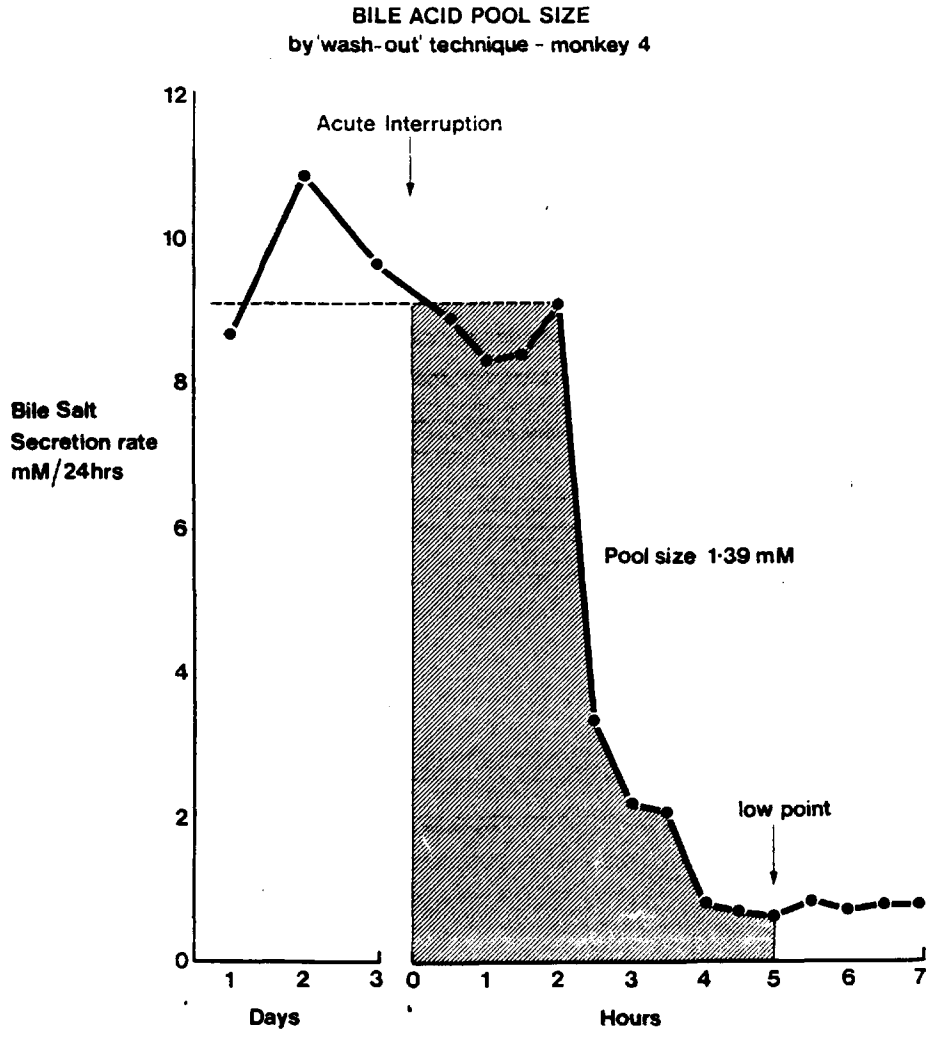


Fig. 9

Bile Acid Pool Size by the 'Washout' Technique

Samples are collected for a few days prior to study to establish the average daily secretion rate represented by the broken line. After complete interruption of the enterohepatic circulation bile acid secretion is initially unaltered as bile acids returning from the intestine maintain the output. There is then an abrupt fall as the bile acid pool is washed out and a low point is reached after 5 hours. The bile acid secretion at this point represents basal synthesis and the area under the curve the bile acid pool size.

return and sample collection pumps are activated. A 1/20 or 5% fraction was thus collected. Although always approximately 5% the fraction collected varied from 1/17 (5.8%) to 1/22 (4.6%). Three factors accounted for these differences. Firstly, there were slight differences between individual stream-splitting pumps. More important were variations in the size and compliance of the tubular silicone pump chambers. It was necessary to calibrate each pair of pump chambers with the stream-splitting pumps. With time the pump chambers tended to stretch and require re-calibration. This alteration only occurred over a period of some months and did not alter during periods of study lasting 5 - 7 days. Sample volumes were corrected to 5% at the time of collection.

(b) Bile Acid Kinetics

i) Validity of Isotope Dilution Technique in the Rhesus Monkey

The use of radio-isotope dilution techniques, first described by Lindstedt, have been well validated in man (Lindstedt, 1957, Hofmann et al., 1970, Hofmann and Hoffman, 1974). Pool size has been measured in monkeys, using isotope dilution techniques, and an exponential decay curve was confirmed (Mok and Dowling, 1975). A preliminary study to confirm the validity of the technique was performed in one monkey. After injection of 10 microcuries of 14-C Cholic acid radio-activity of whole bile was determined in

serial samples. A semi-logarithmic plot of crude radioactivity (as disintegrations per minute) against time (in hours) confirmed that decay of the isotope was exponential (Fig. 10). It was realised that this simple technique assumed constancy of a total bile acid pool size and of the individual bile acid pools. However, with the good fit of the curve by linear regression ($r = 0.98$), it was sufficient to confirm the previously validated technique.

ii) Isotope Administration

The tracer dose of 14-C Cholic acid given to label the bile acid pool can be administered via the T-tube or intravenously. When the isotope label is given via the T-tube a small portion will be lost in the faeces and this may occur before mixing of isotope label with the bile acid pool. If such a loss occurred a reduced quantity of isotope would mix with the pool. Subsequent regression analysis would overestimate pool size. To avoid this source of error all isotope labels were administered intravenously. O'Maille and co-workers have shown that intravenously administered bile acids are rapidly cleared from the systemic circulation (O'Maille et al., 1965). Isotopes were always administered just before a meal so that mixing would be facilitated by the associated period of increased enterohepatic circulation. Allowance was made for the presence of any residual activity from previous studies by collecting an additional bile sample just prior to

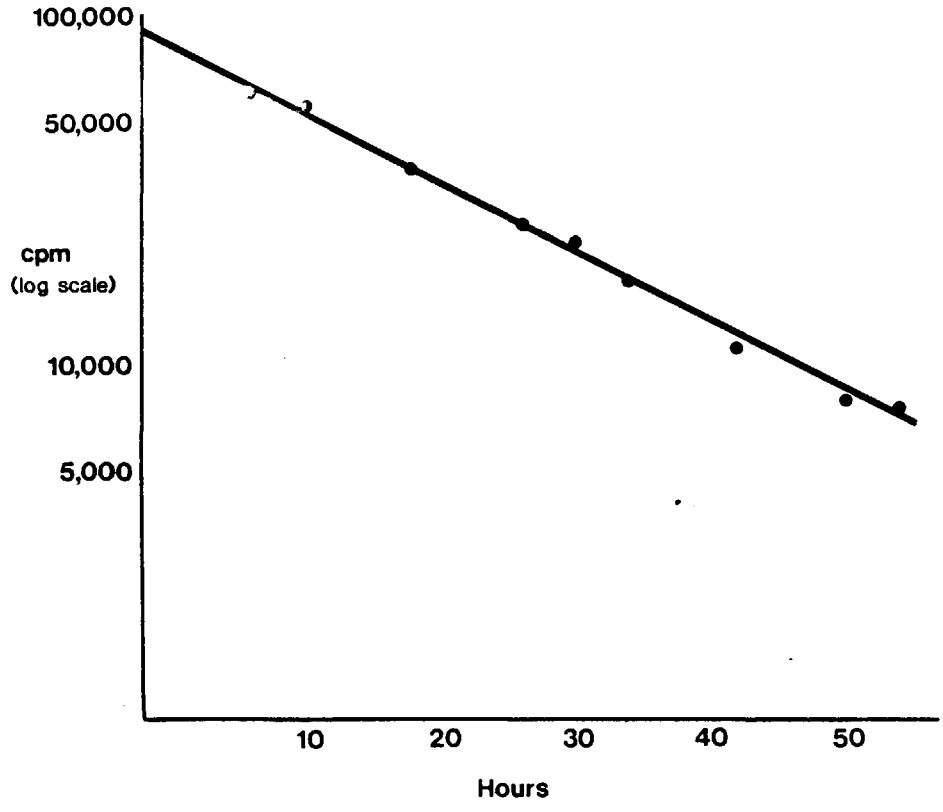


Fig. 10

14-C Cholic Acid Decay Curve

Semi-logarithmic plot of 14-C Cholic acid radioactivity in whole bile showing an exponential decay (r = 0.98)

administration of the isotope label. Values obtained from analysis of these samples were used to correct the specific activity at time zero (SA_{to}), derived from the isotope decay curve, before bile acid pool size was calculated.

(e) Timing of Studies

The effects of surgery on bile secretion and composition has been previously investigated (Herman et al., 1971). These studies showed that bile acid synthesis, bile flow, and biliary lipid secretion were depressed post-operatively but returned to normal within 2 - 4 weeks. All studies were therefore started at least one month post-operatively.

(f) Sample Collection

To ensure representative specimens bile was mixed thoroughly before sample collection. In the studies on altered enterohepatic cycling bile in the reservoir was stirred for 3 - 5 minutes before sampling. In studies during continuous enterohepatic cycling the 5% fraction of bile, collected in a conical tube, was agitated by vortex mixer for 1 - 2 minutes before sampling.

2. BILE ACID KINETIC STUDIES IN MAN

(a) Patient Selection

All the patients investigated were attending for elective surgery for chronic duodenal ulcer. In view of the many factors influencing biliary lipid metabolism, such as age, obesity, diet, drugs, etc., discussed in the literature review above considerable care was taken in choosing patients for this study.

i) Oral Cholecystogram

All prospective subjects were examined with a double dose oral cholecystogram, with tomography as necessary. Two subjects were excluded as a result of positive cholecystographic findings. Neither patient had experienced symptoms suggestive of gallstone disease.

ii) Age, Sex and Race

All patients included in the study were Caucasian males, eliminating any differences due to sex or race. Changes in biliary lipid composition have been noted with increasing age (Valdiviseo et al., 1978). The age range of the patients studied was narrow, 42 - 48 years, and minimised any age effect.

iii) Obesity

None of the patients were obese. All were within 10% of

the normal weight for their age based on height-weight tables.

iv) Drugs

None of the patients were taking any regular medication. Two were taking occasional antacids but these were discontinued for one week prior to, and during, the study.

v) Gallbladder Examination

The gallbladder and biliary tree were carefully examined during the laparotomy for vagotomy and pyloroplasty. One further patient, in whom small stones not shown on cholecystography were palpated, was excluded from the study. Gallbladder and bile ducts were quite normal in the remainder.

The group of patients selected were thus well matched with regard to age, sex, race, obesity and drug intake. Gallbladder disease was excluded by cholecystography and operative examination.

(b) Bile Acid Kinetics

i) Validity of Isotope Dilution Technique in Man

The isotope dilution technique for measurement of bile acid pool size, first described by Lindstedt, has been used extensively for bile acid kinetic studies in man

(Lindstedt, 1957). The validity of the technique has been confirmed by others (Vlahcevic, 1970, Hofmann et al., 1970, Vlahcevic, Miller et al., 1971, Hofmann and Hoffman, 1974.

ii) Isotope Administration

The same arguments, already discussed with regard to isotope administration in the Rhesus monkey, apply. Oral administration may be associated with some losses of the isotope label in the faeces before mixing with the bile acid pool was complete. Intravenous administration, with carrier bile acid, avoids these potential losses and was used in all the studies. The isotope label was given just prior to a meal to facilitate mixing with the bile acid pool.

(c) Duodenal Bile Sampling

The technique of duodenal intubation and collection of bile for bile acid kinetic studies is easily performed. The alternative technique is that of duodenal perfusion with liquid formula diet and non-absorbable marker (Grundy and Metzger, 1972). This technique has the advantage that direct measurements of biliary lipid secretion can be made. It does, however, have certain disadvantages. First, the continuous infusion of formula diet causes sustained cholecystokinin release and gallbladder contraction, almost certainly excluding the gallbladder from the enterohepatic circulation for the remainder of the study. This is desirable in studies before and after cholecystectomy since

it should ensure similar samples are collected each time. However, by causing continuous gallbladder contraction this technique might well affect enterohepatic cycling frequency which was the subject of this study. Secondly, the technique is more complicated and considerably more time consuming, each study lasting 8 - 10 hours. Since cholecystectomy would not be performed during these studies it would be possible to obtain similar (gallbladder) bile samples before and after operation without resort to this more sophisticated technique.

Previous studies have confirmed the validity of using bile-rich duodenal samples as an indicator of gallbladder bile composition. Vlahcevic and co-workers collected duodenal bile samples in patients with and without gallstones (Vlahcevic, Bell et al., 1971). Gallbladder biles were subsequently collected at laparotomy and no differences in composition were detected.

Gallbladder bile was collected following stimulation of gallbladder contraction by intravenous cholecystokinin. This was a uniformly effective stimulus and dark golden bile was obtained in 5 - 10 minutes. Studies in the cholecystectomised Rhesus monkey have shown that cholecystokinin has a choleretic effect (Gardiner and Small, 1976). Whether such an effect occurs in normal man is unknown. Cholecystokinin-stimulated gallbladder bile was collected both pre- and post-operatively. It was assumed that, in

the presence of a normal gallbladder, the major effect of cholecystokinin would be to cause gallbladder contraction. Any choleretic effect on hepatic bile secretion would probably be insufficient to materially change the composition of a large volume of gallbladder bile. Any such effect would affect both pre-and post-operative bile samples equally and, therefore, would not introduce bias into the results.

(d) Vagotomy

The truncal vagotomy was performed using current surgical techniques. In all patients histological studies confirmed that both vagal trunks had been divided. It was realised that this does not, in itself, mean that vagotomy was complete. A careful search was made for any small fibres on the oesophagus and these were divided. If vagotomy does influence enterohepatic cycling frequency, the nerve fibres concerned would be those in the hepatic branch. It is unlikely, with confirmed division of the vagal trunks, that nerve fibres entering these branches would be intact. The branches that may be missed supply the upper part of the stomach and it is unlikely that these would affect enterohepatic cycling. All patients had good symptomatic improvement with regard to their duodenal ulcer disease. Tests of acid production

were not performed as their reliability has been questioned. It was thought that the histological confirmation, together with the symptomatic improvement seen, was sufficient evidence of complete vagotomy for the purposes of this study.

(e) Timing of Post-Operative Studies

Studies in the monkey have indicated that biliary lipid secretion and composition return to normal within 2 - 4 weeks of operation (Hermann et al., 1971). Subsequent studies on patients with indwelling T-tubes after cholecystectomy showed that bile was supersaturated with cholesterol while on free drainage for 4 days. However, within one week of clamping the T-tube biliary lipid secretion and composition were normal (Shaffer and Small, 1972). These studies indicate that the interval of three months before post-operative studies were performed was quite sufficient to prevent the surgery itself from affecting the results.

