STUDIES ON HYDROGEN ION SECRETION BY THE GALLBLADDER EPITHELIUM

BY
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TO MY CHILDREN

NIKOLAS and ATHANASIOS

Life is short and the Art is long, opportunity is fleeting, judgement difficult and experiment dangerous. The Physician must do the right thing at the right time

(First Aphorism of Hippocrates)

DECLARATION

I hereby declare that this thesis is based on the results of experiments performed by myself, except where aknowledged, and that the thesis is exclusively of my own composition. It has not been submitted previously for a higher degree. This work was carried out in the Liver Research Laboratories of the Department of Medicine in collaboration with the Departments of Pathology and Clinical Biochemistry , The Royal Infirmary, University of Edinburgh, Scotland.

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September, 1994

CERTIFICATE

We certify that Ioannis (John) N Plevris has completed nine terms of experimental research and that he fulfilled the conditions of ordinence 39 of the University of Edinburgh, so that he is qualified to submit the following thesis in application for the Degree of Doctor of Philosophy.

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ABSTRACT

Background: In the recent years the contribution of gallbladder mucosa to lithogenesis is increasingly recognised. In man as well as in other species, there is a decline in gallbladder bile pH compared with hepatic bile. Calcium salts are important components of all types of gallstones and changes in the acid-base status of bile, together with other factors, may influence their solubility in bile and play a critical role in the formation of gallstones.

Aim: In this thesis the *in vitro* ability of gallbladder epithelium to secrete hydrogen ions, the mechanism and regulation of the acidification process and its pathophysiological consequences to calcium salt solubility in bile , were studied.

Materials and methods: Fresh human and bovine gallbladder were used for the *in vitro* experiments. The Ussing chamber method was used to investigate hydrogen ion secretion by the human gallbladder epithelium; a whole gallbladder perfusion system was used to study the mechanism and regulation of acid secretion in the normal bovine gallbladder. To study hydrogen ion secretion at cellular level, gallbladder epithelial cell cultures were developed. The effect of acidification on electrolyte and calcium salt solubility was studied in samples of bovine gallbladder bile.

Results: The human gallbladder epithelium was capable of secreting hydrogen ions, through a sodium/hydrogen apical exchange system and its acidification ability was found reduced with inflammation. The normal bovine gallbladder epithelium was also capable of secreting hydrogen ions through a sodium/hydrogen apical exchange system that was ouabain and amiloride sensitive. Acidification was histamine dependent because it was stimulated by histamine and inhibited by H_1 and H_2 antagonists. Cholecystokinin augmented acidification but acetazolamide (carbonic anhydrase inhibitor) did not have any effect on acidification.

Normal bovine gallbladder bile had striking similarities with human and bile acidification significantly improved calcium carbonate solubility but had no effect on calcium phosphate solubility. In addition the anatomical similarities of human and bovine gallbladders were confirmed by pathology studies.

Primary gallbladder epithelial cell short-term cultures were developed to enable a closer study of the biological properties of the gallbladder epithelial cell. Acid production as well as the modulator effect of amiloride and histamine were visualised in gallbladder epithelial cell suspensions with the use of the fluorescent dye acridine orange.

Conclusion: In this thesis it was demonstrated that the gallbladder epithelium secretes hydrogen ions into the bile through a sodium-hydrogen apical exchange and this is a protective mechanism against calcium carbonate precipitation.

AIMS AND STRUCTURE OF THE THESIS

This thesis deals with the mechanisms of production of hydrogen ion (secretion) by the gallbladder mucosa. This concept at the time that the thesis was started was quite novel and has become of increasing importance due to its relevance to calcium salts solubility in bile. Hydrogen ion secretion by the gallbladder mucosa may be an important mechanism of regulating bile pH thus influencing calcium salt solubility in gallbladder bile. Calcium salts are important components of all types of stones in the body and factors which influence their solubility in bile may be critical in the formation of gallstones.

In the ***first part*** of the thesis ***(introduction)***, an overview of the anatomy and the mucosal functions of the gallbladder epithelium is given and also the principles of bile production and gallstone formation ***(chapters 1 to 4)***. This background information provides the basis of understanding the diverse physiological functions of the gallbladder epithelium; when dealing with the mechanisms of gallstone formation it will become apparent that there is a close interaction between gallbladder bile and mucosa and that abnormalities in the mucosal function may directly affect the composition of the gallbladder bile and favour gallstone formation.

The ***second part*** of the thesis ***(experimental work)*** sets several questions regarding the ability of the gallbladder epithelium to secrete hydrogen ions and investigates the effect of inflammation on hydrogen ion production by the human gallbladder epithelium. Although the first part of the experimental work has been carried out on human tissue (preliminary work), it subsequently became necessary to study the regulation of acid production by the normal gallbladder mucosa. Because of lack of adequate normal human gallbladder tissue, the animal model of bovine gallbladder was used. In a series of experiments, the rate and mechanism of acid secretion were investigated, as well as the effect of several pharmacological agents on hydrogen ion production. Subsequently the effect of pH on bovine gallbladder bile, in particular in relation to inorganic calcium salts solubility was investigated. Furthermore, a study of the cellular events associated with acid production was only possible by direct studies on the gallbladder epithelial cells. For this purpose primary gallbladder epithelial cell short-term cultures were developed to enable a closer study of hydrogen ion secretion by using fluorescent pH-sensitive intracellular dyes.

The ***experimental work*** is therefore divided into 5 chapters ***(chapters 5 to 9)***; each chapter is divided into ***background and aim, materials and methods, results and conclusion.***

Chapter 5 deals with studies on human gallbladder epithelium using the Ussing chamber technique; ***chapter 6*** deals with acid secretion by the normal bovine gallbladder epithelium, its mechanism of secretion, regulation and the effect of pharmacological agents; ***chapter 7*** deals with pathology studies on bovine and human gallbladder epithelium; ***chapter 8*** deals with the effects of bile pH on various components of bile and in particular, its influence on inorganic calcium salt solubility; and ***chapter 9*** deals with the development of a method of short-term (up to 2 weeks) culture of gallbladder epithelial cells, their characterisation in culture, and the use of epithelial cell suspensions and fluorescent dyes to visualise acid secretion by the gallbladder.

