INTRODUCTION

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After many years of neglect, disorders of the biliary tract are now exciting clinical and laboratory interest. Clinical interest is due to at least two factors: the increased awareness of gallstones as a major health hazard (Ingelfinger, 1968) and the widespread realisation that gallstones are increasing in frequency both in developed nations and in those which are rapidly developing (Heaton, 1973). Laboratory interest has arisen because the aetiology of cholesterol gallstone formation and gallbladder disease are still confused by conflicting data despite, or due to, the development of sophisticated methodology and model systems.

Bile, an aqueous medium, is the major excretory route for cholesterol, which is insoluble in water. This insoluble sterol is maintained in solution because of the formation of mixed micelles comprising cholesterol, phospholipids and bile salts (Bouchier, 1981). Whether a bile will contain insoluble cholesterol is determined by the relative molar concentrations of bile salts and lecithin (phosphatidylcholine). If the proportion of these two cholesterol solubilising agents falls below a critical level, cholesterol will precipitate from bile or may be present in supersaturated solution. This concept that bile supersaturated with cholesterol (lithogenic bile)

has the potential for gallstone formation has dominated gallstone research since the initial model was proposed by Admirand and Small (1968). These authors defined the upper limit of cholesterol solubility in artificial solutions of bile acids and lecithin under standard conditions of temperature, pH and biliary solid content. Bile from patients with cholesterol gallstones was saturated with cholesterol and fell on or above the line of maximum cholesterol solubility and these authors concluded that saturated bile is a prerequisite for cholesterol stone formation.

This conclusion was supported by an earlier report (Shioda, 1965) who found that saturated bile precedes gallstone formation in experimental hamsters. In gallstone patients, both gallbladder bile and hepatic bile contain excess cholesterol in relation to bile acids and lecithin (Small and Rapo, 1970), while bile supersaturated with cholesterol has been found in young American Indian women who are at a high risk for gallstone formation (Thistle and Schoenfield, 1971). However, supersaturation of bile with cholesterol cannot be the sole factor as Nakayama and van der Linden (1970) and Dam and Hegardt (1971) were unable to distinguish between patients with cholesterol gallstones and health subjects on the basis of bile composition. The problem was further complicated as Danzinger et al (1972) identified patients with cholesterol gallstones and bile which falls within the

normal zone of cholesterol solubilisation and Holzbach et al (1973) demonstrated supersaturated bile in normal subjects, supporting the view that cholesterol supersaturation alone does not adequately explain cholesterol stone formation.

Advances in techniques of studying bile acid metabolism in man have enabled bile acid pool size to be measured using ^{14}C-cholic and ^{14}C-chenodeoxycholic acid. Vlahcevic et al (1970, 1972) studied patients with gallstones and found the bile acid pool size is reduced, suggesting that the lithogenicity of bile may also reflect the reduced size of the circulating bile salt pool. Swell et al (1971) have suggested that there is a critical size of the bile acid pool and a reduction below this size will cause the bile to be lithogenic. Similar observations of reduced bile salt pool size in cholesterol gallstone patients have been made by other investigators (Bell et al, 1972; Pomare and Heaton, 1973) although Mok et al (1980) found no significant difference between patients with and without gallstones. The importance of pool size is also unclear as treatment with cholic acid expands the depleted bile acid pool (La Russo et al, 1975) but neither improves cholesterol solubility in bile (Mok, 1974) nor dissolves gallstones (La Russo et al, 1975). These conflicting results may reflect differences in patient selection as non-obese gallstone patients have a reduced bile salt pool size while in obese patients

the pool size remains constant but cholesterol secretion is increased (Mabee et al, 1976; Shaffer and Small, 1977) or may indicate a biochemical difference between the two primary bile acids.

Other studies showed that without a concomitant increase in hepatic cholesterol synthesis and secretion, a reduced bile acid pool may not be sufficient for the formation of saturated bile. Schwartz et al (1979) demonstrated a marked diminution of bile acid pool size in patients with advanced cirrhosis, but because of an associated decreased cholesterol secretion, the bile of these patients is not saturated with cholesterol. This compensation for the diminished bile acid pool size is not present in patients with gallstones. Instead the biliary cholesterol secretion rate in bile is increased in both Indian (Grundy et al, 1972a) and Caucasian women (Grundy et al, 1972b) with gallstone disease. This may be explained by study of hepatic cholesterol and bile acid synthesis. Hepatic-3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), the rate limiting enzyme of hepatic cholesterol biosynthesis, is activated while cholesterol 7α-hydroxylase, the rate limiting enzyme of bile acid synthesis, is inhibited in gallstone patients, suggesting the pathogenesis of gallstones is related to both increased cholesterol synthesis and decreased bile acid formation (Nicolau et al, 1974; Salen et al, 1975; Coyne et al, 1976). In

both gallstone patients (Coyne et al, 1976) and hamsters with experimental cholelithiasis (Schoenfield et al, 1973) HMG-CoA reductase activity and biliary cholesterol secretion (Adler et al, 1975; La Russo et al, 1975) are decreased during chenic acid therapy.