(f) Sample Collection

A large volume (approximately 30 ml) of bile was drained from the duodenum to ensure a uniform sample and, after thorough stirring, a small aliquot (1.5 ml) was saved for analysis. Bile was siphoned into a container surrounded

by ice to minimise the effect of pancreatic enzyme or bacterial activity. After return of bile to the patient via the duodenal tube the container was rinsed with three volumes of water, which were also returned to the patient via the duodenal tube, to minimise any loss of the isotope-labelled bile acid pool.

3. LABORATORY METHODS

(a) Bile Acids

The use of 3α-hydroxysteroid dehydrogenase for enzymic analysis was first described by Talalay (Talalay, 1960). The enzyme catalyses oxidation of 3α-hydroxyl groups of C_{19}, C_2 and C_{24} steroids to a ketone. In the presence of hydrazine hydrate the ketone is converted to a hydrazone. Thus one atom of hydrogen is released from every steroid molecule in the reaction mixture. Each hydrogen atom reduces one molecule of beta nicatinamide adenine di-nucleotide (NAD) and the quantity of reduced NAD can be measured spectrophotometrically.

Use of the method for quantitative determinations of bile acids has subsequently been reported (Iwata and Yamasaki, 1964) and minor modifications have subsequently been suggested (Admirand and Small, 1968, Engert and Turner, 1973). In this study the modification of Admirand and Small was used.

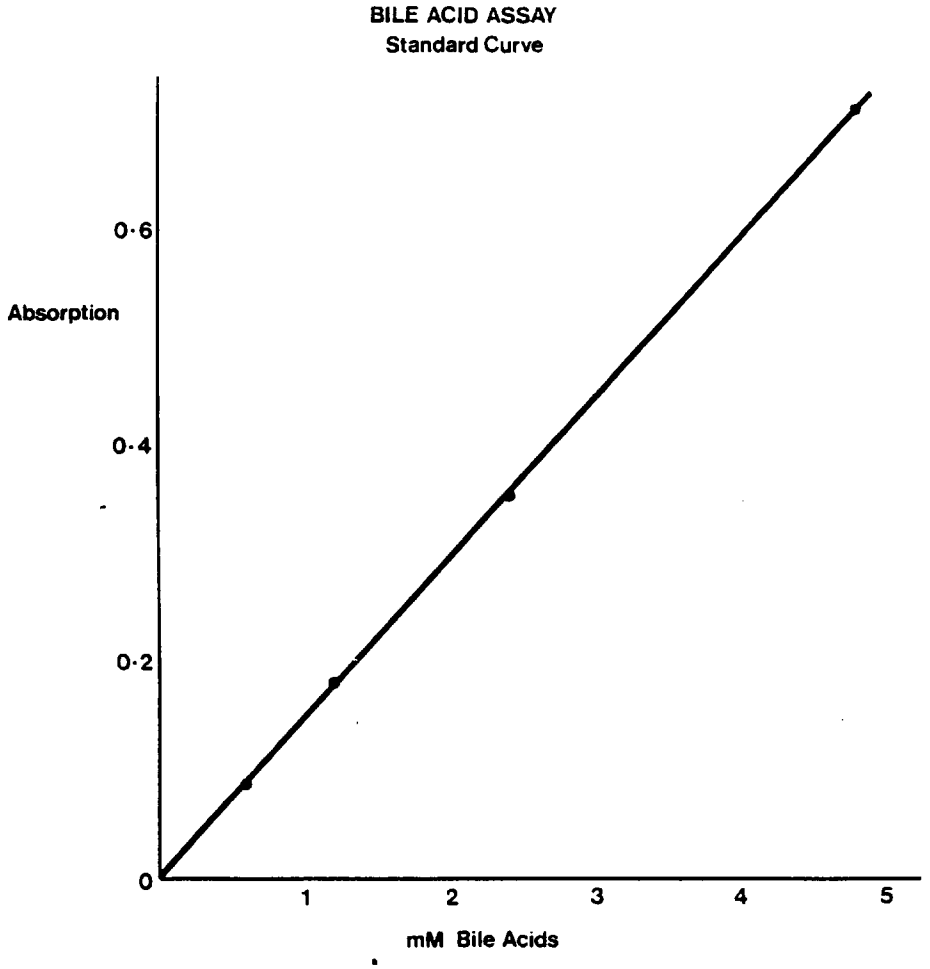


Fig. 11

Bile Acid Assay, Standard Curve

The hydroxysteroid dehydrogenase solution was made with 0.03 M tris buffer (pH 7.2) instead of distilled water. A standard curve was obtained with pure bile salts for each series of determinations. A typical standard curve is shown in Fig. 11. Accuracy of the method was $\pm$3%. All assays were performed in duplicate.

(b) Cholesterol

Several different methods are available for the determination of cholesterol. Most widely used are techniques involving estimation of cholesterol as its digitonide (Schoenheimer and Sperry, 1934, Sperry and Webb, 1950, Babson et al., 1962, Leffler and McDonald, 1963), estimation of cholesterol esters in petroleum ether (Abell et al., 1952), or of free cholesterol (Carr and Drekter, 1956). Previous studies have shown no significant differences between estimations of cholesterol as its digitonide or as esters (Admirand and Small, 1968). The Schoenheimer and Sperry method has been criticised for loss of cholesterol during analysis. A preliminary study was performed to determine the losses, if any, during the process. In the range of concentrations found in bile recovery was 98% or greater. Some losses did occur at much higher concentrations but recovery was always greater than 90%. This method was used in these studies and consistent results were obtained. A typical standard curve is shown in Fig. 12. All assays were performed in duplicate.

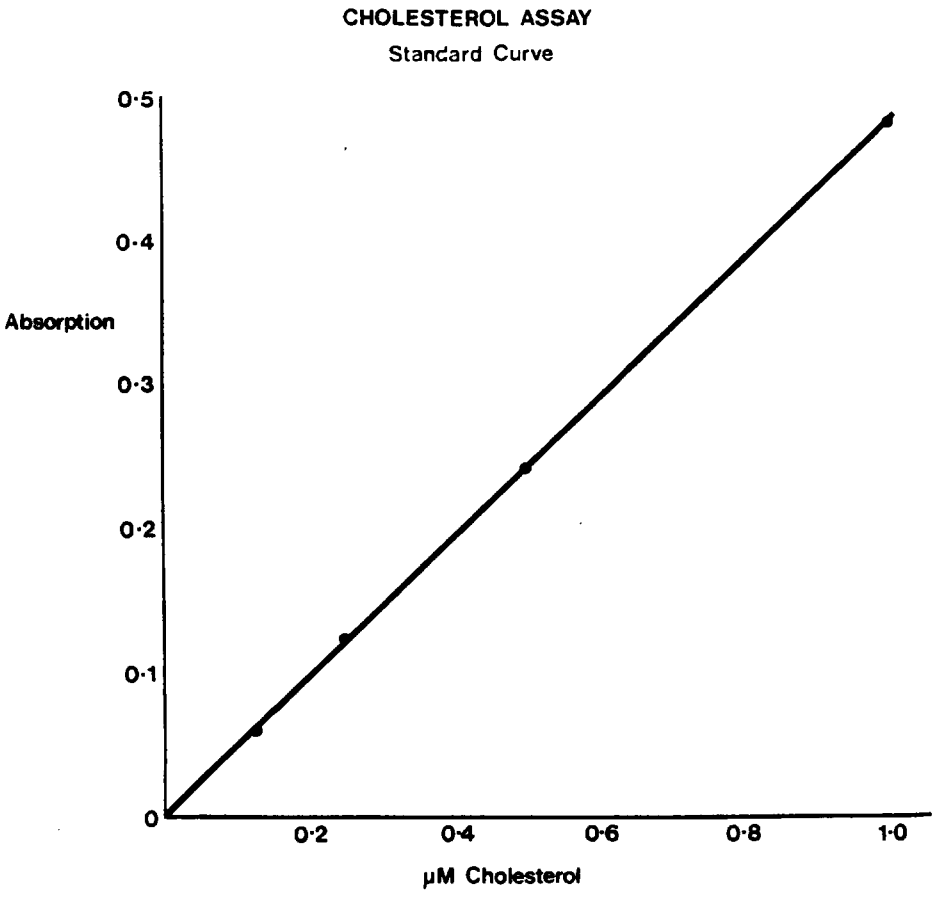


Fig. 12

Cholesterol Assay, Standard Curve

(c) Phospholipids

A method for measuring phospholipids by the colorimetric determination of phosphorus was first described in 1925 (Fiske and Subbarow, 1925). An alternative technique has been described (Dryer et al., 1957) but has not gained widespread acceptance. In the method of Fiske and Subbarow phosphates are converted to ammonium phosphomolybdate which is then reduced with aminonaphtholsulphonic acid which releases blue coloured complexes. Consistent results were obtained with the technique. A typical standard curve is shown in Fig. 13. All assays were performed in duplicate.

(d) Specific Activities

Preliminary thin layer chromatography confirmed that the injected 14-C Cholic acid was more than 99% pure. During its passage through the enterohepatic circulation the labelled bile acid will become conjugated with glycine or taurine. The first step in preparing bile acid samples for specific activity analysis is deconjugation. A taurocholate standard was included with each series of determinations. Thin layer chromatography was then used to confirm that deconjugation was complete. Excellent separation of cholic acid was achieved by thin layer chromatography. Cholic acid formed a well-defined spot. Scintillation counting of eluates derived from silica gel

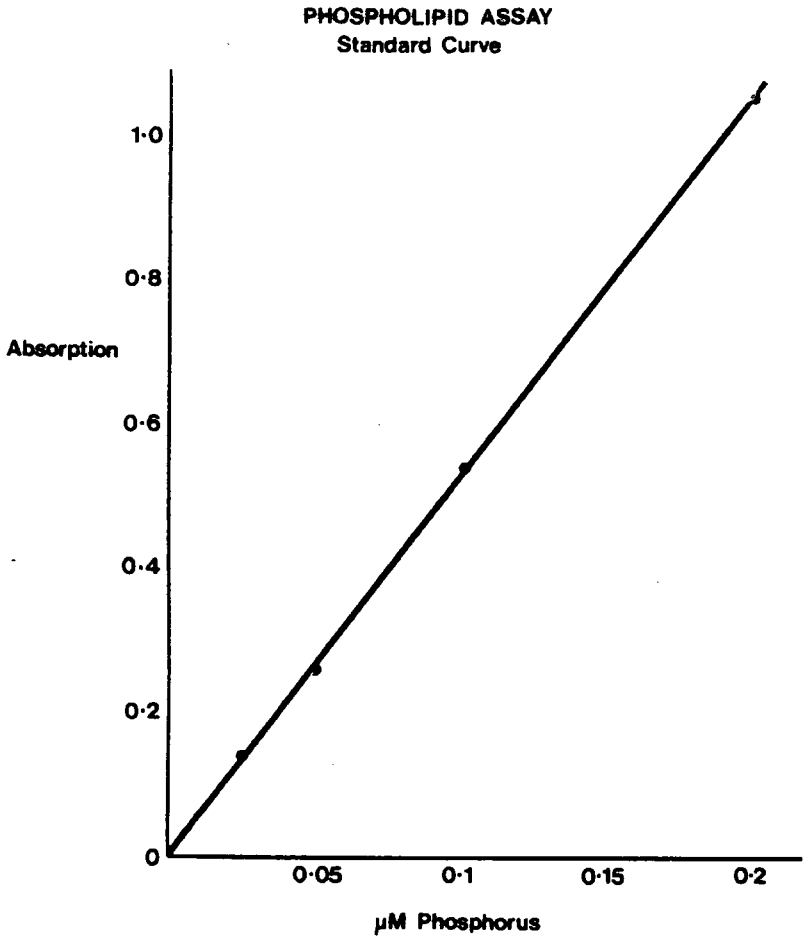


Fig. 13

Phospholipid Assay, Standard Curve

just behind the cholic acid spot had extremely low counts excluding any loss of cholic acid during chromatography. Bile acid assays were performed as described previously. Scintillation counting corrections for quenching and counting efficiency were applied automatically by the liquid scintillation spectrometer used. All decay curves had a good fit by linear regression analysis ($r \geq 0.96$).

SUMMARY

The chronic bile fistula monkey was evaluated. These studies confirmed the suitability of this animal model for studies of biliary physiology and the results were similar to those reported previously (Dowling et al., 1968, Dowling et al., 1970). The well-validated techniques used in the human studies and the possible sources of error have been discussed. The choice of laboratory methods has been justified and the precision of these methods has been demonstrated.

P R E S E N T A T I O N O F R E S U L T S

The results, presented in the following chapter, have been divided into three sections. The first section gives the results of the studies on altered enterohepatic cycling frequency. The second section gives the results of the studies on the effects of vagotomy in man.

(a) Bile Acid Kinetic Data

Cholic acid pool size, cholic acid synthetic rate and daily fractional turnover have all been calculated by regression analysis of the isotope decay curve. When the tracer dose of 14-C Cholic acid is administered it mixes with the bile acid pool. Bile acid pool is therefore defined as the mass of bile acid that dilutes the tracer. Assuming that the bile acid pool is a one-compartment system in a steady state then the specific activity of the isotope will decline exponentially as non-radioactive molecules of cholic acid are synthesised. An increase in synthetic rate will increase the slope of the decay curve (Lindstedt, 1957). The rate of decrease in specific activity is proportional to the specific activity at a given time. This can be expressed mathematically by the equation:

$$\frac{-d(SA)}{dt} = k(SA) \qquad (1)$$

where k is the decay constant ($days^{-1}$), SA is bile acid specific activity (disintegrations per minute per millimole) and t is time (days). Integrating between t_o, the time of isotope administration and some time later t_1 one obtains the equation:

$$k = \frac{\ln_e (SA_{to}) - \ln_e (SA_{t1})}{t_1 - t_o} \qquad (2)$$

From this equation it will be seen that when the natural logarithm of bile acid specific activity is plotted against time the graph will have a slope of k and will intercept the ordinate at the specific activity at t_o. The slope k is the fractional turnover rate which is the fraction of the pool metabolised per day. SA_{to} represents the dilution of the administered isotope-labelled bile acid by the pool of non-radioactive bile acids at that time. By definition specific activity = radioactivity/mass and therefore mass = radioactivity/specific activity.

$$\text{Thus pool} = \frac{\text{dpm injected}}{SA_{to}} \qquad (3)$$

In the steady state metabolism is equal to synthesis. By definition

$$\text{synthetic rate} = \text{pool} \times k \qquad (4)$$

In the studies on altered enterohepatic cycling the cycling frequency is known and controlled, but in the

studies on vagotomy this is not so. The relationship between synthesis and enterohepatic cycling frequency may be defined mathematically by the equation:

$$\text{synthesis} = (1 - k_{abs}) \times (C \times \text{pool}) \quad (5)$$

where k_{abs} is the fraction of the pool absorbed during each enterohepatic cycle and C is the number of cycles per day. It will be seen that with efficient absorption k_{abs} will approach 1 and synthesis will decrease while with inefficient absorption k_{abs} will approach 0 and synthesis will increase. By equating equations (4) and (5) one obtains:

$$k = (1 - k_{abs}) \times C \quad (6)$$

By assuming a value for k_{abs}, which usually exceeds 95% in health, the cycling frequency may be calculated.