The ***discussion*** section is in ***chapter 10,*** and includes an overall critical discussion of the results, the new insights introduced by this thesis regarding hydrogen ion secretion by the gallbladder epithelium, as well as future studies emerging from this work.

References comprise ***chapter 11.***

INTRODUCTION

CHAPTER 1
ANATOMY OF THE GALLBLADDER

1.1 MACROSCOPIC ANATOMY

1.2 HISTOLOGY

1.3 NEUROANATOMY

CHAPTER 1
ANATOMY OF THE GALLBLADDER

1.1 MACROSCOPIC ANATOMY

The gallbladder is a pear-shaped organ attached to the lower surface of the liver. It is about 8 cm long and holds 30-50 ml of bile. It is divided into the fundus, body and neck. The fundus is rounded and projects below the inferior margin of the liver. The body lies in contact with the visceral surface of the liver and is directed upward at the left. The neck becomes continuous with the cystic duct which turns into the lesser omentum to join the right side of the common hepatic duct to form the common bile duct. The arterial supply of the gallbladder is from the cystic artery, a branch of the right hepatic artery. The cystic vein drains directly into the portal vein. A number of very small arteries and veins also run between the liver and the gallbladder. The nerves to the gallbladder are derived from the coeliac plexus (Snell R S, 1981).

The cystic duct which is derived from the bile duct is commonly associated with anatomical variations which are of surgical importance. The gallbladder itself can rarely be absent or buried in the liver or occasionally suspended from the liver by an acquired mesentery. Occasionally it has a sacculation in its neck (Hartman's Pouch) and sometimes the fundus is congenitally folded upon itself within its serous or peritoneal coat. Bilobed gallbladders have rarely been reported in man (Boyton E A, 1926) but they are very common in animals. The wall of the cystic duct forms a spiral valve (of Heister) which controls the movement of bile and prevents the bending and obstruction of the cystic duct. Anatomical variations of the cystic artery which is a branch of the right hepatic artery are common and usually only 50-60% of the gallbladder receive blood from the cystic artery only (Basmajian J V et al, 1989). The innervation of the gallbladder is from sympathetic and parasympathetic fibres (vagus). The sympathetic fibres exist in the hepatic branch of the anterior right parasympathetic trunk and both efferent and afferent fibres are present. Sympathetic adrenergic fibres innervate the smooth muscle of the gallbladder and the cholinergic ganglia. The afferent fibres responsible for pain in the biliary tree pass through the sympathetic trunk to the abdominal ganglia and finally to the spinal cord (Lundgren O et al, 1989).

1.2 HISTOLOGY

The wall of the gallbladder consists of the following layers: ***a) Mucosa*** which is composed of a simple columnar epithelium and lamina propria, ***b)*** a layer of ***smooth muscle, c)*** a well developed ***peri muscular connective tissue*** layer and ***d) a serous membrane.***

The morphology of the mucosa of the gallbladder has been studied in detail (Evett R D et al, 1964). It has been adapted to perform the absorptive and secretory functions of this organ. The mucosa is a simple columnar type epithelium possessing surface microvilli measuring *15-25 μm* in height and *2.5-7 μm* in width. When observed under a microscope the columnar epithelial cells show several *0.2-0.5 μm* long microvilli usually presenting an apical tuft *(30-70 nm tall)* of glycocalyx (Gilloteaux J et al, 1989). Two other types of cells are present in the mucosa in very small numbers. The first type is the pencil cells which are thinner and stain darker. These cells always appear richer in mitochondria and have fewer mucous vacuoles in the cytoplasm. The second type of cells is the small basal *ZIO*-philic cells (stained with *Z*inc-*I*odine-*O*smium tetroxide) which resemble lymphocytes and are positioned just above the basement membrane. These cells appear less electron dense than the other types of epithelial cells and display abundant rough endoplasmic reticulum as well as smooth endoplasmic reticulum, lipid inclusions and mitochondria (Gilloteaux J et al, 1989).

Mast cells and polymorphonuclear leucocytes are seldom found within the surface of the epithelium but when detected they are in close vicinity to the basement membrane (Hudson I and Hopwood D, 1986).

Often the gallbladder mucosa forms crypts which are gland-like pits with a covering surface epithelium composed of cell types similar to those found in the typical epithelial lining of the gallbladder. This type is called diverticular or *Rokitansky-Aschoff* crypt epithelium. These formations are more common near the cystic duct. The cells of these glands have characteristics of mucous secreting cells and are largely responsible for production of most of the mucous present in bile.

The adjacent epithelial cells are joined by the *zonula occludens* or *tight junctions* which represent the site of fusion between adjacent membranes at the apex of the cell (Farquhar M G and Palade G E, 1963). The *tight junctions* extend vertically for approximately *100-200 nm* and circumferentially around the entire cell and form a ring around the neck of the cell at which all adjacent cells are closely opposed. Although the term *tight junction* implies a zone of complete cell fusion it is now well recognised that this area is permeable to several ions and compounds and represents a paracellular

pathway of epithelial transport (Machen T E et al, 1972). The biliary epithelium lies at the basement membrane which is around *300 Å* thick and this in turn rests on the lamina propria which is composed of loose bundles of collagen fibrils and fibroblasts surrounding a rich plexus of capillaries. In the lamina propria mast cells are noticed. Capillaries are found in close relationship to the basement membrane and are characterised by a fenestrated endothelium. There is no muscularis mucosa in the gallbladder and the mucosa rests on a thin layer of smooth muscle which is equivalent to the muscularis externa of the intestine. Outside the muscle coat is the sub serosal layer which contains arteries, veins, lymphatics and nerves to the gallbladder.

1.3 NEUROANATOMY

The gallbladder develops as an outgrowth of the duodenal mucosa (Lundgren O et al, 1989, Cai W et al, 1983). The innervation of this organ is similar to that found in the intestine but our current knowledge of its physiological importance is small compared to that of the intestinal tract. Intramural nerves and smooth muscle in the gallbladder wall are arranged as in the intestinal mucosa. Nerves are present in all three main layers of the gallbladder and are arranged as ganglia associated with the intramural nerve plexus. Three main plexuses are recognised; a *subserosal,* a *myenteric* which is connected with branches from this subserosal plexus and a *fine meshed mucosal nervous network* which is connected with branches from the other two plexuses. The nerve terminals are located close to blood vessels and smooth muscle cells and are also found in the basement membrane of the epithelial layer. The extrinsic nerves and sympathetic fibres which reach the gallbladder originate mainly from the coeliac ganglion (Paumgardner G et al, 1969). The sympathetic fibres innervate the vascular smooth muscle and are in close contact with non-adrenergic nerve cells of the plexuses but they do not contact the epithelial cells. Parasympathetic fibres reach the gallbladder through vagal fibres and are present in the ganglia of the lamina propria (Kyosola K et al, 1977).