In contrast, other reports indicate a dissociation between hepatic HMG-CoA reductase activity and biliary cholesterol synthesis. In patients with T-tubes, intestinal infusion of chenodeoxycholate (Lindblad et al, 1977) or ursodeoxycholic acid (Schersten and Lindblad, 1979) caused bile to become unsaturated within four hours, a change too rapid to be mediated by change in HMG-CoA reductase activity. Furthermore, the incorporation of acetate into cholesterol (Nervi et al, 1981) demonstrated that hepatic cholesterogenesis is not related to hypersecretion of biliary cholesterol. In addition, it has been demonstrated that lipoproteins transport cholesterol to the liver (Nervi and Dietschy, 1975; Andersen et al, 1977), which led to the recognition that most bile cholesterol originates from plasma lipoproteins, particularly high density lipoprotein fraction (Schwartz et al, 1978a, 1978b).

However, the hepatocyte may not be the sole factor determining bile composition as early reports demonstrated improved bile lithogenicity after cholecystectomy. Hepatic T-tube bile was found to be less saturated with cholesterol than hepatic bile obtained

before surgery (Shaffer et al, 1972), a finding confirmed by others (Simmons et al, 1972; Nahrwold and Rose, 1976). On the other hand, other investigators (Almond et al, 1973; Adler et al, 1974; Kimball et al, 1976) were unable to demonstrate any improvement in biliary lipid composition after cholecystectomy. Again, these conflicting results may reflect differences in patient selection as non-obese patients show improvement after cholecystectomy while obese patients with cholesterol hypersecretion do not (Shaffer and Small, 1977). If cholecystectomy leads to improvement of bile lipid composition in non-obese patients then it is possible that the gallbladder may play a role in the pathogenesis of gallstones.

The diurnal cycle of feeding and fasting in subjects with and without gallstones is associated with saturated hepatic and gallbladder bile during fasting in all subjects (Metzger et al, 1973). Similar observations have been made on rhesus monkeys by Redinger et al (1973). Bile saturation was attributed to sequestration of bile salts in the gallbladder during fasting which interrupts the enterohepatic circulation and causes a reduction in hepatic secretion rates of bile acids (Redinger et al, 1973). As biliary cholesterol output is not always coupled to bile acid secretion, particularly at lower rates, an interruption of the enterohepatic circulation could lead to increased saturation of bile (Thureborn, 1962; Mok et al, 1978).

Evidence has been presented that the reduced bile acid pool size in patients with gallstones is simply the result of increased frequency of the enterohepatic circulation in these patients (Northfield and Hofmann, 1973, 1975) caused by impaired gallbladder storage or enhanced gallbladder emptying (Northfield and Hofmann, 1973).

Cholesterol cholelithiasis has been more common among women than men in every population examined (Nakayama and Miyake, 1970; Holland and Heaton, 1972), a difference believed to be increased by pregnancy (Friedman et al, 1966). Oral contraceptives in women in the child-bearing age (Boston Collaborative Drug Surveillance Programme, 1973) and oestrogen in the post menopausal women (Boston Collaborative Drug Surveillance Programme, 1974) have been shown to increase significantly the incidence of surgically confirmed gallbladder disease in comparison with matched control populations. Furthermore, a recent analysis in males found that gallstone formation is a risk whenever oestrogen is prescribed for coronary artery disease (Coronary Drug Project Research Group, 1977). While there is evidence, then, that sex hormones may play a pathogenic role, the mechanism remains obscure. Riegel et al (1935) found that cholesterol was increased and bile salts decreased in gallbladder bile obtained from 34 patients undergoing Caesarean section. Bennion et al (1976) demonstrated

increased cholesterol saturation in women taking oral contraceptives, a change reversed when the oral contraceptives were discontinued. Studies in the pregnant baboon by McSherry et al (1977) found decreased bile acid synthesis and bile acid pool size, but there was also a decreased cholesterol concentration in bile, with a net result that the lithogenic index was decreased in the third trimester of pregnancy, when oestrogen levels are maximal.

While the association of oestrogen with an increased prevalence of gallstones may be the result of a metabolic alteration, other explanations are feasible. One might also consider the possibility that sex hormones affect bile composition through alterations in gallbladder motility. Sex steroids have been shown to alter motility in a wide variety of smooth muscle preparations besides uterus (Daniel, 1960) and may therefore affect the gallbladder. Potter (1936), in 309 Caesarean sections, found 75% of patients to have larger, atonic, distended gallbladders, an observation in agreement with cholecystographic studies which have suggested impaired gallbladder emptying and concentrating ability in late pregnancy (Levyn et al, 1933; Gerdes and Boyden, 1938). These observations were supported by a recent study using real time ultrasonography (Braverman et al, 1980) which showed incomplete emptying of the gallbladder in late pregnancy. This, of course, might

cause retention of cholesterol crystals and subsequent stone formation.