Because of the considerable variations in pool size between individuals the kinetic data is tabulated for each individual, as well as in tables and histograms for the group as a whole. Cholic acid pool size, cholic acid synthesis and fractional turnover rate, were derived directly from the decay curve. Total bile acid pool size was calculated from the relative concentration of total bile acids to cholic acid and the cholic acid pool size. The specific activities from which the decay curves were derived are tabulated in Appendix II.

(b) Biliary Lipid Composition

The concentrations of the three biliary lipids, cholesterol, bile acids and phospholipids have been tabulated in two ways. In each table biliary lipid composition is first expressed as concentrations in millimoles $\pm$1 standard error of the mean. In the lower half of each table the same data is expressed as the relative molar composition.

Relative molar composition is derived by dividing the molar concentration of each biliary lipid by the sum of the molar concentrations of all three lipids and expressing the fraction as a percentage. The relative molar composition data have also been plotted on the triangular co-ordinates of Admirand and Small. The principle constituents of bile are water, cholesterol, bile acids and phospholipids. All relative concentrations of these four substances can be defined within the bounds of a tetrahedron. If the concentration of the water is assumed to be constant then the relative concentration of the three solid components can be defined within a triangle (Fig. 14) (Admirand and Small, 1968). The relative concentrations of cholesterol, bile acids and phospholipids are such that all data points fall within the lower left hand corner of the triangle.

This corner of the triangle has been enlarged in figure 15 and its relationship to complete triangle (Fig. 14) is shown in the inset. The heavy line crossing the triangle

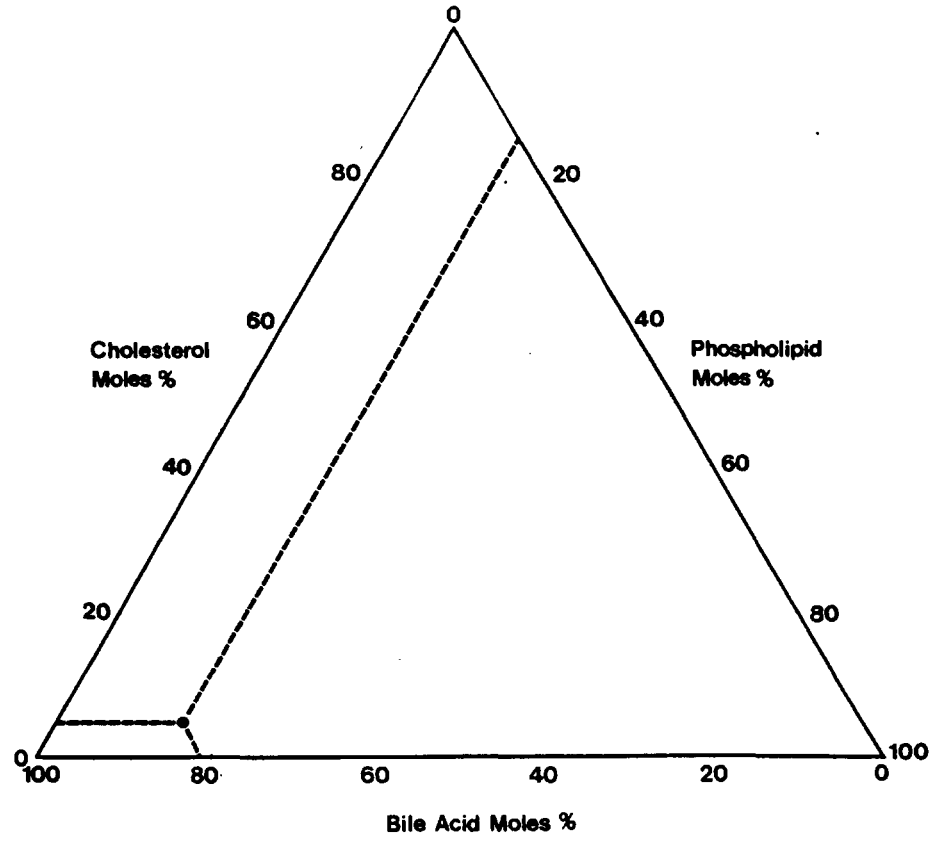


Fig. 14

The Triangular Plot of Biliary Lipid Composition

For a given concentration of water, in this case 90%, the relative concentrations of the three biliary lipids may be defined within the bounds of a triangle. The per cent of the total moles of bile acid, phospholipid, and cholesterol are shown on the scales along the sides of the triangle. Since the sum of bile acid, phospholipid and cholesterol, equals 100% the composition of any bile can be represented as a single point within triangular coordinates. Thus a bile containing 80% bile acids, 15% phospholipids, and 5% cholesterol is represented as the single point at the intersection of the broken lines in the figure.

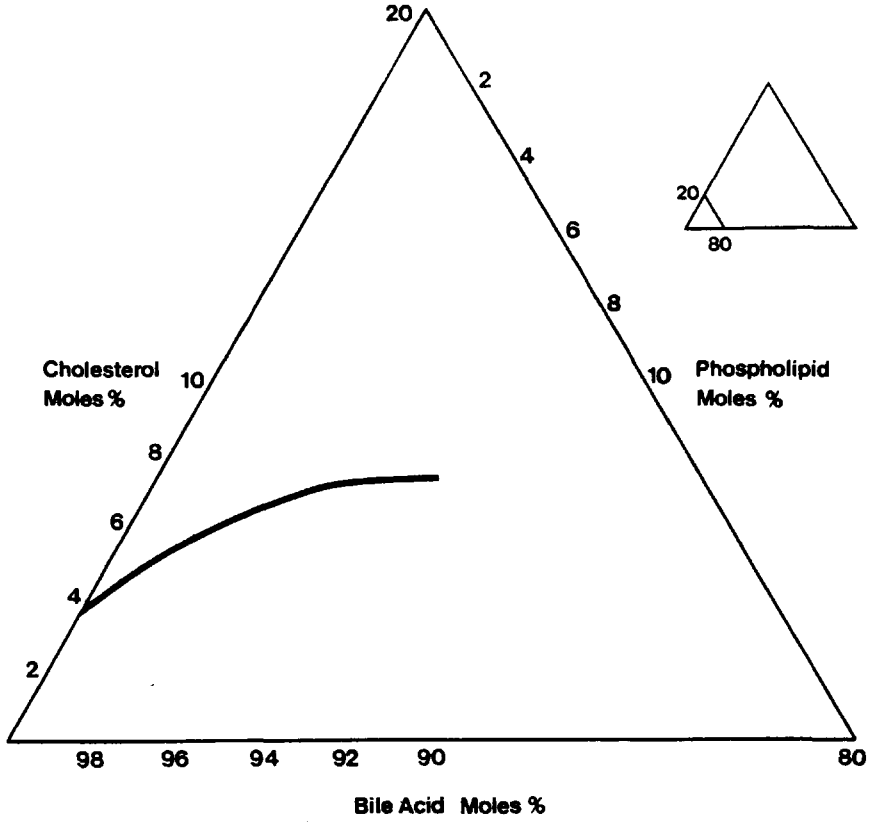


Fig. 15

The Triangular Plot of Biliary Lipid Composition

As bile acids are the predominant constituent the data points are situated in the lower left hand corner of the triangle. The triangle above is an enlargement of this corner of the triangle and its relationship to Fig. 14 is shown in the inset. Most of the results are plotted on this triangle. The solid line represents the maximum cholesterol solubility for any combination of bile acids and phospholipids (Admirand and Small, 1968).

is the line of maximum solubility. The triangular plots in the results are all of the area shown in figure 15, with the exception of figure 23 in which a larger area is displayed.

Biliary lipid composition can also be expressed in terms of a lithogenic index (Metzger et al., 1972). The method is based on the triangular phase diagram of Admirand and Small and numerically relates the actual molar percentage of cholesterol in a given bile sample to the maximum molar percentage that could be dissolved in that sample. This ratio of actual cholesterol to maximum possible cholesterol is termed the "lithogenic index". It will be seen that undersaturated samples will have a lithogenic index of less that 1, a saturated sample will have an index of 1, and a supersaturated sample an index greater than 1. Three such samples are shown in figure 16.

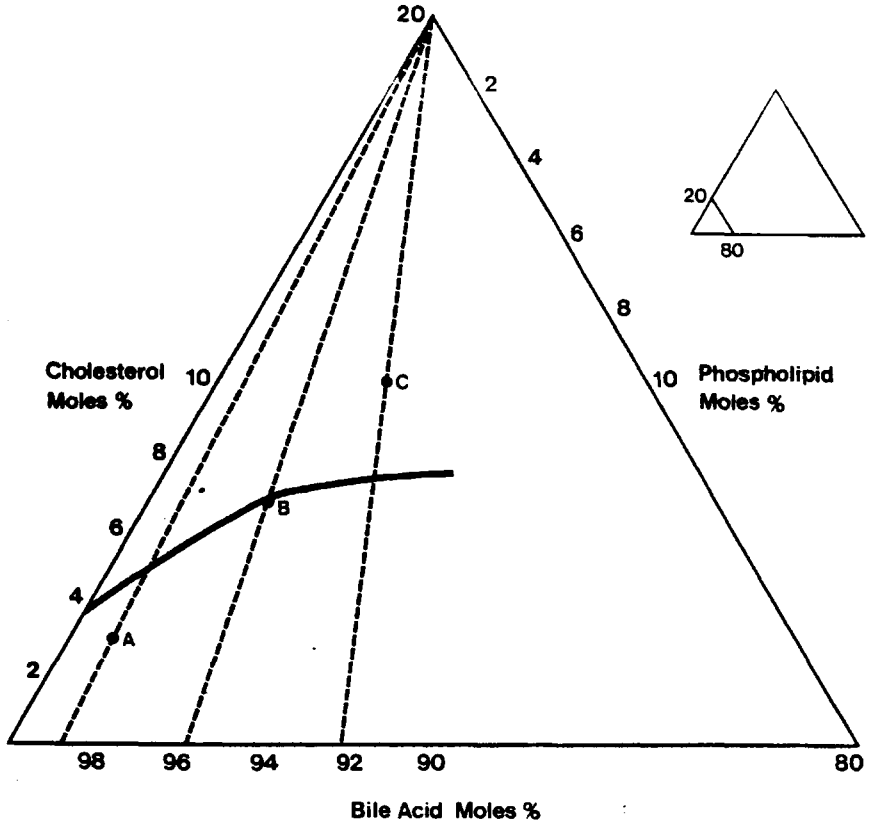


Fig. 16

The Lithogenic Index

The degree of cholesterol saturation of a given bile sample may be expressed numerically by the ratio of the cholesterol concentration in that sample to the maximum amount of cholesterol that could be dissolved in that sample. Points A, B and C represent unsaturated, just saturated and supersaturated samples.

	Cholesterol Concentration	Maximum Cholesterol Solubility	Lithogenic Index
A	3.0	4.9	0.61
B	6.8	6.8	1.00
C	10.0	7.2	1.39

R E S U L T S - S E C T I O N 1

EFFECTS OF ALTERED ENTEROHEPATIC CYCLING IN THE RHESUS MONKEY

1. BILE ACID KINETICS

(a) Bile Acid Pool Size

In all three monkeys each increase in cycling frequency produced a corresponding decrease in bile acid pool size. With the bile acid pool making one enterohepatic cycle/day the mean cholic acid pool size was 1.76 mM and the total bile acid pool, calculated from the relative concentration of cholic acid to total bile acids was 3.62 mM. When cycling frequency was increased to two cycles/day cholic acid and total bile acid pools decreased to 1.23 mM and 2.33 mM respectively. A further increase in cycling frequency to four cycles/day resulted in a further contraction of cholic and total bile acid pools to 0.59 mM and 1.50 mM. When cycling frequency was increased to 8 cycles/day pool sizes reached their lowest values. Cholic acid pool was 0.37 mM and total bile acid pool was 0.87 mM. When the pool was allowed to cycle normally through an uninterrupted enterohepatic circulation both cholic and total bile acid pools expanded to 0.48 mM and 1.04 mM respectively. There was a significant inverse correlation between pool size and cycling frequency in all three animals. Linear regression analysis gave values

for r, the regression coefficient, of 0.94, 0.89, 0.81 in monkeys 1, 2 and 3 respectively. These changes are shown graphically in figure 17. Values for individual animals are presented in Tables 1 - 3 and mean values for the group as a whole are presented in Table 4.

This close correlation between cycling frequency and bile acid pool size strongly suggests that cycling frequency is a major determinant of bile acid pool size. The pool size during continuous enterohepatic cycling corresponds to a cycling frequency of 6.5 cycles/day. This is a reasonable figure for animals taking two meals per day, since the bile acid pool normally circulates 2 - 3 times with each meal and some enterohepatic circulation continues to occur between meals.