Intrinsic Nerves

These nerves are believed to be *cholinergic* and *peptidergic* (Cai W et al, 1983). Each layer of the gallbladder is densely innervated by peptidergic nerves, ganglia and terminals which are present on the outer side of the muscle layer in close relationship to the basement membrane of the epithelium and the gallbladder smooth muscle. Blood vessels in the gallbladder wall are supplied with nerves which are immunoreactive for several peptides such as *vasoactive intestinal peptide (VIP), substance P (SP)* and *NPY.*

The epithelium also is supplied by nerves which are immunoreactive for *VIP* and *SP*, while in the smooth muscle nerves immunoreactive for *VIP*, *SP* and somatostatin are found (Plevris J N et al, 1994). *Enkephalinergic* nerve fibres have also been described in the myenteric plexus in the human gallbladder (Polak JN et al, 1977).

CHAPTER 2
PHYSIOLOGY OF THE GALLBLADDER EPITHELIUM

CHAPTER 2
PHYSIOLOGY OF THE GALLBLADDER EPITHELIUM

The gallbladder mucosa has one of the highest reported rates of water transport. It offers a particularly durable *in vitro* preparation as it tolerates a pH range of *2.5-11*, osmolarities from one tenth to triple that of plasma and alcohol concentrations of up to at least *8% v/w* (Diamond J M, 1968). The gallbladder epithelium possesses specific physiological properties such as symmetrical permeability characteristics and absence of electrical potential differences associated with ion transport. The exact number of physiological functions that the gallbladder serves is not known. Primary functions include:

a) Storage of bile, during interdigestive periods, ***b)*** concentration of stored bile by removal of water and inorganic ions, ***c)*** evacuation of the gallbladder by contraction of smooth muscle in response to *CCK* and the moderation of hydrostatic pressure in the biliary tree.

Several other properties of the gallbladder have recently been investigated and it is now known that the gallbladder epithelium is capable of absorbing to a limited extent organic components of bile such as *bilirubin, cholesterol, fatty acids* (Rose R C et al, 1981; Jacyna M R et al, 1987; Conter R L et al, 1981) and is also capable of secreting *mucous glycoproteins* and *several biliary proteins.* The gallbladder epithelium has also a role in the acidification of bile. It is of some interest that not all animal species have gallbladders although an explanation has been proposed by correlating intermittent eating habits with the presence of a gallbladder as in sheep, rabbit, guinea pig, human and continuous eating habits with the absence of the gallbladder as in rat, horse, pigeon etc. However, it should not be necessarily concluded that animals without a gallbladder are unable to concentrate bile or store significant quantities of it, as in several species the biliary tree serves such functions.

2.1 ABSORPTIVE FUNCTIONS OF THE GALLBLADDER

2.1.1 Fluid Transport

The liver secretes over a litre of bile per day. The gallbladder concentrates hepatic bile 4-5 fold. Although it has been shown by Toth JL et al, 1990, that the human

gallbladder absorbs about 1 ml of water per hour *in vitro*, it is believed that the rate of absorption *in vivo* is three times that of the *in vitro* rate. In addition, the gallbladder epithelium absorbs large amounts of inorganic electrolytes, in particular sodium and it is the coupling between active sodium transport and passive water absorption that allows the mucosa to absorb water against an osmotic gradient (Whitlock R T and Wheeler H O, 1964). In the gallbladder, the absorbed fluid is isotonic to plasma and although the mechanism by which net solute transport (sodium) results in net water transport is an osmotic one, an osmotic gradient is not established between the two sides of transport. To explain this, Curran P and McIntoch J 1962, have proposed the double membrane model by which there is a continuous supply of actively transported solute into a space between the two membranes, one of which has a much higher reflection co-efficient (R) than the other. Solvent will flow out of this double membrane system away from the high reflection co-efficient membrane towards the low reflexion co-efficient membrane. In a typical epithelium such as the gallbladder the high reflection co-efficient membrane corresponds to certain components of the epithelium such as the basolateral membrane. The low reflection co-efficient membrane could correspond to the basement membrane when the tissue is *in vitro* or the capillary membrane under conditions *in vivo*. The compartment between the membranes could then assume the structure of the lateral intercellular space and solute (sodium chloride) is transferred into this space by moving across the cell membrane under the influence of an active transport system.

Tormey J and Diamond J R have shown in 1967 that the gallbladder epithelium has an important morphologic characteristic in that it has a long narrow convoluted channel between adjacent epithelial cells. These lateral intercellular spaces are closed by the tight junctions at the luminal surface of the epithelium but are open at the serosal surface of the tissue (Figure 1).

A model of transport proposed by Diamond J R and Tormay J 1966, has the same basic features as Curran's model, but in addition it proposes that the apical end of the lateral intracellular space is the initial site of solute accumulation. Water absorption follows osmosis resulting in an increase in hydrostatic pressure which bulges the membranes of adjacent cells apart and fluid is prevented from flowing back into the luminal solution by the tight junctions. The absorbed fluid is forced to pass along the intracellular space gradually equilibrating osmotically with intracellular fluid. The contents of this space thereby maintain an osmotic gradient concentrated at the apical end and approaching isotonicity at the serosal end. Experiments in Necturus and rabbit gallbladder revealed the presence of leaky tight junctions to water and favour the

paracellular pathway for transport of various known electrolytes across the gallbladder *in vitro* (Stewart M C, 1989).