Thus, some reports indicate that sex steroids may play an aetiological role through a metabolic mechanism; others suggest that the effects of sex hormones on gallbladder motility may play a role.

A role of the gallbladder in stone formation is supported by the effects of vagotomy. Thirty years ago, truncal vagotomy was reported to increase the resting volume of the human gallbladder (Johnson and Boyden, 1952), a finding confirmed later (Inberg and Vuorio, 1969), while disturbed emptying of the gallbladder has also been observed after vagotomy (Rudick and Hutchinson, 1965). Furthermore, while human gallstones placed in a canine gallbladder usually dissolve promptly, some studies have shown that truncal vagotomy significantly retards this dissolution (Barnett and Hilburn, 1966). Since then truncal vagotomy has been associated with an increased prevalence of gallstones (Clave and Gaspar, 1969; Tompkins et al, 1972) or increased bile lithogenicity (Cowie and Clark, 1972). However, it is not certain that vagotomy leads to deterioration in bile lipid composition as Mujahed and Evans (1971) found no correlation between vagotomy and the incidence of gallstones. This is in accordance with Stempel and Duane (1978) who demonstrated an increased bile acid pool size and improvement of biliary lipid composition after vagotomy and concluded that any

increased incidence of gallstones after vagotomy must be due to mechanisms not presently appreciated.

These conflicting reports indicate that cholesterol solubility in bile cannot be the sole factor determining cholesterol gallstone formation and consequently the micellar theory must be expanded.

Additional factors have been proposed. Holan et al (1979) confirmed that current saturation indices cannot discriminate between bile without stones and bile with cholesterol gallstones. Moreover, measuring the nucleation time (the time required for formation of cholesterol crystals) these authors showed a large difference in stone patients, who formed crystals much quicker than controls. In controls, there was a correlation between saturation of bile and nucleation time, but in patients with stones the saturation characteristics of bile showed no correlation with the formation of crystals, a finding strongly suggesting that factors other than cholesterol saturation are involved in cholesterol stone formation.

Gallbladder bile of gallstone patients contains a high level of glycoproteins (Bouchier et al, 1965) and experimental models of lithogenesis are associated with an excess of glycoproteins in bile or biliary tract tissues (Bouchier, 1971a). Furthermore, in those animal models where it has been possible to study the composition of the bile during the development of gallstones it appears that an increased secretion of

glycoproteins precedes gallstone formation (Freston et al, 1969; Womack, 1971). The origin of the biliary glycoprotein was attributed to budding of the liver plasma cell membrane during the process of bile formation (Bouchier, 1971b). The most interesting subsequent study (Wahlin et al, 1974) demonstrated that mouse gallbladder epithelium discharged glycoproteins in the presence of olive oil in the stomach. It was subsequently found that a basal secretion of glycoprotein takes place irrespective of the nutritional state, but fasting diminished and refeeding increased the population of glycoprotein granules (Wahlin et al, 1976a). Moreover, cholecystokinin-pancreozymin (Wahlin et al, 1976b) and cholinergic drugs (Axelsson et al, 1979) stimulate glycoprotein secretion while feeding a lithogenic diet to mice was associated with increased glycoprotein secretion by the gallbladder epithelium, suggesting that increased mucous secretion preceded and accompanied gallstone formation (Wahlin, 1976). This is in accordance with the previous observation of Freston et al (1969) and Womack (1971), although the precise role of the glycoproteins in lithogenesis remains uncertain.

Direct evidence that the gallbladder may influence biliary lipid composition was reported by Niederhiser et al (1976) who found that significant quantities of cholesterol were absorbed by the guinea pig gallbladder.

This absorbed cholesterol can be converted to cholesterol ester in the gallbladder wall and these authors concluded that absorption of cholesterol may be a protective mechanism which prevents precipitation of cholesterol. Absorption of cholesterol might be relevant to cholesterolosis since, in this disorder, large quantities of cholesterol esters accumulate in the gallbladder wall (Boyd, 1923; Rains, 1964). An equally interesting recent study showed that the mucosa of human gallbladder synthesises effectively both cholesterol and triglycerides (Tilvis et al, 1982). The significance of lipid absorption and metabolism for cholesterol stone formation is not clear and further studies are required to investigate this question.