(b) Cholic Acid Synthesis

There was little difference in cholic acid synthetic rate when the bile acid pool was cycling at either one or two cycles/day: 0.43 mM/day and 0.42 mM/day respectively. Synthetic rate decreased to 0.25 mM/day when cycling frequency was increased to four cycles/day but the magnitude of change (42%) was less than the corresponding change in bile acid pool size (52%). A further decrease, to 1.7 mM/day, occurred when cycling frequency increased to eight cycles/day but the magnitude of change was less (36%) and seemed to

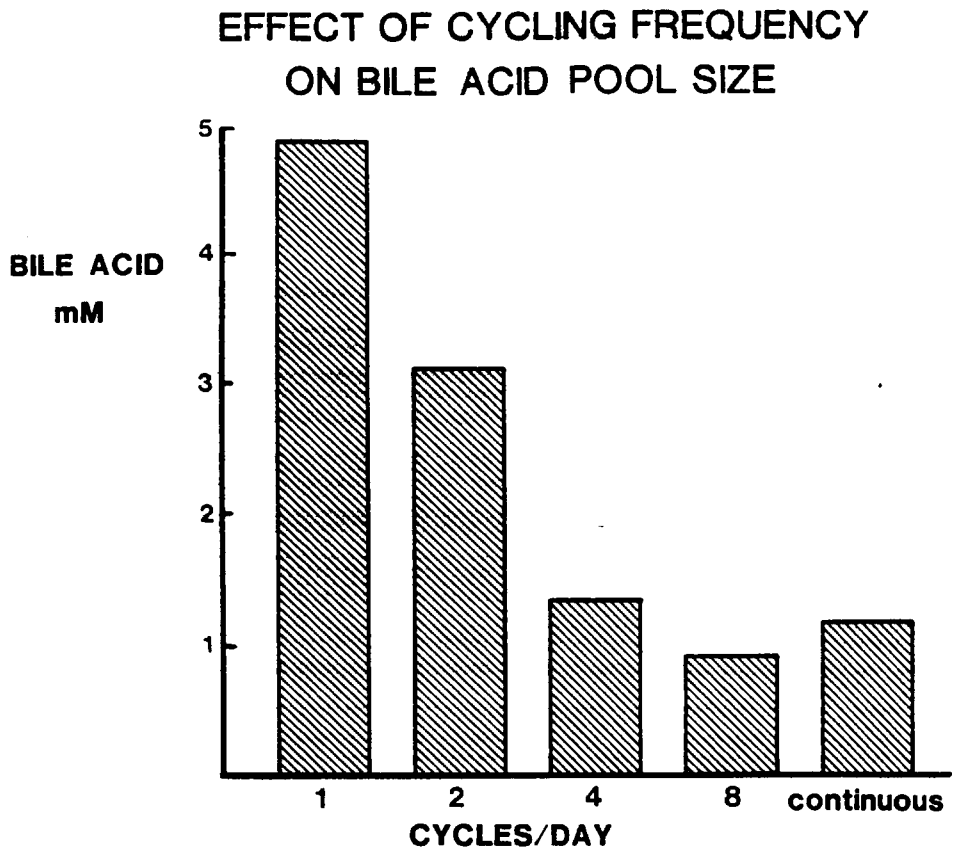

EFFECT OF CYCLING FREQUENCY
ON BILE ACID POOL SIZE
BILE ACID
mM
5
4
3
2
1
1
2
4
8
continuous
CYCLES/DAY

Fig. 17

parallel the change in bile acid pool size (37%). No significant changes occurred when the pool was continuously cycled. There was a significant correlation between cycling frequency and cholic acid synthetic rate. Linear regression analysis gave values for r of 0.97, 0.82, and 0.90 in monkeys 1, 2 and 3 respectively. These changes are shown graphically in figure 18. Values for individual animals are presented in Table 1 - 3 and mean values for the group as a whole are presented in Table 4.

In the hypothetical model, on which this thesis is based, the increases in cycling frequency were exactly balanced by a decrease in bile acid pool size and, therefore, hepatic bile acid flux remained constant. Hepatic bile acid flux appears to be a major determinant of bile acid synthesis which should, therefore, also remain constant. The serial decreases in cholic acid synthesis were unexpected. It seems likely that the observed changes are due to an inexact balance between pool size and cycling frequency. Thus, in general, when the cycling frequency doubles, pool sizes decrease by less than half. Hepatic flux consequently increases and synthesis decreases.

(c) Daily Fractional Turnover Rate

There were modest increases in daily fractional turnover rate with each increase in cycling frequency. At one

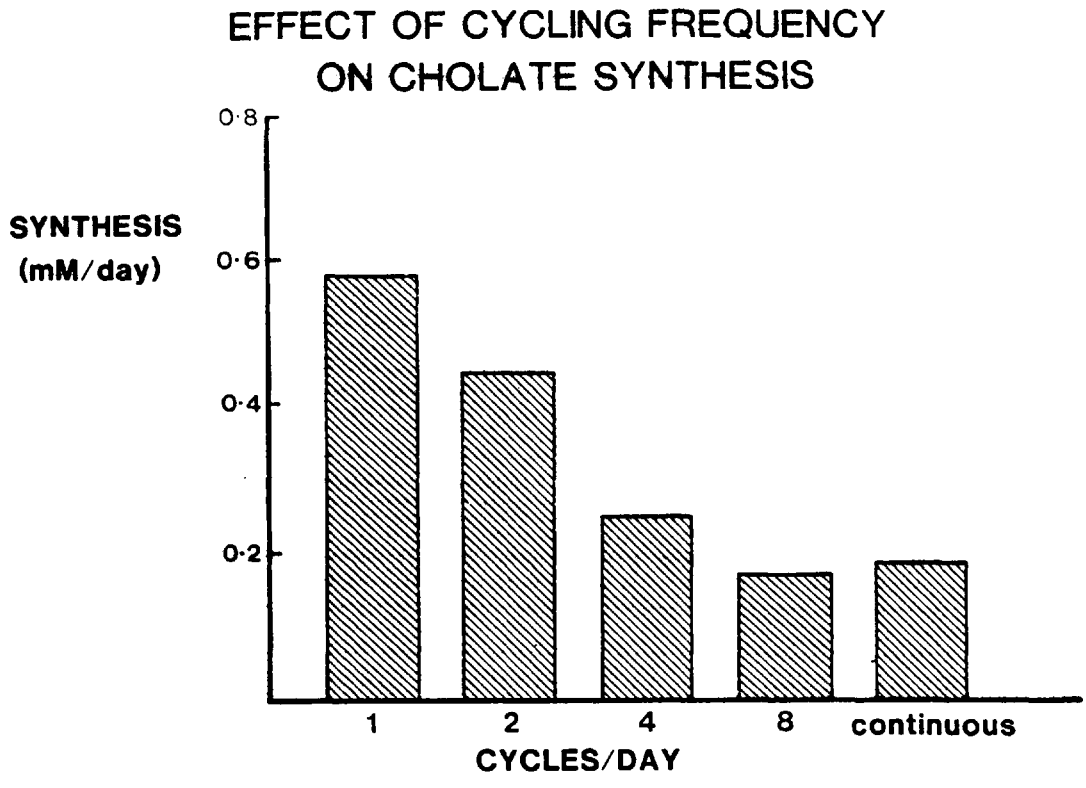

EFFECT OF CYCLING FREQUENCY
ON CHOLATE SYNTHESIS
SYNTHESIS
(mM/day)
0·8
0·6
0·4
0·2
1
2
4
8
continuous
CYCLES/DAY

Fig. 18

cycle/day the fractional turnover rate was 0.26. This increased to 0.35 at two cycles/day and to 0.43 at four cycles/day. A further small increase, to 0.47, occurred when cycling frequency was increased to eight cycles/day and a small decrease, to 0.41, occurred when the pool was continuously cycled. There was reasonably close correlation between cycling frequency and daily fractional turnover rate. Linear regression analysis gave values for r of 0.99, 0.78 and 0.83 in monkeys 1, 2 and 3 respectively. These changes are shown graphically in figure 19. Values for individual animals are presented in Tables 1 - 3 and mean values for the group as a whole in Table 4.

The increases in daily fractional turnover rate were expected. If a small pool circulates more frequently than a large pool then the daily flux of bile acids through the intestine will be similar. Assuming absorption is unchanged then the faecal losses will be equal. However, the same quantitative loss will represent a larger fraction of the smaller pool and fractional turnover rate, which represents the fraction of the pool lost each day, will be greater.

2. BILIARY LIPID COMPOSITION

Because of the importance of the relative molar composition of cholesterol, bile acids and phospholipids in relation to the degree of cholesterol saturation of bile, the results

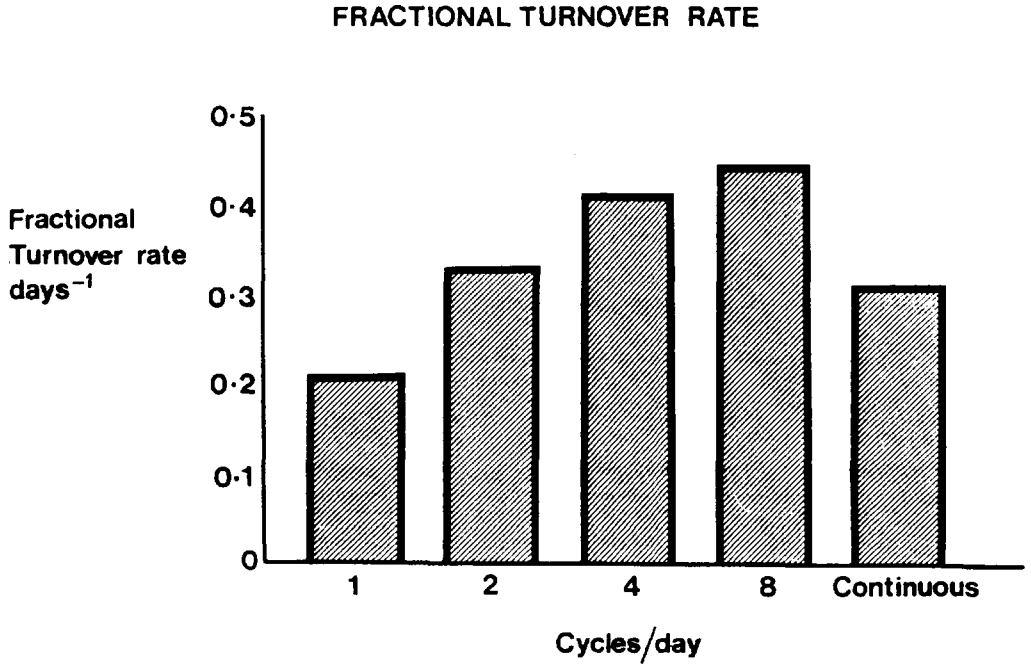

FRACTIONAL TURNOVER RATE
Fractional Turnover rate days^{-1}
0·5
0·4
0·3
0·2
0·1
0
1
2
4
8
Continuous
Cycles/day

Fig. 19

of the biliary lipid analyses have been tabulated together. Alterations in enterohepatic cycling frequency did produce some of the changes in bile acid kinetics associated with cholesterol gallstone disease. However, these alterations did not produce the changes in biliary lipid composition, namely increased cholesterol saturation, of cholesterol gallstone disease. Biliary lipid composition remained remarkably constant throughout the study and the changes between one cycling frequency and another were small and insignificant. The composition data for individual animals is presented in Tables 5 - 7, and mean values for the group as a whole are presented in Table 8. When the relative molar composition of biliary lipids is plotted on the triangular co-ordinates of Admirand and Small all points remain in the stable micellar zone (Fig. 20), and the Lithogenic Index is less than 1 at all cycling frequencies (table 9). These results lend support to the view that there is a hepatic defect which accounts for the abnormal biliary lipid composition and that cycling frequency does not significantly alter composition.

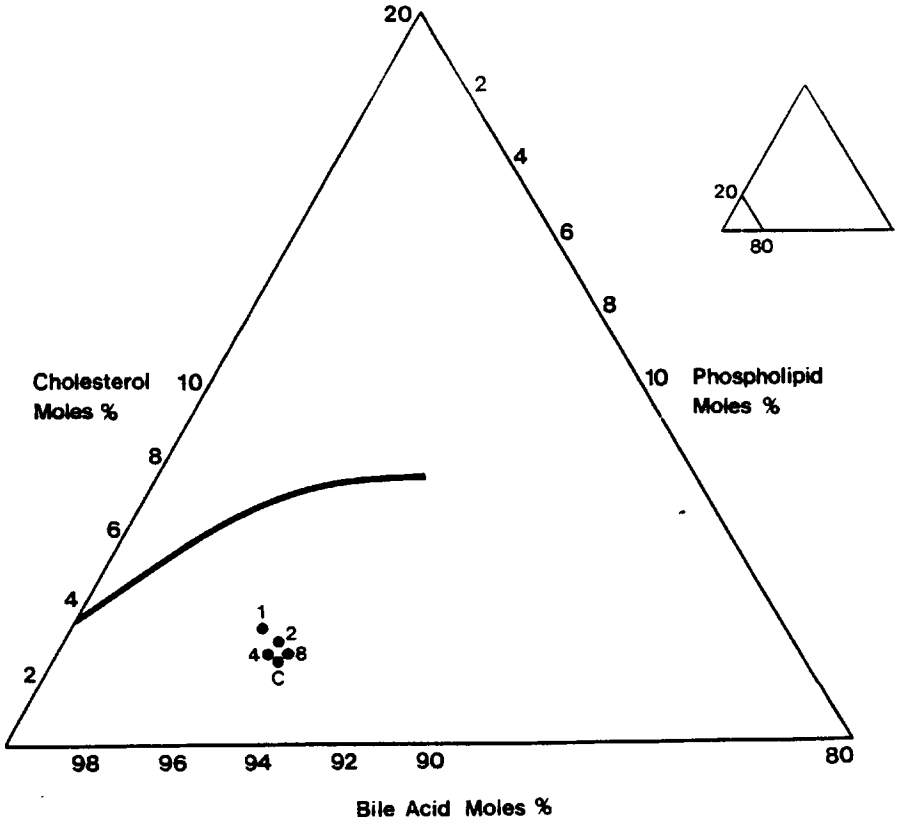


Fig. 20

Triangular plot of biliary lipid composition at 1,2, 4 and 8 cycles/day and with continuous enterohepatic cycling (c)

T A B L E 1

BILE ACID KINETICS

Monkey 1

Cycles/day	Cholic Acid Pool mM	Cholic Acid Synthesis mM/day	Fractional Turnover Rate $days^{-1}$	Bile Acid Pool mM
1	1.69	0.41	0.24	3.83
2	1.40	0.44	0.31	2.33
4	0.72	0.28	0.38	1.55
8	0.30	0.15	0.51	0.84
C	0.34	0.16	0.47	0.87

C = continuous cycling

T A B L E 2

BILE ACID KINETICS

Monkey 2

Cycles/day	Cholic Acid Pool mM	Cholic Acid Synthesis mM/day	Fractional Turnover Rate $days^{-1}$	Bile Acid Pool mM
1	0.91	0.30	0.33	2.70
2	0.91	0.37	0.41	2.07
4	0.46	0.23	0.49	1.40
8	0.39	0.19	0.48	0.89
C	0.52	0.23	0.44	1.12

C = continuous cycling

T A B L E 3

BILE ACID KINETICS

Monkey 3

Cycles/day	Cholic Acid Pool mM	Cholic Acid Synthesis mM/day	Fractional Turnover Rate $days^{-1}$	Bile Acid Pool mM
1	2.66	0.56	0.21	4.32
2	1.37	0.45	0.33	2.58
4	0.58	0.23	0.41	1.55
8	0.42	0.18	0.44	0.82
C	0.59	0.19	0.31	1.12

C = continuous cycling

T A B L E 4

BILE ACID KINETICS

Pooled Data for 3 Monkeys

Cycles/day	Cholic Acid Pool mM	Cholic Acid Synthesis mM/day	Fractional Turnover Rate $days^{-1}$	Bile Acid Pool mM
1	1.76	0.43	0.26	3.62
2	1.23	0.42	0.35	2.33
4	0.59	0.25	0.43	1.50
8	0.37	0.17	0.48	0.85
C	0.48	0.19	0.41	1.04

T A B L E 5

BILIARY LIPID COMPOSITION

Monkey 1 — Mean ± 1 S.E.M.