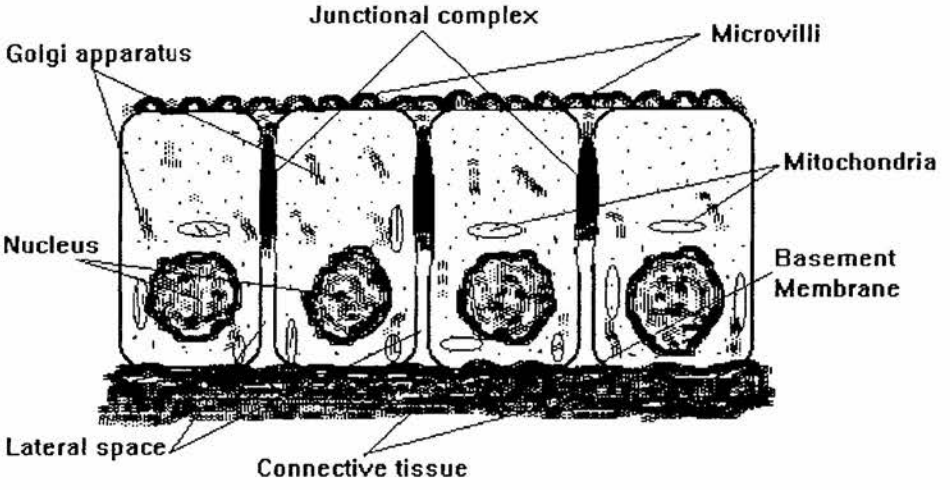

Figure 1: Ultrastructural features of gallbladder epithelium

2.1.2 Electrolyte transport

Sodium transport

Sodium transport by the gallbladder mucosa has been extensively studied in animals and in the human gallbladder. It is well documented that the gallbladder epithelium is capable of transporting sodium chloride across the apical membrane by an electroneutral process. The mechanism of the entry process remains controversial, but it has been attributed by some to simple *symport* of sodium and chloride while others propose the operation of a parallel exchange *(antiport process)* i.e. *sodium / hydrogen* and *chloride / bicarbonate*. Weinman S A and Reuss L 1982, concluded that double exchange is the predominant mechanism of apical sodium / chloride entry. In the porcine gallbladder, sodium and chloride transport are mediated both by *sodium / hydrogen* and *chloride / bicarbonate* exchange mechanism and in addition by sodium bicarbonate co-transport.

At the basolateral membrane sodium transport is active involving a *sodium / potassium activated ATP-ase.*

Potassium Transport

Net secretion of potassium by the rabbit gallbladder (although only 6% of the rate of sodium absorption and an intracellular potassium activity of 1.5 times that for an equilibrium distribution across the cell membrane) gives evidence of an active transport process. This is considered to take place through a *sodium / potassium* exchange pump in the basolateral cell membrane. Segal Y et al 1990, have shown that Ba^{++} and tetraethylammonium block the large conductance *(Maxi) potassium* channels in the apical membrane of necturus gallbladder as well as voltage-activated potassium conductance channels. It appears that in the basolateral side *potassium and chloride channels* exist.

Calcium Transport

Calcium is regarded as an important mediator of intestinal fluid absorption and calcium channel antagonists alter water and electrolyte flux. Scheeres DE et al 1990, suggested that gallbladder absorption is controlled by intracellular calcium concentrations as it is inhibited by verapamil, but not by changes in extracellular calcium concentration. Mucosal but not serosal addition of verapamil caused profound inhibition of short circuit current and transepithelial potential difference in the *prairie dog* gallbladder *in vitro*, and this provides evidence of free cytosolic calcium as a regulator of active ionic transport.

pH changes across the Gallbladder Epithelium

It has been recognised for years that the gallbladder bile is more acidic as compared to the hepatic bile (pH of the hepatic bile around 8 and of the gallbladder bile 6.5). *Rabbit* gallbladder *in vivo* and *in vitro* acidifies the luminal content by at least 1 pH unit with respect to serosal fluid. Traditional teaching would attribute this acidification to bicarbonate absorption. However, a single description of how gallbladder mucosa handles hydrogen ions cannot be given because significant species differences are evident from various studies and because a distinction between the hydrogen transport

and bicarbonate transport is difficult to make. Work by Moore EW et al 1987 in *dog* gallbladder suggested that acidification is secondary to active hydrogen ion secretion, but no data has been available for the human situation. A currently proposed mechanism of bile acidification in the *necturus* gallbladder by Altenberg GA et al 1990, would involve an apical sodium hydrogen exchange which is amiloride sensitive. The mechanism and of acidification of gallbladder bile has not been studied in detail and its pathophysiological significance largely remains unknown. In the human situation, acidification of bile has been proposed as a protective mechanism against calcium precipitation and gallstone formation .

Regulation of Ion Transport

Regulation of gallbladder mucosal function is complex and involves neural, humoral and luminal factors. It is known that the rate of fluid absorption in the gallbladder has diurnal variation. More recently, considerable progress has been made in the understanding of gastrointestinal peptides and neural influences on cellular transport. Vasoactive intestinal polypeptide *(VIP)* appears to act as a neuro-transmitter in the neurones who innervate the gallbladder epithelial cells (O'Grady SM et al, 1989). It promotes fluid secretion and reverses sodium potassium and bicarbonate transport from absorption to secretion. These changes are mimiced by a *cAMP* analogue, *8BR cAMP*, and are not affected by the nerve toxin called tetrodotoxin. The effects of *VIP* are inhibited by noradrenaline acting through α_2 receptors. A possible mechanism of action of *VIP* includes activation of the adenyl cyclase and increase of cellular *cAMP* which stimulates chloride secretion by opening apical chloride channels and inhibiting sodium absorption. Jacyna MR et al , 1989, have shown that secretin can reverse sodium absorption to net secretion in the human gallbladder *in vitro.*

Prostaglandins have been found to inhibit sodium and water absorption and turn it to net secretion (Kaminski DL, 1989). Prostaglandins are usually released from the gallbladder by local mucosal damage.

Sympathetic stimulation increases water reabsorption while vagal stimulation reduces net water reabsorption in atropinised animals through a non-cholinergic mechanism which is possibly mediated by *VIP.* Roslyn JJ et al 1989, have demonstrated that taurodeoxycholate can cause an inhibition of transepithelial potential difference and short circuit current in the *prairie dog* gallbladder and this effect can be modified by the addition of phosphatidylcholine. This suggests that the ratio of biliary bile acids to

phospholipids may regulate gallbladder fluid transport and electrolyte absorption. Another important regulatory function in gallbladder epithelium is the control of cellular volume. When the *necturus* gallbladder is exposed to hypotonic solutions, the epithelial cells swell due to rapid water influx and then shrink due to volume regulatory decrease in size. The volume changes are regulated by intracellular calcium and possibly modulated by calmodulin and the microfilaments of the cellular cytoskeleton which activate the potassium and chloride channels through the basal lateral membrane (Furlong TJ et al ,1990).