Lipid absorption may be important for the 25% of gallstone patients at risk of acute inflammatory reaction (acute cholecystitis) (Sjödahl et al, 1978). Most authors agree that acute cholecystitis is aseptic in its initial stage (Gottfries, 1968; Sjödahl et al, 1978) and a chemical factor may be implicated (Sjödahl and Wetterfors, 1974). Among the chemical agents considered as possible mediators of the inflammatory reaction is lysolecithin (lysophosphatidylcholine) (Niederhiser et al, 1973; Sjödahl et al, 1975; 1978; Tagesson et al, 1978) as lysolecithin instilled into the guinea pig gallbladder was associated with severe histological damage (Niederhiser et al, 1973) and an increased proportion of this phospholipid is present in the bile

of patients with acute cholecystitis (Sjödahl and Wetterfors, 1974). The exact source of lysolecithin in bile is not known. Lysolecithin is a hydrolytic product of lecithin formed through the action of phospholipase A (Niederhiser et al, 1973). Where this enzymatic conversion occurs is also not known. One hypothesis is that pancreatic secretions which contain the enzyme phospholipase A (Vogel and Zieve, 1960) regurgitate into the gallbladder, although Tagesson et al (1978) demonstrated phospholipase A_2 activity in human gallbladder mucosa and postulated that this is the source of lysolecithin in bile. Niederhiser and Harmon (1978) failed to demonstrate the presence of this enzyme in animal and human gallbladder mucosa.

These studies are complicated by the difficulties of developing a suitable model and consequently development of a culture technique for gallbladder mucosa appears a logical development in such studies. This, if successful, would provide a model system, allowing precise control of experimental conditions to study such factors as lipid biochemistry, hormone effect and metabolic activity. It is towards this end this project was designed.

The morphology of cultured tissues is easily studied by light and electron microscopy. Cellular activity <u>in vitro</u> can be directly observed and recorded on film. However, tissue cultures do have disadvantages. No matter how much attention is paid to experimental

technique, the tissue is still exposed to an unnatural environment. In particular, the tissue loses its vascular network and nerve supply which may have effects leading to misinterpretation of results if experimental work is inadequately controlled.

There are three main culture methods, known as tissue culture, organ culture and cell culture. In both tissue and organ culture very small fragments of tissue are placed in medium and allowed to develop, but in organ culture special measures are taken to prevent the tissue from becoming disorganised. In tissue culture no such precautions are taken and cells begin to migrate from the tissue fragment which soon becomes disorganised (Paul, 1975). In cell culture, the tissue is intentionally disorganised at the beginning by disrupting it into individual cells (Paul, 1975) which are then allowed to grow as a monolayer on treated plastic or as a cell suspension (Reid and Rojkind, 1979).

The brief report by Hosono in 1935, describing the cultivation of guinea pig gallbladder epithelium in blood plasma or Ringer's solution, and two recent reports (Koyama et al, 1980; Morgan et al, 1981) describing the development of two cell lines from human gallbladder carcinoma, constitute the reported experience of culture of normal or diseased gallbladder cells. Therefore, studies of intestinal epithelial cultures were used as a basis for gallbladder culture

because the gallbladder is embryologically related to the intestinal tract (Tilvis et al, 1982). Although intestinal epithelial cells can be isolated readily by a number of established techniques, their survival time *in vitro* is limited (2-3 hours, Trier, 1980) and attempts to establish short- and/or long-term cultures met with complete lack of success (Quaroni and May, 1980). Similarly, organ cultures of small intestinal mucosa from human and experimental animals preserve viable tissue for 24-48 hours (Browning and Trier, 1969; Ferland and Hugon, 1979; Trier, 1980). On the other hand, tissue culture of intestinal mucosa maintains viable and differentiated cells for 10-12 days (Lichtenberger et al, 1979) allowing adequate scope for experimental study. Also, the preservation of tissue architecture in tissue culture may permit retention of tissue-specific functions and pharmacological responses as some interactions within the tissue matrix and basement membrane are critical for normal cellular physiology (Reid and Rojkind, 1979).

Cell types differ in their growth characteristics and therefore there is no universal medium or clearly defined guidelines for selection of media. Consequently, a variety of media successfully used for culture of intestinal mucosa were selected and these media assessed both alone and in combination. The use of foetal calf serum, buffers, hormones and antibiotics have also been used in tissue culture of intestinal cells and these

were incorporated into the experimental design to define conditions necessary for the successful culture of gallbladder. In common with the intestinal work, successful culture would be assessed initially by morphology and subsequently by biochemistry, using established marker enzymes for some cell organelles.

In summary, the aims of this study were:-

1. To develop tissue culture of guinea pig gallbladder cells and optimise culture conditions.
2. To observe the morphological changes which this tissue undergoes during culture.
3. To assess the cellular activity morphologically and biochemically.
4. To assess the effect of bile on cultured cells.
5. To attempt human gallbladder culture.