Cycles/ Day	Number of Samples	Bile Acid mM	Cholesterol mM	Phospholipid mM
1	13	36.94 ± 1.80	1.73 ± 0.37	3.73 ± 0.36
2	5	50.30 ± 6.61	1.19 ± 0.20	3.92 ± 0.75
4	11	54.26 ± 1.87	1.16 ± 0.08	2.66 ± 0.21
8	11	53.90 ± 2.11	1.72 ± 0.13	3.94 ± 0.20
C	10	50.60 ± 1.24	1.23 ± 0.11	2.39 ± 0.18

Cycles/ Day	Number of Samples	Bile Acid Moles %	Cholesterol Moles %	Phospholipid Moles %
1	13	87.12 ± 1.14	4.08 ± 0.72	8.80 ± 0.70
2	5	90.78 ± 0.71	2.15 ± 0.27	7.07 ± 0.69
4	11	93.42 ± 0.33	2.00 ± 0.15	4.58 ± 0.27
8	11	90.49 ± 0.38	2.89 ± 0.27	6.62 ± 0.29
C	10	93.32 ± 0.31	2.27 ± 0.20	4.41 ± 0.30

C = continuous cycling

TABLE 6

BILIARY LIPID COMPOSITION

Monkey 2 — Mean ± 1 S.E.M.

Cycles/ Day	Number of Samples	Bile Acid mM	Cholesterol mM	Phospholipid mM
1	13	52.44 ± 3.61	1.37 ± 0.14	2.79 ± 0.24
2	11	58.95 ± 2.67	1.93 ± 0.42	3.61 ± 0.34
4	11	64.24 ± 4.83	1.72 ± 0.14	3.00 ± 0.23
8	12	81.87 ± 3.46	1.82 ± 0.28	4.26 ± 0.26
C	11	70.96 ± 1.41	1.47 ± 0.19	2.44 ± 0.12

Cycles/ Day	Number of Samples	Bile Acid Moles %	Cholesterol Moles %	Phospholipid Moles %
1	13	92.65 ± 0.67	2.42 ± 0.29	4.93 ± 0.53
2	11	91.41 ± 0.39	2.99 ± 0.17	5.60 ± 0.39
4	11	93.16 ± 0.33	2.49 ± 0.15	4.35 ± 0.27
8	12	93.09 ± 0.38	2.07 ± 0.27	4.84 ± 0.29
C	11	94.78 ± 0.21	1.96 ± 0.24	3.26 ± 0.17

C = continuous cycling

TABLE 7

BILIARY LIPID COMPOSITION

Monkey 3 — Mean ± 1 S.E.M.

Cycles/ Day	Number of Samples	Bile Acid mM	Cholesterol mM	Phospholipid mM
1	11	41.33 ± 1.50	0.83 ± 0.25	1.97 ± 0.59
2	10	29.77 ± 3.21	0.97 ± 0.10	1.80 ± 0.21
4	12	43.29 ± 2.89	1.34 ± 0.87	1.93 ± 0.12
8	10	50.33 ± 2.7	1.49 ± 0.17	3.22 ± 0.40
C	11	43.93 ± 2.19	0.84 ± 0.21	1.32 ± 0.16

Cycles/ Day	Number of Samples	Bile Acid Moles %	Cholesterol Moles %	Phospholipid Moles %
1	11	93.66 ± 0.84	1.88 ± 0.46	4.46 ± 0.57
2	10	91.49 ± 1.38	2.98 ± 0.56	5.53 ± 0.93
4	12	92.97 ± 0.38	2.88 ± 0.17	4.15 ± 0.30
8	10	91.44 ± 0.88	2.71 ± 0.32	5.85 ± 0.84
C	11	95.32 ± 0.30	1.82 ± 0.33	2.86 ± 0.38

C = continuous cycling

TABLE 8

BILIARY LIPID COMPOSITION

Pooled Data for 3 Monkeys — Mean ± 1 S.E.M.

Cycles/ Day	Number of Samples	Bile Acid mM	Cholesterol mM	Phospholipid mM
1	37	43.69 ± 1.50	1.57 ± 0.16	2.04 ± 0.21
2	26	46.09 ± 3.30	1.42 ± 0.12	2.98 ± 0.28
4	34	53.62 ± 2.38	1.41 ± 0.07	1.68 ± 0.13
8	33	62.99 ± 3.31	1.69 ± 0.12	3.84 ± 0.18
C	32	55.18 ± 2.29	1.34 ± 0.14	2.04 ± 0.13

Cycles/ Day	Number of Samples	Bile Acid Moles %	Cholesterol Moles %	Phospholipid Moles %
1	37	92.37 ± 0.64	3.32 ± 0.33	4.31 ± 0.42
2	26	91.29 ± 0.59	2.81 ± 0.25	5.90 ± 0.42
4	34	94.55 ± 0.20	2.49 ± 0.11	2.96 ± 0.16
8	33	91.93 ± 0.37	2.47 ± 0.17	5.60 ± 0.31
C	32	94.23 ± 0.19	2.29 ± 0.16	3.48 ± 0.20

C = continuous cycling

T A B L E 9

BILE LITHOGENIC INDEX

Cycles/day	Lithogenic Index
1	0.46
2	0.41
4	0.36
8	0.37
C	0.33

C = continuous cycling

R E S U L T S S E C T I O N I I

EFFECTS OF VAGOTOMY ON BILE ACID KINETICS AND BILIARY LIPID COMPOSITION IN MAN

1. BILE ACID KINETICS

(a) Bile Acid Pool Size

After vagotomy the cholic acid pool increased in two patients and remained unchanged in two patients. Total bile acid pool increased in three of the 4 patients and decreased in the fourth patient. When the results are considered together there was a mean increase in cholic acid pool of 88% from 1.67 mM before vagotomy to 3.15 mM after. Total bile acid pool size almost doubled from 4.61 mM before vagotomy to 9.12 mM after. Pool size data for each patient before vagotomy are presented in Table 10 and after vagotomy in Table 11. The mean data for the four patients before and after vagotomy are presented in Table 12 and figure 21.

(b) Cholic Acid Synthesis

Mean cholic acid synthetic rate was unchanged after vagotomy, 0.51 mM/day, compared with 0.52 mM/day before. When the patients are considered individually there were increases in two and decreases in two. A constant cholic acid synthetic rate in the presence of an altered bile acid

BILE ACID POOL SIZE BEFORE AND AFTER VAGOTOMY IN MAN

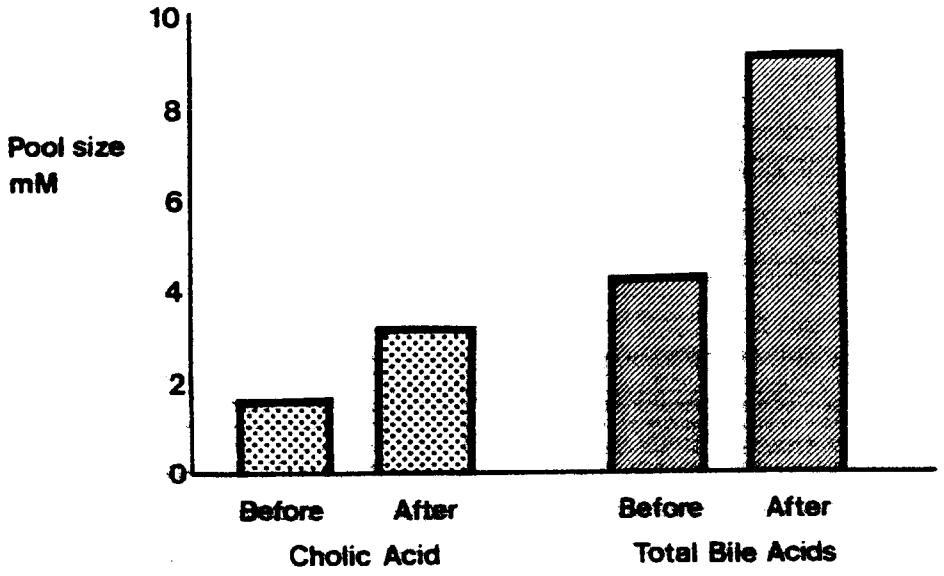


Fig. 21

pool size is most easily explained by an alteration in cycling frequency maintaining hepatic flux of bile acids more or less constant. This will be considered more fully in conjunction with the daily fractional turnover rates in the next paragraph. Cholic acid synthetic rates for the individual patients are presented in Table 10 (before vagotomy) and Table 11 (after vagotomy). Mean values before and after vagotomy are presented in Table 12.

(c) Fractional Turnover Rate

Fractional turnover decreased in three of the patients and increased slightly in the fourth. Mean value before vagotomy was 0.33 $days^{-1}$ and after vagotomy was 0.18 $days^{-1}$ (Table 12). Individual values for each patient are presented in Table 10 (before vagotomy) and Table 11 (after vagotomy).

A decrease in fractional turnover rate in the presence of a large pool is consistent with a decrease in cycling frequency. The larger pool circulating more slowly presents the same mass of bile acids to the ileum each day and similar mass is therefore lost, assuming absorption is equal before and after vagotomy. The same mass represents a smaller proportion of the larger pool and hence daily fractional turnover rate is decreased.

An approximation of cycling frequency may be made from equation (6) discussed in the previous chapter

$$k = (1 - k_{abs}) \times C \qquad (6)$$

This equation may be rearranged so that

$$C = \frac{k}{(1 - k_{abs})} \qquad (7)$$

where C is cycling frequency, k is fractional turnover rate and k_{abs} is the fraction of the pool absorbed during each enterohepatic cycle. Cycling frequency was calculated using several different values for k_{abs}. A curvilinear relationship was demonstrated between pool size and cycling frequency with a regression coefficient of r = 0.76. This correlation was constant regardless of the chosen value for k_{abs}. The curve for an absorption coefficient of 0.95 is presented in Fig. 22.

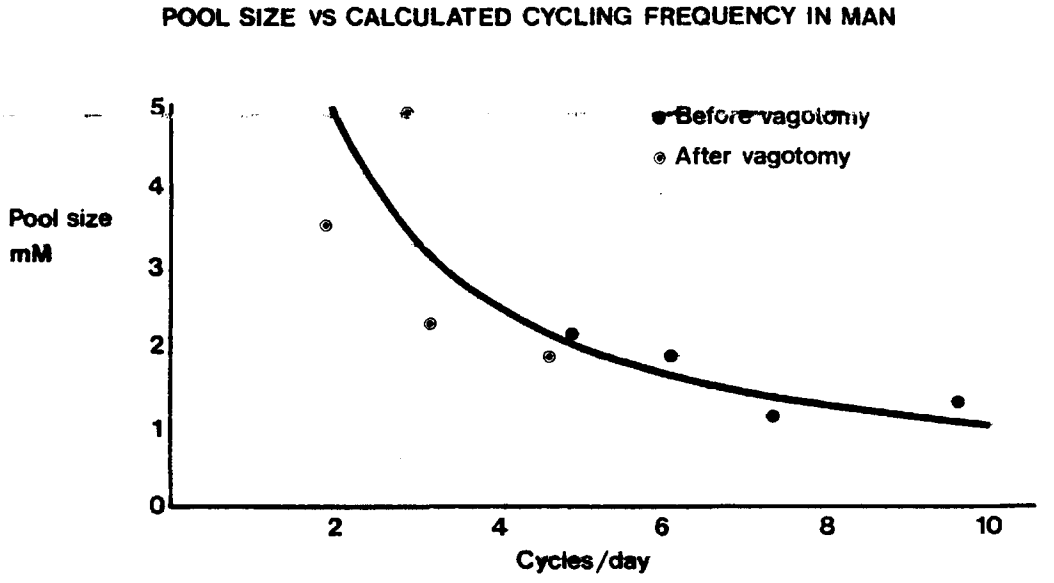

POOL SIZE VS CALCULATED CYCLING FREQUENCY IN MAN
Pool size
mM
5
4
3
2
1
0
2
4
6
8
10
Cycles/day
Before vagotomy
After vagotomy

Fig. 22

2. BILIARY LIPID COMPOSITION

As in the previous sections the results of the biliary lipid analyses have been tabulated both as millimoles and moles %. In all the studies in patients the concentration of phospholipids was higher than normal. The reason for this is unclear. The analyses were performed in a different laboratory to the analyses of bile samples from the Rhesus monkey. As in the studies on Rhesus monkeys there was a relative increase in bile acids after vagotomy. Although the concentrations of phospholipid were high both before and after vagotomy, the relative concentration decreased after vagotomy. Cholesterol concentration increased after vagotomy but was much less marked when the relative compositions are considered. When the relative composition is plotted on the triangular co-ordinates of Admirand and Small the bile lies within the stable micellar zone before and after vagotomy (Fig. 23). Biliary lipid composition before vagotomy is presented in Table 13 and after vagotomy in Table 14. Overall changes are summarised in Table 15, and the lithogenic indices in Table 16.

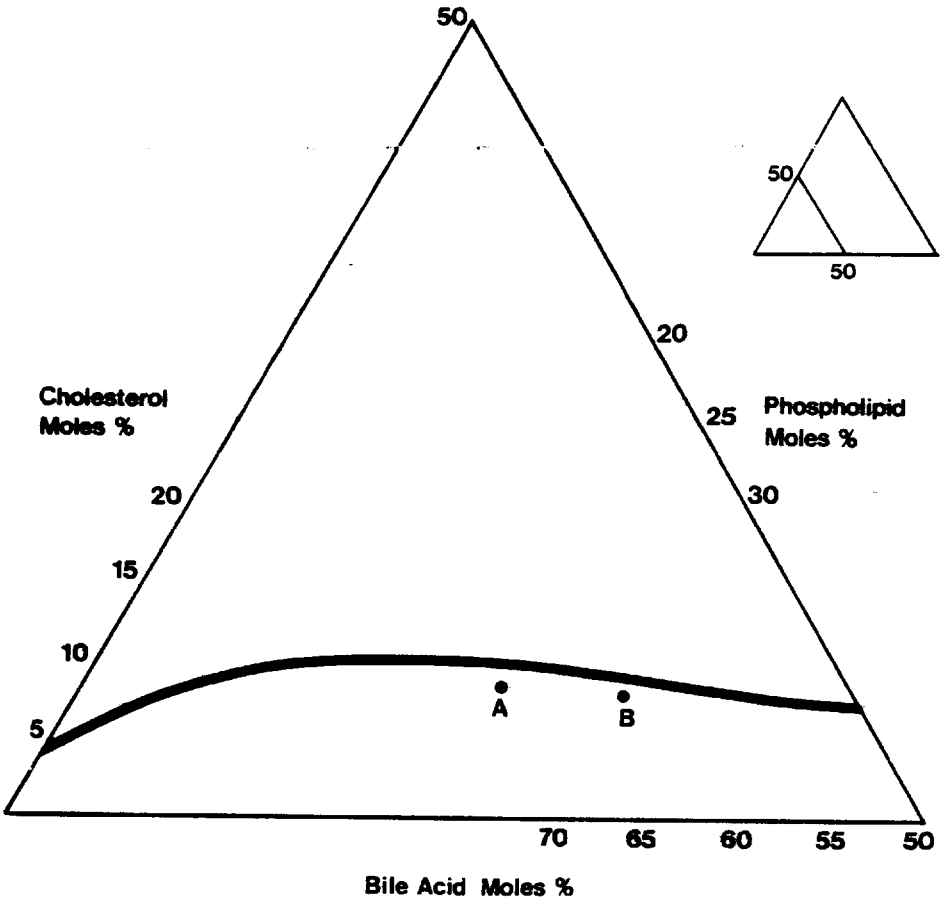


Fig. 23

Triangular plot of biliary lipid composition before (B) and after (A) vagotomy in Man.