Several changes in gallbladder mucosal transport happen in relation to mucosal inflammation and gallstone formation and will be discussed in the next chapter.

2.1.3.Absorption of Organic Components of Bile

The gallbladder maintains a considerable concentration gradient of bile salts and bile pigments across its wall due to its concentrating ability and therefore the permeability of the mucosa to these organic substances is of particular interest. The normal *guinea pig* gallbladder is impermeable to highly ionised organic substances such as taurocholate sulphobromophthalein *(BSP)*, but is much more permeable to weakly ionised compounds such as unconjugated bilirubin and chenodeoxycholic acid. The gallbladder epithelium allows a reasonably high rate of diffusion of substances which dissolve in the lipoidal membrane. It is thought that the gallbladder, which contains gallstones, has decreased permeability because of its thickened mucosa due to chronic inflammation. In acute cholecystitis, however, increased permeability to water and highly ionised substances is seen (Svanvic J et al, 1986).

The gallbladder mucosa is injured by direct exposure to conjugated and unconjugated bile salts. Absorption of bile salts is linearly increased to the luminal concentration and in *in-vitro* experiments it has been shown that pure solutions of bile salts cause injury at concentrations significantly lower to those which are normally achieved in gallbladder bile. It is possible that the presence of bilirubin and cholesterol and the fact that most bile salts are conjugated, protect the epithelium from damage. Chenodeoxycholic acid has a *pKa* of *6.4* and although it has the same molecular weight as cholate, which has a *pKa* of *5.5*, it is absorbed much more rapdily at a pH of *8*. This suggests that absorption is related to the fraction that is non-ionised and lipid-soluble. The deconjugation of bile salts by bacterial action may cause acceleration of bile salt absorption with subsequent injury to gallbladder mucosa and this may have relevance to gallstone formation. Similarly unconjugated bilirubin has been shown in the *guinea pig* gallbladder to be

absorbed faster than conjugated bilirubin and is linearly related to the concentration of the pigment (Heuman DM et al, 1990). Cholesterol has been shown to be absorbed by the gallbladder mucosa and the rate of absorption may be a factor of importance in gallstone formation (Jacyna MR et al 1987). The majority of cholesterol absorbed is found in the mitochondrial in microsomal fractions. Absorption of cholesterol from the mucosa may provide a protective mechanism against supersaturation and precipitation of this compound.

2.2 SECRETORY FUNCTIONS OF THE GALLBLADDER EPITHELIUM

Estimations of human gallbladder bile obtained at surgery from patients with cholelithiasis have indicated a protein concentration of between *5-50 mg per ml* of bile, which makes protein the third most abundant solid constituent in bile (LaRusso NF,1984). Hepatic bile has a lower protein concentration of about *3-5 mg per ml.*

A significant variety of proteins are present in bile and can be broadly divided into three main groups: ***a) transport proteins*** such as albumin, transferrin, ceruloplasmin, apolipoproteins, ***b) immunoglobulins*** such as secretory IgA, IgM, IgG and ***c) hormones*** such as insulin, epidermal growth factor, *CCK* and several enzymes such as glutathione S-transferase, alkaline phosphatase, amylase. Albumin represents the major protein which has been derived from the plasma pool. The majority of proteins produced by the liver enter the plasma compartment and it is only *1%* of plasma proteins which appear in bile. The majority of the biliary proteins which exist in plasma have a ratio of bile to plasma concentration of less than *0.02.* Apolipoproteins in bile may reach a significant percentage of their levels in plasma, whilst secretory IgA is usually found in higher concentrations in bile than in plasma.

An important source of biliary protein is the liver cell. The bile duct epithelium and that of the gallbladder contribute some proteins in bile either directly or from plasma via transepithelial transport (Reuston RH et al, 1980). In particular the gallbladder secretes glycoproteins which are considered important in the pathogenesis of gallstone formation. Glycoproteins can either be derived from plasma and these would include A_1 acid glycoprotein, small amounts of carcinoembryonic antigen and other proteins with carcinoembryonic antigen-like immunoreactivity. Mucin glycoproteins represent a population of high molecular weight glycoproteins that have a common basic structure but with a great variety with respect to molecular size, sulphate content and internal sugar residues. These proteins have a potential role in gallstone formation. The mucin

glycoproteins which are produced from the gallbladder are chemically and structurally similar to those produced by other parts of the gastrointestinal tract. Galactose N-acetyl galactosamine, glycosamine and fructose are the major sugars. The carbohydrate content of mucin glycoprotein (up to 85%) is significantly higher than in plasma glycoproteins which is around 50% (Reuben A,1984). Mucins in their vast majority are produced by the gallbladder epithelium and bile ducts rather than the hepatocytes and this has been confirmed in gallbladder tissue culture studies. Patients with gallstones and animals with experimentally induced gallstones hypersecrete mucus and this may promote cholesterol crystal nucleation (LaMont JT et al , 1984). Purified human gallbladder mucins can induce nucleation of supersaturated bile. In the *prairie dog* model of cholesterol gallstones, high dose aspirin prevents mucus hypersecretion and cholesterol crystal nucleation (Lee SP et al, 1981). In a recent study , aspirin was found to inhibit mucus glucoprotein synthesis in the human gallbladder (Rhodes M et al, 1992).

The other important group of biliary proteins is immunoglobulins. Secretory IgA may have a role in the humoral defence of the biliary tree. Other transport proteins may also function by removing potentially toxic substances from the body such as copper. Apolipoproteins A_1 and A_2 are anti-nucleating agents which stabilise the mixed micelles (Reuben A,1984).

2.3 GALLBLADDER MOTILITY

Gallbladder motility has been studied extensively and abnormalities in motility have been associated with both acalculous and calculous gallbladder disease. Physiologically one of the most important layers of the gallbladder is the smooth muscle layer which is formed by thick densely packed muscle cells in the fundus which become less closely assembled in the neck and cystic duct (Severi C et al, 1988). During inter-digestive periods the gallbladder of humans and animal species stores and concentrates the bile produced by the liver. During that period, the gallbladder diameter increases several times but with little change in intra-gallbladder pressure and this is achieved by combination of the great absorptive capacity of the mucosa and the elasticity of its wall.

The gallbladder has a continuous cyclic motor activity, which is probably mediated by the hormone motilin, and during the fasting phase it appears that there is a continuous to and fro movement of bile which might serve to thoroughly mix its contents to avoid relative stasis and stone formation (Howard PJ et al, 1991). It is also possible that

during this phase cholecystokinin, secretin and pancreatic polypeptide do not significantly affect the gallbladder motility. After a meal the gallbladder empties at a slow steady concentration in response to both neuronal and humoral influences. Again there is little change in intra-gallbladder pressure during emptying, and gallbladder emptying appears to co-incide with a rise in gallbladder pressure of only a few mmHg and in association with phase 2 of the motor migratory complex of the small intestine (Scott RB et al, 1988).

There is evidence of gastro-biliary reflex by which gastric distension increases the pressure in the gallbladder and the sphincter of Oddi motility. After blocking with atropine the muscarinic receptors, gallbladder and sphincter activity were abolished. Vagotomy delays and decreases gallbladder emptying and this adds to the evidence that gallbladder motility is primarily mediated by the vagus nerve (Masclee AAM et al, 1990).

Gastrointestinal hormones play an important role in gallbladder motor response to distension of the stomach and small intestine with food. *CCK* promotes contraction possibly by two ways; first, by releasing acetylcholine from cholinergic neurones (action to M_1 muscarinic receptor), hence the reduced sensitivity of the gallbladder to *CCK* after vagotomy and second, by direct myogenic action on the gallbladder smooth muscle (Grider JR et al, 1990, Pozo MJ et al, 1990). More recently, Schjoldager B et al, 1988, identified the *CCK receptor* which is a plasma membrane protein with a molecular weight between *70,000* and *85,000 Dalton* and it represents the binding sub unit of the *CCK receptor.* Fat acts as a stimulus for gallbladder emptying and the mechanism of the response appears to be through release of cholecystokinin.

Other gastrointestinal peptides have been associated with gallbladder motility. Vasoactive intestinal polypeptides *(VIP)* is thought to be involved in relaxation of the gallbladder. In a more recent study a selective competitive inhibitor to *VIP* did not modify the gallbladder contractile response, while a growth hormone releasing factor analogue which interacts with both pancreatic *VIP* receptors and receptors of glucagon secreting family of peptides, produced an increase in gallbladder motility (Greco AV et at, 1990). These observations suggest that the main neurotransmitter which is involved in the gallbladder inhibitory response is a member of the glucagon secreting family of peptides. Peptide *YY* and pancreatic polypeptide have been identified as modulators of motor activity in the gallbladder (Lillemoe K et al, 1988). Peptide *YY* may exert anti-*CCK* effects in the *prairie dog* gallbladder and associated relaxation, but in other studies this was not demonstrated. The actions of pancreatic polypeptide may be similar although not fully clarified. Neurotensin induces a dose dependent contraction of the

gallbladder. Diabetic patients with autonomic neuropathy have a decreased sensitivity to *CCK* and an atonic gallbladder, and in patients with severe pancreatic disorders the relationship between the gallbladder emptying and *CCK* secretion may be disrupted (Stone BG et al, 1988). This may be due to the fact that exocrine pancreatic insufficiency may result in a reduction of fat digestion and triglyceride absorption, finally leading to an insufficient CCK secretion and poor gallbladder emptying.

Gallbladder motility may also be influenced by extra- intestinal factors such as age, race and nutritional status. Elderly people may empty their gallbladders faster than younger individuals. Gallbladder volumes in response to feeding are consistently lower in blacks, though fasting gallbladder volumes are similar in black and white subjects. Obese individuals are most prone to have abnormal gallbladder emptying, and are at high risk of cholesterol gallstone formation (Parodi JE and Becker JM, 1990).

2.4 ELECTRICAL PROPERTIES OF THE GALLBLADDER EPITHELIUM

It has already been mentioned that the gallbladder epithelium is able to effect a net absorption of an isotonic sodium chloride solution. Most of the initial studies of electrolyte transport to the gallbladder *in vitro* have been done on gallbladders from *fish, rabbits or guinea pig* (Diamond JM, 1964, Dietschy JM, 1964 &1966). Electrolyte absorption in those gallbladders was almost nearly electrically neutral with transmural potential differences less than *1 mV*. However, more recent information on gallbladders of *man, goose, rabbit* and *monkeys* have demonstrated a significant serosa positive potential difference (Rose RC et al 1973, Gelarden RT et al 1974). In particular, several studies have been done in human gallbladder and we know that the normal human gallbladder epithelium develops an average serosa positive transmural potential difference of *7.6 mV* with a average short circuit current of *136 mA/cm*^{2} (Rose RC, 1975). Variable serosa positive potential differences have been reported (in monkeys *1.5-2.5 mV*, goose *3.6 mV*, while the rabbit expresses a serosa negative potential difference *-0.4 mV).*

Elimination of oxygen from the bathing solution in *in vitro* experiments results in a prompt reduction of the short circuit current. The effect of anaerobiosis is reversible if oxygen supply is restored within 10 minutes (Rose RC, 1975). Tissue resistance appears to increase during treatment with nitrogen which may be attributed to swelling of the epithelial cells. Cardiac glycosides (ouabain) reduce the short circuit current by inhibiting active transport of sodium. Elimination of sodium from the bathing solutions

would eliminate potential difference. Similarly, tissues with acute or chronic inflammation would present a lower circuit current and potential difference (Rose RC, 1973). Studies on the electrical properties of the gallbladder epithelium, not only have given important information regarding ion transport, but also can be used as a measurement of viability of the tissue during *in vitro* experiments with this tissue.

CHAPTER 3
PHYSIOLOGY OF BILE

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PHYSIOLOGY OF BILE

In this chapter the mechanisms of production of the major components of bile will be considered in brief.