T A B L E 1 0

BILE ACID KINETICS

Before Vagotomy

Patient	Cholic Acid Pool mM	Cholic Acid Synthesis mM/day	Fractional Turnover Rate $days^{-1}$	Bile Acid Pool mM
R.D.	1.28	0.48	0.37	4.11
J.P.	1.31	0.63	0.48	3.19
G.G.	2.17	0.53	0.25	5.65
J.R.	1.92	0.45	0.23	5.49

T A B L E 1 1

BILE ACID KINETICS

After Vagotomy

Patient	Cholic Acid Pool mM	Cholic Acid Synthesis mM/day	Fractional Turnover Rate $days^{-1}$	Bile Acid Pool mM
R.D.	4.97	0.74	0.15	14.00
J.P.	3.45	0.34	0.10	11.15
G.G.	2.28	0.37	0.16	7.51
J.R.	1.89	0.57	0.30	3.82

T A B L E 1 2

BILE ACID KINETICS

Pooled Data

	Cholic Acid Pool mM	Cholic Acid Synthesis mM/day	Fractional Turnover Rate $days^{-1}$	Bile Acid Pool mM
Before Vagotomy (4 men)	1.67	0.52	0.33	4.61
After Vagotomy (4 men)	3.15	0.51	0.18	9.12

T A B L E 1 3

BILIARY LIPID COMPOSITION

Before Vagotomy — Mean ± 1 S.E.M.

Patients	Number of Samples	Bile Acid mM	Cholesterol mM	Phospholipid mM
R.D.	4	53.45 ± 6.04	7.22 ± 0.88	32.15 ± 4.60
J.P.	4	45.80 ± 3.16	7.58 ± 0.91	21.20 ± 3.92
G.G.	4	12.43 ± 1.32	1.62 ± 0.44	3.83 ± 0.71
J.R.	4	49.55 ± 4.21	4.03 ± 0.35	17.58 ± 3.56

Patients	Number of Samples	Bile Acid Moles %	Cholesterol Moles %	Phospholipid Moles %
R.D.	4	58.73 ± 1.73	8.01 ± 0.68	33.26 ± 1.43
J.P.	4	61.59 ± 1.87	10.30 ± 1.46	28.11 ± 1.46
G.G.	4	70.99 ± 2.67	8.77 ± 1.54	20.24 ± 1.60
J.R.	4	70.15 ± 3.08	5.77 ± 0.53	24.09 ± 3.36

TABLE 14

BILIARY LIPID COMPOSITION

After Vagotomy — Mean ± 1 S.E.M.

Patient	Number of Samples	Bile Acid mM	Cholesterol mM	Phospholipid mM
R.D.	4	99.63 ± 10.41	8.02 ± 1.00	27.55 ± 3.30
J.P.	4	50.57 ± 2.30	11.87 ± 0.90	22.20 ± 2.08
G.G.	4	33.68 ± 6.48	4.54 ± 0.70	16.30 ± 2.92
J.R.	4	85.69 ± 20.60	9.11 ± 2.49	20.65 ± 4.64

Patient	Number of Samples	Bile Acid Moles %	Cholesterol Moles %	Phospholipid Moles %
R.D.	4	73.78 ± 1.10	5.93 ± 0.58	20.29 ± 0.58
J.P.	4	59.77 ± 0.86	14.07 ± 1.24	26.15 ± 2.10
G.G.	4	61.52 ± 2.80	8.53 ± 0.88	29.96 ± 3.49
J.R.	4	74.05 ± 1.97	7.77 ± 0.47	18.19 ± 1.61

TABLE 15

BILIARY LIPID COMPOSITION

Pooled Data — Mean ± 1 S.E.M.

	Number of Samples	Bile Acid mM	Cholesterol mM	Phospholipid mM
Before Vagotomy (4 men)	16	39.94 ± 4.70	4.95 ± 0.69	18.52 ± 3.10
After Vagotomy (4 men)	16	66.51 ± 8.84	8.19 ± 0.84	21.64 ± 1.87

	Number of Samples	Bile Acid Moles %	Cholesterol Moles %	Phospholipid Moles %
Before Vagotomy (4 men)	16	65.61 ± 1.82	8.07 ± 0.65	26.31 ± 1.73
After Vagotomy (4 men)	16	67.78 ± 1.96	8.74 ± 0.83	23.48 ± 1.62

T A B L E 1 6

BILE LITHOGENIC INDEX

Man	Lithogenic Index
Before Vagotomy	0.86
After Vagotomy	0.86

S U M M A R Y O F R E S U L T S

1. Increasing enterohepatic cycling frequency in the Rhesus monkey has been shown to:

a) decrease cholic acid and total bile acid pool size.

b) decrease cholic acid synthetic rate.

c) increase daily fractional turnover rate.

2. No significant alterations in biliary lipid composition occurred when cycling frequency was increased.

3. Bile acid pool size increased following vagotomy in man and an inverse curvilinear relationship existed between cholic acid pool size and calculated cycling frequency.

4. No significant alterations in the relative composition of biliary lipids occurred after vagotomy although the absolute concentrations of all three lipids increased.

D I S C U S S I O N

Our understanding of the physiology of biliary lipid metabolism and bile acid kinetics stems mostly from research work carried out within the last 15 years. A major advance was made when Admirand and Small found that cholesterol solubility in bile could be defined in terms of the relative molar composition of the three biliary lipids, bile acids, cholesterol and phospholipids (Admirand and Small, 1968). They showed that there was an excess of cholesterol in relation to the solubilising lipids, bile acids and phospholipids, in patients with gallstones. The supersaturated bile was an unstable solution in which cholesterol precipitation was likely, leading to stone formation and growth. Two years later it was shown that this abnormal bile was secreted by the liver (Small and Rapo, 1970). This discovery, confirmed by another group (Vlahcevic, Bell and Swell, 1970), challenged the long-accepted role of the gallbladder in the pathogenesis of gallstones. It now seemed that the gallbladder provided a suitable environment in which the supersaturated bile is disturbed and precipitation occurs. How this occurs is uncertain but it seems likely that water reabsorption locally concentrating the solid constituents of bile close to the gallbladder wall (Cussler et al., 1970), and mucus secreted by the gallbladder mucosa or bile pigments may be responsible for initiating nucleation

of a gallstone.

In the same year Vlahcevic and co-workers found a significant decrease in pool size in patients with gallstones (Vlahcevic et al., 1970). They postulated that the abnormal biliary lipid composition, described by Admirand and Small, was the result of reduced bile acid secretion rate which was in turn the result of the smaller bile acid pool (Swell et al., 1971). They made the assumption that cycling frequency was similar in patients with gallstones and normals.

This thesis has tested an alternative hypothesis to explain the reduced bile acid pool size seen in patients with cholesterol gallstones: that the reduced pool size, increased daily fractional turnover and the decreased cholic acid synthesis are the result of increased enterohepatic cycling frequency.

The results of these experiments, in which cycling frequency has been directly controlled, have shown a significant inverse correlation between pool size and cycling frequency. Are these findings valid and can they be extrapolated to human patients with gallstones? The evidence to date suggests that studies of biliary physiology in primates are good indicators of biliary physiology in man. The normal and abnormal enterohepatic circulation of the Rhesus monkey has been

extensively examined by Dowling and co-workers. Their findings of increased cholesterol saturation of bile at low bile acid secretion rates (Dowling et al., 1971) have also been demonstrated in man (Nilsson and Schersten, 1969, Soloway and Schoenfield, 1975). Partial interruption of the entero-hepatic circulation by ileal resection increases bile acid turnover both in man (Austad et al., 1967) and in the monkey (Dowling et al., 1970). Biliary lipid composition is not greatly dissimilar to man though always less saturated with cholesterol. One problem that can disturb physiological studies of the enterohepatic circulation is blockage or dislodging of the bile fistula tube. These problems were minimised by housing the animals in the laboratory, so that they were frequently observed, rather than in a separate animal house. The animals were also examined late each evening minimising the time that they were left unattended. Despite these precautions some studies had to be discarded following loss of bile or abnormal regression analysis of the 14-C Cholic acid decay curve. It might be suggested that the large bile acid pool size found at the cycling frequency of 1/day was artificially high. On the first day of study the intravenously injected 14-C Cholic acid would pass through the liver and be secreted in bile that accumulated in the reservoir without being exposed to the intestine. This would tend to increase the slope of the decay curve and the value of the y intercept. However, the decay curve was

recalculated with the first point omitted and there was no significant difference in the slope or the y intercept. It is therefore felt that this potential source of error has not contributed to the size of the bile acid pool. Another factor which could increase bile acid pool size at a cycling frequency of 1/day is hepatic biosynthesis. The prolonged withholding is effectively a complete interruption of the enterohepatic circulation. Previous studies have shown that the liver responds to such an interruption by increasing hepatic bile acid biosynthesis (Dowling et al., 1970). Increased synthesis begins between 12 and 24 hours after the acute interruption in response to the decreased hepatic bile acid flux. Although this may have contributed to the increased pool size the amount is probably small. Maximum synthetic rate is only reached towards the end of twenty-four hours and the maximum rate will be constant regardless of pool size. Maximum synthesis would be inhibited when the pool is returned and hepatic flux increases. The amount of bile acid synthesised will form a smaller proportion of the enlarged pool than it would of a normal pool. Secondly it is possible that it may take longer for the large pool to be washed out and the low-point of the wash-out curve reached. The onset of increased synthesis may therefore also be delayed and the amount of increased synthesis thus reduced. Furthermore synthetic rates were very similar at 1 and 2 cycles/day. Increased synthesis would contribute no more

than a tiny fraction to the bile acid pool circulating at 2 cycles/day since synthesis does not increase until approximately 12 hours after interruption. It therefore seems that increased synthesis resulting from prolonged withholding of the pool does not contribute more than a small amount to the increased pool size once a steady state has been reached. At the other extreme, it might be argued that when the bile acid pool is cycled at 8 cycles/day the bile in the reservoir is returned to the liver before the pool has completely traversed the intestine. The cycling frequency of 8 cycles/day is well within the normal physiological range. It seems likely that the small pool observed at this frequency could be washed out within the three hour period before bile was returned.

The changes in bile acid kinetics observed in these studies are extremely similar to those observed by Vlahcevic in patients with gallstones (Vlahcevic et al., 1970). These results suggest that their assumption of a constant cycling frequency may have been invalid since cycling frequency was the only variable in the studies and wide changes of pool size were observed. Previous studies have suggested a relationship between pool size and cycling frequency. Following studies in patients with coeliac disease and post-cholecystectomy subjects Low-Beer and Pomare suggested an inverse relationship between pool size and cycling frequency (Low-Beer and Pomare, 1973). Patients with coeliac

disease have a sluggish enterohepatic circulation and their pool sizes were large. The post-cholecystectomy subjects, in whom the pool might be expected to circulate more frequently in the absence of a gallbladder, had small pools. Further support for this view was provided by Hepner who showed that pool size increased when a diet designed to minimise gallbladder contraction was given (Hepner, 1975). Northfield and Hofmann measured biliary lipid outputs and pool sizes in gallstones and controls and demonstrated a correlation between pool size and cycling frequency (Northfield and Hofmann, 1975). An unusual feature of this study was the absence of differences between their gallstone patients and controls. Bile acid pool size and bile acid outputs were not reduced in the patients nor was cholesterol output increased. There is a close similarily to the curvilinear regression between cycling frequency and pool size in the studies before and after vagotomy and the curve described by Hofmann and Northfield in their gallstone patients and controls.

Since these experiments have been completed independent studies in man have confirmed the relationship between pool size and cycling frequency (Shaffer and Small, 1977, Mok et al., 1977). In both these studies the cycling frequency has been calculated from measurements of bile acid outputs and bile acid pool size. Cycling frequency was not controlled in these studies and the perfusion techniques used effectively

exclude the gallbladder from the enterohepatic circulation but did ensure identical bile samples were collected. The inverse correlation between pool size and cycling frequency was demonstrated in a large number of subjects with and without cholesterol gallstone disease. In two other studies the intestinal phase of the enterohepatic circulation was shortened by regular ingestion of Sorbitol (Duane, 1978, Hardison et al., 1979). Since the circulation of bile acids through the portal circulation and liver was rapid the time spent in the intestinal lumen accounted for most of each enterohepatic cycle. Shortening the intestinal transit time should therefore increase cycling frequency. In both these studies bile acid pool size was reduced again indicating an inverse relationship between pool size and cycling frequency. There is no evidence of altered intestinal transit in patients with gallstones and alteration in gallbladder function may therefore be the principle determinant of enterohepatic cycling frequency.

The absence of any significant change in biliary lipid composition with alterations of cycling frequency during this study was striking. Biliary lipid composition is determined by the secretion rates of the three lipids. Phospholipid secretion is closely related to bile acid secretion (Nilsson and Schersten, 1969, Wheeler and King, 1972). Cholesterol secretion is also related to bile acid secretion rates but

decreases proportionately less at low bile acid secretion rates increasing cholesterol saturation of bile (Dowling et al., 1971, Hardison and Apter, 1972). Bile acid secretion is dependent on hepatic biosynthesis and the recirculation of bile acid returning from the enterohepatic circulation. The relative constancy of composition suggests a reasonably constant hepatic bile acid flux, which results from the decreases in pool size compensating for the increased cycling frequency. Although altered enterohepatic cycling frequency can produce the bile acid kinetic abnormalities of cholesterol gallstone disease it does not cause the secretion of hepatic bile supersaturated with cholesterol and, therefore, cannot be the causative defect.