3.1 BILE COMPOSITION

Bile represents the exocrine secretion of the liver. It provides the major excretory pathway for toxic metabolites, pigment material, cholesterol and lipid waste products. Bile is also necessary for digestion and absorption of dietary lipids in the small intestine. Water is the main solute in bile while solids represent 5-10% of the total weight of bile. The predominant organic solids in bile are: bile salts, lecithin, cholesterol. The inorganic electrolytes present in hepatic bile closely reflect plasma concentrations. Sodium is the predominant cation, whilst calcium concentration is generally higher in bile, in particular the gallbladder bile as compared to plasma. The osmolality of bile is approximately *300 mosm/l* and very similar to that of plasma. The composition of human hepatic bile and cholesterol bile is given in the following table:

TABLE 1

	Hepatic bile	*Gallbladder bile*
Sodium mmol/l	150-160	152.1-211.7
Potassium mmol/l	2.7-4.9	5.1 - 12.8
Calcium mmol/l	2.5-4.8	from trace to 10
Magnesium mmol/l	1.4-3.0	
Chloride mmol/l	100-105	
Bicarbonate mmol/l	25- 40	27-55
Bilirubin mmol/l	0.25-1.2	2.3-18.5
Protein mg/dl	90-200	100
Total Bile Acids mmol/l	41.3-102	32.17-38
Phospholipids mmol/l	5.9-10.3	28-48
Cholesterol mmol/l	2.6-5.2	7.6-12.2

(Data from several studies presented in the Anatomy and Physiology of the Biliary Tract Bocus-Burk, Gastroenterology 1985).

The human gallbladder bile has been obtained by puncture at laparotomy from patients without biliary tract disease.

3.2 BILE ACIDS

3.2.1. Bile acid synthesis

Bile acids in normal human bile are divided into *primary* and *secondary* (figure 2). *Primary* bile acids are synthesised in the liver whilst the *secondary* bile acids are those formed by the intestinal bacterial action on the primary bile acids. Bile acids are synthesised from cholesterol in the liver.

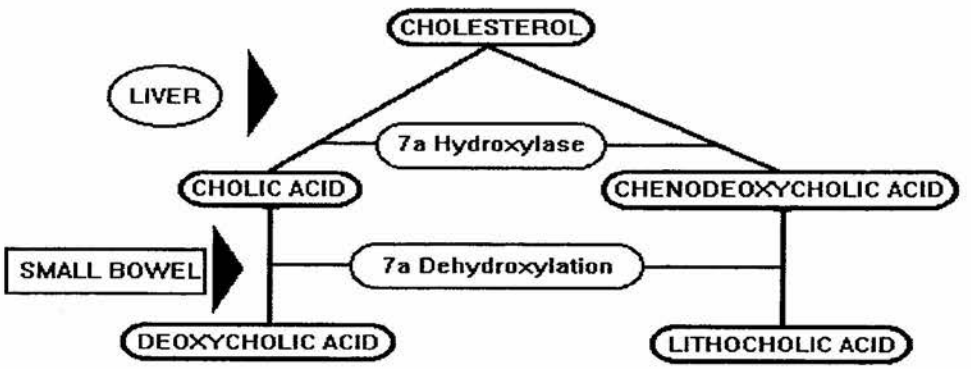

Figure 2: Bile salt synthesis from cholesterol.

The first and rate limiting step is controlled by the enzyme *cholesterol 7a hydroxylase.* This is a microsomal enzyme and belongs to the *cytochrome P450* family (Jelinek DF et al 1990). . Hepatic *cholesterol 7a hydroxylase* activity exhibits considerable diurnal variation and has a short half life of 2-3 hours. Increased activity of this enzyme often parallels the increased activity of ^{3}*hydroxy-* ^{3}*methyl-glutaric coenzyme (HMG COA)* which is an intermediate substrate to cholesterol. Induction of

hepatic cholesterol 7a hydroxylase activity may be achieved by cholestyramine administration.

The main primary bile acids are cholic acid and chenodeoxycholic acid both derived from cholesterol. Cholic acid has three *hydroxyl 3,7,12* groups while the chenodeoxycholic acid has two *3,7.* Intestinal bacterial dehydroxylation at 7a position results in the formation of deoxycholic acid (from cholic acid) and lithocholic acid (from deoxycholic acid). All these bile acids are reabsorbed from the intestine, carried to the liver in the portal blood, taken up from the sinusoids and finally secreted into the bile (the so- called *enterohepatic circulation)* (Bouchier IAD, 1983). Before secretion the bile acids are conjugated with either glycine or choline in the liver so that there are two subtypes of each bile acid. Again bacteria in the intestine promote deconjugation of the bile acids.

The secreted bile acids within bile are stored in the gallbladder during the interdigestive period. In the jejunum, bile acids act as detergents to promote fat absorption. Their active absorption occurs later in the terminal ileum. Hepatic clearance and intestinal absorption are rapid and efficient and first pass clearance of bile acids by the liver is extremely high, although there is a small spilling into the plasma compartment. The total concentration of bile salts within the interhepatic circulation represents the bile acid pool. The recycling system is very efficient and therefore a small pool of bile acids recirculates 5-15 times a day with a loss of 3-5% at every circuit via faecal excretion. These losses are accurately being replaced because a low rate of return stimulates synthesis of bile acids from cholesterol, maintaining therefore a stable bile acid pool (Aronchick CA and Brooks FP, 1985).

The bile acid molecules carry the *hydroxy groups* in the *3,7* and *12* positions and these groups are all orientated on the same side of the bile acid molecule. The non-polar water insoluble aspect of the molecule is on the opposite surface. When the bile acids reach a critical concentration they form aggregates known as *micelles* in solution (figure 3). In a *micelle* the bile acid molecule is orientated in such a fashion that the water soluble part faces outwards towards the water phase of the solution, while the lipid soluble portion (water insoluble) faces inwards. It therefore follows that if the molecules are oriented in a radial or rectangular manner, the interior of the bile acid complex is occupied by the water-insoluble groups and can therefore retain water insoluble molecules such as cholesterol or phospholipids.Therefore these molecules can be maintained in solution forming with the bile salts *a mixed micelle.* The evidence so far is that in normal bile, bile acids, phospholipids and cholesterol exist as *mixed micelles* (Aronchick CA and Brooks FP, 1985).