Secretion of such abnormal bile remains the corner stone of our current understanding of cholesterol gallstone disease. However, it is now realised that a number of secretory defects exist, all of which produce abnormal bile. At one extreme are the grossly obese who may have larger than normal bile acid pools but a vastly increased cholesterol secretion (Bennion and Grundy, 1975). Obese patients with gallstones also have increased cholesterol secretion and slightly decreased bile acid secretion (Grundy et al., 1974). American Indian women with gallstones, many of whom are obese, have a mixed defect with increased cholesterol and decreased bile acid secretion (Grundy et al., 1972). Lastly, non-obese Caucasians have normal cholesterol secretion but

decreased bile acid output (Shaffer and Small, 1977). In these experiments, in which cycling frequency was controlled, pool size altered but composition did not. It therefore seems likely that the reduced bile acid pool size seen in patients with gallstones is due to a decreased hepatic bile acid secretion rate rather than altered enterohepatic cycling. In other words, the original assumption by Vlahcevic and co-workers that cycling frequency was the same in their gallstone patients and controls was in fact true (Vlahcevic et al., 1970). However, the small bile acid pool which they observed in their gallstone patients was the result, not the cause, of decreased hepatic bile acid secretion. This has now been confirmed by Shaffer and Small who showed that non-obese patients with gallstones have small bile acid pools, low bile acid secretion rates, and thus low enterohepatic cycling frequency.

The effect of cholecystectomy on biliary lipid composition has been controversial. Several groups have reported improvement in the relative composition of bile following cholecystectomy (Simmons et al., 1972, Shaffer et al., 1972, Kernahan and Danziger, 1975), and studies on subjects after the loss of gallbladder function also showed improvement in composition (Antsaklis et al., 1975, Redinger, 1976). However, others have reported that biliary lipid composition was unchanged (Almond et al., 1973, Adler et al., 1974,

McDougall et al., 1975, Kimball et al., 1976). It seems likely that cholecystectomy, or loss of gallbladder function, will increase the enterohepatic cycling frequency. Normally the gallbladder sequesters a major portion of the bile acid pool during fasting. Studies in the baboon have shown that up to 85% of the pool is in the gallbladder at the end of a 12 hour fasting period (Gardiner and Conaway, 1976). After removal of the gallbladder the bile acid pool will presumably circulate more or less continuously. If the experimental findings in the Rhesus monkey can be extrapolated to man the increase in cycling frequency should cause a further decrease of the already small bile acid pool in patients with gallstones. Biliary lipid composition would presumably be unaffected if the increase in cycling frequency was balanced by the decrease in pool size. Duodenal perfusion studies have now been performed in non-obese Caucasian patients with gallstones before and after cholecystectomy (Shaffer and Small, 1977).

These studies have confirmed that there is an increase in enterohepatic cycling frequency and that a further reduction in pool size occurs, as predicted by the studies in the Rhesus monkey. However, biliary lipid composition was improved because the decrease in pool size was less than the increase in cycling frequency. The net effect was to increase hepatic bile acid secretion rate and, since cholesterol secretion was unchanged, improve relative composition.

These findings support the interpretation of the experimental results that a hepatic defect of bile acid secretion is the principle aetiological factor in cholesterol gallstone disease in non-obese subjects. After cholecystectomy the hepatic defect remains but is masked by the increased entero-hepatic cycling. Biliary lipid composition was virtually unaltered by truncal vagotomy in man and all bile samples remained within the micellar zone described by Admirand and Small. The high concentrations of phospholipids in all the samples in the study of patients was an unexpected finding. Absolute concentrations of bile acids and cholesterol were within the normal range. The presence of the abnormality in all samples suggests a constant abnormality in the analytical process, in which case the effect will have been to shift the data points to the right on the triangular plot. This will not disturb the ratio of cholesterol to bile acid or the relationship of the data points to the curve of maximum cholesterol solubility which is relatively constant for phospholipid concentrations ranging from 7% to 30%.

Although there have been a number of studies in experimental animals there is surprisingly little data regarding biliary lipid composition before and after vagotomy. Smith and co-workers investigated duodenal ulcer patients before and after vagotomy, using the same intubation technique for bile collection as in this study (Smith et al., 1973). There were no significant changes in composition 10 days after

vagotomy. Further samples were collected one year after vagotomy and, although the absolute concentrations of all three lipids had increased, there was no significant alteration in relative composition. More recently Duane and Stempel have examined patients who had undergone vagotomy 2 - 5 years previously and a group of well matched controls (Duane and Stempel, 1977). Relative concentration of cholesterol was lower in the vagotomy group than controls. The absence of any increase in cholesterol saturation of bile in these studies or in the present study suggests that vagotomy does not predispose to cholesterol gallstone disease. Studies in animals have produced conflicting results. Most of the studies have been in the dog, which has extremely low cholesterol saturation of bile and some have used chronic cannulation techniques which may affect the results. Cholesterol saturation was found to be increased in two studies by the same group but in one this was the result of decreased bile acid concentration and in the other was the result of increased cholesterol concentration (Fletcher and Clark, 1969, Cowie and Clark, 1972). These differences were probably due to greatly differing secretion rates in the two studies. Machleder also found an increase in cholesterol concentration (Machleder, 1971) while Amdrup and Griffith found no change in biliary lipid composition (Amdrup and Griffith, 1970). Few firm conclusions can be drawn from the widely differing results of the animal studies.

The bile acid kinetic studies in man demonstrated an increase in bile acid pool size and it is possible that this was due to a decreased enterohepatic cycling frequency. The observed changes suggest that vagotomy does not affect hepatic bile acid synthesis and, indeed, one would not expect it to. Much of the evidence linking vagotomy and gallstone disease was based on the hypothesis that the gallbladder was the cause, as well as the site, of gallstone formation (Nielsen, 1964, Nobles, 1966, Miller, 1968, Clave and Gaspar, 1969). Many other reports related gastric surgery in general to an increased incidence of gallstones (Majoor and Suren, 1947, Griffiths and Holmes, 1964, Lundman et al., 1964, Horwitz and Kirson, 1965, Sapala et al., 1979). While branches of the vagus nerve may be divided during gastrectomy the increased incidence observed in these cases cannot be attributed to the vagotomy alone. It has been suggested that there is an association between duodenal ulcer disease and gallstones (Schreiber, 1968) and in most series the incidence of gallstone disease was higher after Polya than Billroth I gastrectomy. Others have failed to find an association with vagotomy (Bouchier, 1970, Mujahed and Evans, 1971, Engel and Spellberg, 1978) or gastrectomy (Turungen and Antila, 1964, Fletcher and Clark, 1968).

The observed increase in bile acid pool size and decreased enterohepatic cycling frequency could be due to dilatation of the denervated gallbladder. Increased quantities of

bile would be stored in the gallbladder transiently reducing hepatic bile acid flux and increasing synthesis until a new steady state was reached with an enlarged bile acid pool. Dilatation of the gallbladder after vagotomy was first reported over 30 years ago (Johnson and Boyden, 1947) and since then numerous radiographic studies have been performed. Some groups have found no change in gallbladder size (Glanville and Duthie, 1964, Williams and Irvine, 1966, Kramhoft, 1972) but the majority have confirmed Johnson and Boyden's findings both in man (Rudick and Hutchison, 1965, Inberg and Vuorio, 1969, Fagerberg et al., 1970, Parkin and Johnston, 1972) and in the dog (Carter and Sawyer, 1969, Schein and Gliedman, 1970). It is now known that at times normal subjects may secrete a supersaturated bile (Smallwood et al., 1972). An increase in stasis which might occur in a denervated dilated gallbladder might favour cholesterol precipitation at these times. There have been few direct measurements of bile acid pool size. White and co-workers have measured pool size in dogs using similar isotope dilution techniques to this study (White et al., 1974). They also found bile acid pool size to be increased and fractional turnover rate to decrease. Making the same assumptions with regard to absorption that were used in this study, cycling frequency can be calculated from their data. Cycling frequency fell from 9 cycles/day before vagotomy to 3 cycles/day after. Arnesjo and Stahl found taurocholate half-life

decreased after vagotomy but neither cholic acid or total bile acid pools were measured (Arnesjo and Stahl, 1974). More recently Duane and Stempel compared bile acid pool size of post-vagotomy subjects to well-matched controls and have also found that pool size expanded after vagotomy (Duane and Stempel, 1977).

This and other studies in man suggest that increased cholesterol saturation of bile does not occur after truncal vagotomy. It seems possible that the observed increase in bile acid pool size is due to reduced enterohepatic cycling frequency which, in turn, is the result of prolonged or increased sequestration of the bile acid pool in the denervated dilated gallbladder. If vagotomy does predispose subjects to the subsequent development of gallstones it may be related to increased stasis with stratification of gallbladder bile.

C O N C L U S I O N

The chronic bile fistula monkey is a most suitable model for the study of the enterohepatic circulation.

In this preparation serial increases of enterohepatic cycling frequency caused serial decreases of bile acid pool size. With each increase in cycling frequency cholic acid synthesis decreased and fractional turnover increased. Biliary lipid composition was unaffected by any changes in cycling frequency. It is concluded that cycling frequency is an important determinant of bile acid pool size but not of biliary lipid secretion and composition, and it is unlikely that altered cycling frequency has a role in gallstone pathogenesis. Truncal vagotomy did not produce the increase in biliary cholesterol saturation normally associated with cholesterol gallstone disease. Bile acid pool size increased. This may be due to dilatation of the gallbladder after vagotomy.

C O N T R I B U T I O N

T O C L I N I C A L S U R G E R Y

Cholesterol gallstone disease and its complications account for a large part of general surgical practice today. Cholecystectomy is now the commonest reason for laparotomy and the number of such operations performed exceeds 0.5 million in the United States alone. A further increase may be anticipated as a result of the lithogenic effects of oestrogens used in the manufacture of oral contraceptive tablets. Operative surgery remains the most effective treatment of the disease at present and alternative modalities such as oral bile acids are unlikely to prove suitable for widespread use.

It was the aim of this thesis, therefore, to attempt to contribute to our understanding of the physiology of the enterohepatic circulation with particular reference to enterohepatic cycling frequency. Prevention of cholesterol gallstone disease cannot take place until the mechanisms which cause secretion of abnormal bile are understood.

Truncal vagotomy and pyloroplasty has been widely used in the treatment of duodenal ulcer disease. Its often-stated aetiological role in gallstone formation has been based largely on retrospective studies. It was thought that this thesis, primarily involved with the enterohepatic circulation, might also contribute to our understanding of this problem. Vagotomy does not appear to produce the biochemical abnormalities associated with cholesterol gallstone disease.

S U M M A R Y

Because of the limitations of investigating the enterohepatic circulation in man, due to the inaccessibility of the biliary tree, the chronic bile fistula monkey was used to study the effects of altered enterohepatic cycling.

In these experiments, in which cycling frequency was carefully controlled, it was found that there was a significant inverse correlation between cycling frequency and bile acid pool size. It was thought likely that the increased hepatic bile acid flux induced by an increase in cycling frequency reduced hepatic synthesis until a new steady state, with a smaller bile acid pool, was reached. As the new steady state was achieved hepatic bile acid flux declined though not quite to its original level. These small increases in hepatic bile acid flux, acting via the negative feedback mechanism, produced small decreases in cholic acid synthetic rate with each increase in cycling frequency. Fractional turnover rate increased with increasing enterohepatic cycling frequency since, although daily faecal loss decreased, the amount lost represented greater proportions of the contracted bile acid pool.

The absence of any changes in biliary lipid composition with alterations in enterohepatic cycling frequency supports the view that the principle aetiological factor in cholesterol gallstone disease is decreased hepatic bile acid synthesis

leading to decreased bile acid secretion and the formation of bile supersaturated with cholesterol. The findings challenge the view that the reduced bile acid secretion is the result of a reduced bile acid pool size.

Truncal vagotomy in man did not change the biliary lipid composition significantly. In the bile acid kinetic studies after vagotomy pool size increased and calculated cycling frequency decreased. These findings are comparable with dilatation of the denervated gallbladder.

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A P P E N D I X I

Detailed Laboratory and Analytical Methods

B I L E A C I D A N A L Y S I S

(Talalay, P. 1960, Iwata and Yamasaki, 1964, Admirand and Small, 1968)

I REAGENTS

1. Hydroxysteroid Dehydrogenase

Pseudomonas testosteroni (Worthington Biochemical Corporation, Freehold, New Jersey). Acetone-dried cells stored frozen and fresh solution made daily.

5 mg cells and 10 ml of cold 0.03 M Tris-HCl Buffer (pH 7.2) containing 0.001 M EDTA per litre are ground in a Potter-Evelyheim tissue grinder immersed in ice.

The resulting suspension is transferred to centrifuge tubes and centrifuged at 20,000 rpm, 5°C, for 20 minutes.

The supernatant, kept on ice, is hydroxysteroid dehydrogenase solution.

2. Nicatinamide Adenine Dinucleotide (NAD)

A 5.6 mM solution of NAD is required. 41 mg of B NAD (Sigma Chemical Company, St. Louis, Missouri) is dissolved in 6 - 7 ml of cold distilled water.

Adjust pH to 7 using saturated sodium bicarbonate.

Dilute to 10 ml with distilled water.

3. Hydrazine Hydrate

A 1 M hydrazine hydrate solution (pH 9.5) is required.

12.5 ml hydrazine hydrate (Matheson, Coleman and Bell, Chicago, Illinois) is added to 20 ml of cold distilled water.

3.75 ml of 2 N sulphuric acid is added to the mixture and diluted to 250 ml.

4. Buffer Solution

O.I.M. glycine is titrated to pH 9.5 with sodium hydroxide. 250 ml solution made up.

II STANDARDS

Pure bile acids (Calbiochem, La Jolla, California), recrystallized and free from impurities on thin layer chromatography are dissolved in methanol. 1.96 mg bile acid are dissolved in 10 ml methanol to make a 4.8 mM solution. Standards of 2.4 mM, 1.2 mM and 0.6 mM are produced by serial dilution.

III PREPARATION OF SAMPLE

0.1 ml of whole bile is diluted with 2.0 ml of methanol. This dilution may be altered for very dilute bile samples. Duplicate all samples.

IV DETERMINATION

1. Prepare a reaction mixture with 0.5 ml distilled water, 1.0 ml glycine buffer, 1.0 ml hydrazine hydrate solution, 0.3 ml NAD solution and 0.1 ml of either standard bile acid solution, methanolic bile sample or, in the case of the blank, methanol.

2. 0.1 ml of the hydroxysteroid dehydrogenase enzyme solution was added to each of the standards, the unknowns and the blank.

3. The blanks are transferred to quartz cuvettes (4 ml. capacity, 10 mm light path) and the optical density at a wavelength of 340 mμ determined to correct the spectrophotometer for background.

4. The contents of each of the standards and unknowns are transferred to quartz cuvettes and the optical density determined 90 minutes after the addition of the enzyme solution.

5. A standard curve is constructed by plotting the optical density of the four standards against their concentration in millimoles per litre. The concentrations of the unknowns are derived from the standard curve and multiplied by a factor of 21 to correct for the initial dilution.

C H O L E S T E R O L A N A L Y S I S

(Schoenheimer and Sperry, 1934, Sperry and Webb, 1950)

I REAGENTS

1. Lieberman-Burchard Reagent

250 ml of acetic anhydride are mixed with an equal volume of glacial acetic acid and stirred well.

250 ml of this mixture are placed in a 500 ml volumetric flask which is then packed in ice.

41 ml of concentrated sulphuric acid are then slowly added to the flask and mixed well.

Bring to room temperature.

Add more of the acetic anhydride/glacial acetic acid mixture to bring the volume up to 500 ml.

2. Digitonin Solution

Digitonin is hygroscopic and must be stored frozen with tape-sealed screw cap.

1 g digitonin (Nutritional Biochemicals Corporation, Cleveland, Ohio) is dissolved in 100 ml of methanol.

II STANDARDS

Dissolve 387 mg of pure cholesterol (Calbiochem, La Jolla, California) in 50 ml of glacial acid, warming gently while mixing.

Transfer the solution to a 100 ml volumetric flask and dilute to 100 ml with glacial acetic acid to make a 10 mM solution. Standards of 5 mM, 2.5 mM and 1.25 mM are produced by serial dilution.

III PREPARATION OF SAMPLE

Whole bile is used in this assay. Depending on the amount available 0.5 ml or 0.2 ml of bile may be used. If 0.2 ml are used 4.8 ml of isopropyl alcohol should be used in the first step of the determination below and not 4.5 ml.

Duplicate all samples.

IV DETERMINATION

PART 1

1. Place 4.5 ml of isopropyl alcohol into a screw-capped test tube and close cap.

2. Open caps, one at a time, and add 0.5 ml of bile sample and close caps tightly.
3. Vortex for 30 seconds.
4. Wait 5 minutes and vortex again.
5. Centrifuge at 1,500 rpm at room temperature for 10 minutes.
6. Pipette 3 ml of supernatant into conical centrifuge tubes.
7. Add 1.5 ml of the 1% digitonin solution.
8. Add 1.5 ml of distilled water.
9. Vortex for 5 seconds, close tubes with a cork and allow to stand overnight at -4°C.

PART 2

1. Centrifuge at 2,500 rpm at room temperature for 10 minutes. The precipitated cholesterol should be packed at the bottom of the tube. If supernatant is not clear repeat centrifuging.
2. Pour off supernatant, invert and allow to dry for approximately 1 hour.
3. Add 0.1 ml of glacial acetic acid to each sample, but not to the standards or blanks.
4. Add 3 ml of the Lieberman-Burchard reagent to each tube, one sample at a time, and vortex cholesterol clump immediately.
5. When the reagent has been added to all tubes, vortex all the tubes.
6. Incubate in a 37°C waterbath for 20 minutes.

7. Remove from the waterbath and place tubes in ice.

8. The blanks are transferred to quartz cuvettes (4 ml. capacity, 10 mm light path) and the optical density at a wavelength of 625 mμ determined to correct the spectrophotometer for background.

9. The contents of each of the standards and unknowns are transferred to quartz cuvettes and the optical density determined.

10. A standard curve is constructed by plotting the optical density of the four standards against their concentration in millimoles per litre. The concentrations of the unknown bile samples are derived from the standard curve.

P H O S P H O L I P I D A N A L Y S I S

(Fiske and Subbarow, 1925)

I REAGENTS

1. Fiske-Subbarow Reducing Agent

Dissolve 30 g of sodium bisulphite in water to a final volume of 200 ml. Mix until solution is clear.

Add 500 mg of 1-amino-2-naphthol-1-sulphonic acid (Sigma Chemical Co., St. Louis, Missouri). The solution will become milky in appearance.

Add 10 ml of anhydrous sodium sulphite. The solution will clear, with mixing, after 5 - 10 minutes.

Filter through No. 40 Whatman filter paper and store in a brown bottle.

This solution should be used within 10 days of making and should not be mouth-pipetted.

2. Ammonium Molybdate

A 0.22% solution is prepared by dissolving 2.2 g of ammonium molybdate in one litre of distilled water.

3. Sulphuric Acid 10 N

4. Sodium Phosphate Tribasic

5. Doubly Distilled Water

II STANDARDS

38 mg of Tribasic sodium phosphate (Sigma Chemical Co., St. Louis, Missouri) in 100 ml of distilled water. The concentration of phosphorus in this solution is 1 μM/ml. 0.2 ml, 0.1 ml, 0.05 ml and 0.025 ml quantities are used as standards.

III PREPARATION OF SAMPLES

Dilute bile samples with doubly distilled water. Usual dilution is 1:20 but concentrated bile samples may need to be diluted to 1:100. Cork with new corks and vortex.

Duplicate all samples.

IV DETERMINATION

All glassware used in this assay must be washed in N/100 hydrochloric or nitric acid and must not be used for any other assay.

1. Pipette 0.1 ml of the diluted bile sample into a Folin

'blood sugar' tube and incubate at 100°C until dry. Include the standards in this process.

2. To the dried specimen add 0.5 ml of 10 N sulphuric acid and mix on vortex until the specimen is dissolved. Place 0.5 ml of 10 N sulphuric acid in an additional tube as a blank to be carried through the remainder of the procedure.
3. Place all tubes in a 160°C sand bath under a clean phosphorus-free fume hood for 3 hours. Temperature is checked with a thermometer in 12 ml of concentrated sulphuric acid.
4. Add two drops of hydrogen peroxide to each tube and replace in sandbath for a further 1 - 1½ hours. The reaction is complete when the solution is colourless.

Ensure all the remaining steps can be completed before proceeding further.

5. Add 4.6 ml of the 0.22 ammonium molybdate solution to each tube.
6. Add 0.2 ml of the Fiske-Subbarow reducing agent to each tube.
7. Vortex all samples.
8. Cover each tube with a glass tear or marble, heat in a boiling waterbath for 8 minutes, and then allow to cool for 20 minutes. A blue colour develops.
9. The blanks are transferred to quartz cuvettes (4 ml

capacity, 10 mm light path) and the optical density at a wavelength of 830 mμ determined to correct the spectrophotometer for background.

10. The contents of each of the standards and unknowns are transferred to quartz cuvettes and the optical density determined.

11. A standard curve is constructed by plotting the optical density of the four standards against their concentration in micromoles per 0.1 ml. The concentration of phosphorus in the samples is derived from the standard curve and multiplied by 20 to correct for the initial dilution. Since only 4% of the phospholipid molecule is phosphorus, the concentration of phosphorus must be multiplied by 25 to obtain the concentration of phospholipid.

B I L E A C I D

S P E C I F I C A C T I V I T Y

(Vlahcevic et al., 1970, Hofmann et al., 1970)

I REAGENTS

1. Moving Phase for Thin Layer Chromatography

Add 50 ml of ethyl acetate and 10 ml of glacial acetic acid to 50 ml of iso-octane.

2. Liquid Scintillation Cocktail

In this study Aquasol (New England Nuclear, Boston, Massachusetts) was used.

II STANDARDS

A taurocholate standard is run through the deconjugation and chromatographic processes to ensure completeness of deconjugation.

A cholic acid standard (Calbiochem, La Jolla, California) is used during the chromatographic process to identify the spots.

III METHOD

1. 0.1 ml of whole bile is added to 2 ml of 2 N sodium hydroxide in a screw-topped 15 ml round-bottomed test tube and capped tightly.
2. Autoclave for 3 hours at 2 atmospheres pressure.
3. Extract cholesterol with three volumes, 6 ccs, of ether three times pooling the ether extracts.
4. Backwash the ether extracts with alkaline water (0.001 N sodium hydroxide) and add to the original sample.
5. Acidify to pH1 with concentrated hydrochloric acid.
6. Extract the bile acids with three volumes of ether three times, pooling the extracts.
7. Evaporate to dryness.
8. Add 0.1 ml of methanol, vortex thoroughly and spot across a silica gel thin layer chromatographic plate. Add further 0.1 ml quantities of methanol and overspot across the plate, five times in all.
9. Run the thin layer plate, identify the cholic acid spots with iodine vapour and scrape off into a centrifuge tube.
10. Add 1 cc methanol and centrifuge. Add further 1 cc quantities of methanol, centrifuge and pool the supernatants, four times in all.
11. Evaporate to dryness and take up bile in a measured amount (1 ml) of methanol for liquid scintillation counting or bile acid analysis.

12. For bile acid analysis see previous section.

For liquid scintillation counting 0.1 ml of methanolic bile solution is placed in a clean dry counting vial. Allow the methanol to evaporate and add 10 cc of aquasol and count in a liquid scintillation spectrometer.

A P P E N D I X I I

Data derived by linear regression analysis of 14-C Cholic Acid decay curves

B I L E A C I D K I N E T I C S A T 1 C Y C L E / D A Y

Monkey	14-C Cholic Acid Administered dpm x 10^{-6}	Specific Activity to dpm x 10^{-6}	Fractional Turnover Rate $days^{-1}$	Cholic Acid Pool mM	Cholic Acid Synthesis mM/day	%Bile Acids as Cholic Acid	Bile Acid Pool mM	r
1	26.07	11.71	0.236	2.226	0.525	53.4	4.168	0.96
1	20.47	17.69	0.250	1.157	0.299	33.1	3.496	0.97
2	26.07	28.56	0.332	0.913	0.303	33.8	2.701	0.95
3	26.07	9.79	0.212	2.663	0.564	61.7	4.316	0.97

BILE ACID KINETICS AT 2 CYCLES/DAY

Monkey	14-C Cholic Acid Administered dpm x 10^{-6}	Specific Activity to dpm x 10^{-6}	Fractional Turnover Rate $days^{-1}$	Cholic Acid Pool mM	Cholic Acid Synthesis mM/day	%Bile Acids as Cholic Acid	Bile Acid Pool mM	r
1	20.47	14.58	0.310	1.404	0.435	60.2	2.332	0.97
2	18.10	20.98	0.389	0.863	0.336	44.8	1.925	0.99
2	18.10	19.03	0.430	0.951	0.409	43.0	2.212	0.95
3	18.10	13.23	0.330	1.368	0.452	53.0	2.581	0.95

* Specific activities corrected for residual counts from previous study.

B I L E A C I D K I N E T I C S A T 4 C Y C L E S / D A Y

Monkey	14-C Cholic Acid Administered dmp x 10^{-6}	Specific Activity to dmp x 10^{-6}	Fractional Turnover Rate $days^{-1}$	Cholic Acid Pool mM	Cholic Acid Synthesis mM/day	%Bile Acids as Cholic Acid	Bile Acid Pool mM	r
1	20.47	28.27	0.380	0.724	0.275	46.8	1.547	0.99
2	20.47	51.19	0.562	0.399	0.225	26.8	1.492	0.96
2	20.47	39.16	0.431	0.523	0.225	40.2	1.300	0.99
3	20.47	34.68	0.377	0.590	0.223	33.1	1.783	0.99
3	20.47	38.15	0.435	0.564	0.245	42.6	1.323	0.95

* specific activities corrected for residual counts from previous study

B I L E A C I D K I N E T I C S A T 8 C Y C L E S / D A Y

Monkey	14-C Cholic Acid Administered dpm x 10^{-6}	Specific Activity to dpm x 10^{-6}	Fractional Turnover Rate $days^{-1}$	Cholic Acid Pool mM	Cholic Acid Synthesis mM/day	%Bile Acids as Cholic Acid	Bile Acid Pool mM	r
1	18.41	61.78	0.510	0.298	0.152	35.5	0.839	0.99
2	18.41	49.10	0.505	0.375	0.189	37.7	0.995	0.95
2	18.41	45.85	0.462	0.402	0.186	51.7	0.777	0.98
3	18.41	49.39	0.534	0.373	0.199	45.9	0.812	0.97
3	18.41	39.20	0.346	0.470	0.163	57.4	0.818	0.95

* specific activities corrected for residual counts from previous studies

B I L E A C I D K I N E T I C S W I T H C O N T I N U O U S C Y C L I N G

Monkey	14-C Cholic Acid Administered dpm x 10^{-6}	Specific Activity to dpm x 10^{-6}	Fractional Turnover Rate $days^{-1}$	Cholic Acid Pool mM	Cholic Acid Synthesis mM/day	%Bile Acids as Cholic Acid	Bile Acid Pool mM	r
1	18.41	53.83	0.470	0.342	0.162	39.5	0.866	0.95
2	18.41	35.54	0.443	0.518	0.229	46.2	1.124	0.98
3	18.41	31.26	0.311	0.589	0.185	52.7	1.118	0.99

* specific activities corrected for residual counts from previous studies

B I L E A C I D K I N E T I C S B E F O R E V A G O T O M Y I N M A N

Patient	14-C Cholic Acid Administered dpm x 10^{-6}	Specific Activity to dpm x 10^{-6}	Fractional Turnover Rate $days^{-1}$	Cholic Acid Pool mM	Cholic Acid Synthesis mM/day	%Bile Acids as Cholic Acid	Bile Acid Pool mM	r
R.D.	51.4	40.15	0.373	1.281	0.478	31.16	4.111	0.95
J.P.	51.4	39.29	0.480	1.309	0.629	41.01	3.192	0.98
G.G.	51.4	23.72	0.246	2.169	0.534	38.42	5.645	0.98
J.R.	51.4	26.86	0.232	1.915	0.445	34.86	5.493	0.99

B I L E A C I D K I N E T I C S A F T E R V A G O T O M Y I N M A N

Patient	14-C Cholic Acid Administered dpm x 10^{-6}	Specific Activity to dpm x 10^{-6}	Fractional Turnover Rate $days^{-1}$	Cholic Acid Pool mM	Cholic Acid Synthesis mM/day	%Bile Acids as Cholic Acid	Bile Acid Pool mM	r
R.D.	51.4	10.35	0.149	4.973	0.740	35.51	14.001	0.97
J.P.	51.4	14.91	0.099	3.451	0.342	30.94	11.154	0.99
G.G.	51.4	22.56	0.161	2.281	0.368	30.38	7.508	0.96
J.R.	51.4	27.18	0.303	1.8925	0.574	49.55	3.824	0.96

A P P E N D I X I I I

Ethical Approvals

A protocol for the series of animal experiments was submitted to the ethical committee of the University of California, San Francisco, and their approval obtained prior to the start of the experiments.

A protocol for the studies in humans was submitted to the ethical committee of the Norfolk and Norwich Hospital and their approval obtained prior to the start of the studies.

In view of the use of radio-isotopes in the study, approval was also sought, and obtained, from the Isotope Advisory Panel, Department of Health and Social Security, London.