The detergent property of bile acids is important in stabilising the physical state of bile and in promoting fat digestion and absorption. Conjugation of bile acids enhances their hydrophilicity and the acidic strength of their side chain. These features decrease

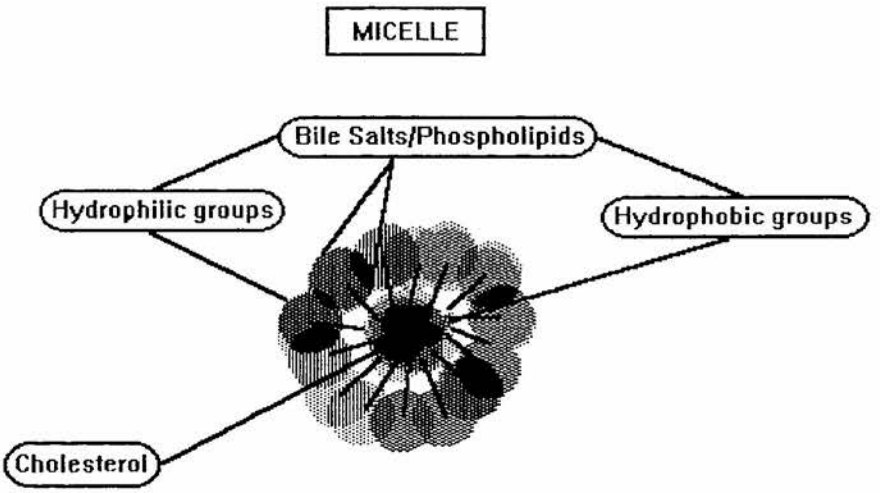

Figure 3: Diagrammatic illustration of the ultrastructure of a micelle.

their ability to cross cell membranes by passive diffusion during their transit down to the biliary tract and small intestine.

Glycine and taurine conjugates demonstrate selective resistance to hydrolysis by pancreatic enzymes during the small intestinal transit. Conjugation with taurine is the preferred pathway as taurine conjugates are more hydrophilic as compared to glycine conjugates and intracellular taurine depletion may alter the proportion of bile acids conjugates and effect bile flow. Sulphation and glycuronidation of bile acids are minor metabolic pathways of bile acid metabolism (Moseley RH, 1992).

3.2.2. Bile acid transport

The hepatic translocation of bile acids from plasma to bile involve three distinct steps; *a) hepatocellular uptake* at the sinusoidal and basal lateral membrane surface of the hepatocyte, *b) intracellular* processing and *c) excretion* across the canalicular membrane.

Hepatocellular uptake.

Bile acid uptake by the hepatocyte is regarded as a secondary active process dependent on basal lateral sodium, potassium, adenosine, phosphatase *(ATP-ase activity).* However, Ziegler K et al 1992, have shown that taurocholate and cholate are taken up by adult rat hepatocytes by both sodium dependent and independent transport. The uptake of unconjugated bile acids appears to be primarily mediated by a sodium independent process involving non ionic diffusion. It appears that a *54 KD* protein may mediate sodium independent bile acid transport while a *48 KD* is the sodium dependent bile transport protein (Howard PJ and Murphy GM, 1993).

Intracellular events.

Once the uptake across the basolateral membrane has occurred, bile acids are translocated across the cell to the apical canalicular membrane by two intracellular transport systems. One system involves mainly protein binding and the other vesicular transport. Protein binding mainly occurs in the cytosol and the involved proteins belong to the group Y' of *glutathione S-transferases* which are a unique family of cytosolic proteins involved in several cellular reactions such as detoxification and binding of non substrate compounds such as bilirubin and bile acids (Stolz A et al 1989) . Such protein binding might not only serve the intracellular transport of bile acids, but also to protect intracellular organelles by reducing the detergent activity and therefore the toxicity of these salts.

Vesicular transport

. Vesicular transport involves formation of a complex between the bile acids and individual receptors of the sinusoidal membrane. The complexes form aggregates which are encased in an endocytic vesicle which soon becomes internalised into the cytoplasm.

These vesicles concentrate the receptor molecules while excluding other plasma proteins. Several vesicles containing bile salts fuse with and discharge their contents across the canalicular membrane by exocytosis. The *Golgi apparatus* is important for intracellular transport of bile acids (Simion FA et al 1984).

Canalicular bile acid transport

Canalicular excretion represents the rate limiting step in the transport of bile acid from sinusoidal blood into the bile. This is largely mediated by a sodium independent carrier mechanism which is driven by a negative transmembrane potential and involves *454 aminoacid transport protein (100KD)* which is localised in the bile canalicular domain (Ruetz S et al 1988). Since conjugation confers a high negative charge, conjugated bile acids being more negatively charged are more efficiently secreted compared with uncharged or positively charged bile acid derivatives. However, other mechanisms may co-exist for carrier mediated secretion at the canalicular membrane.

3.3 CHOLESTEROL AND LECITHIN TRANSPORT

The secretion of biliary lipids such as cholesterol and phospholipids is closely linked to that of bile acids (Duane WC, 1992). At low rates of bile salt secretion there is a small output of lecithin or cholesterol while with increasing bile salt flux through the liver there is a corresponding rise in lecithin and cholesterol secretion. The hepatic lecithin which is available for transport is predominantly synthesised with preformed choline. Secretion and synthesis of lecithin or choline parallel the newly synthesised biliary lecithin consitututes only a small fraction of total of the hepatic lecithin synthesis and most of lecithin is predominantly mobilised from a preformed hepatic pool. Lecithin output depends on the types of bile salts secreted. It is well known that in man, replacement of bile salts by ursodeoxycholic or cholic acid reduces phospholipid secretion.

Cholesterol secretion into bile originates from either newly synthesised cholesterol or from free cholesterol. Newly synthesised cholesterol constitutes 30% of biliary cholesterol while the remainder comes from lipoproteins which originate from dietary cholesterol or from the endogenous synthesis of cholesterol from various body tissues. Bile salt secretion directly influences biliary lipid secretion probably during transcellular passage of these compounds. The most possible site of interaction could be the *Golgi* apparatus where vesicles containing bile salts and biliary lipids could form.

3.4 BILIRUBIN TRANSPORT

Bilirubin is the major pigment in bile. The major source of bilirubin is from degradation of erythrocytes in the reticulo-endothelial system. Into the circulation bilirubin is reversibly bound to albumin and transported to the liver. The albumin and bilirubin complex binds to the plasma membrane and subsequently bilirubin is transferred to one or more cytosolic proteins. The main two proteins involved are the *Y protein or ligandin* which belongs to the glutathione S-transferase group and the *Z protein* which is a fatty acid binding protein. These proteins facilitate the transfer of unconjugated bilirubin from the inside of the sinusoidal membrane to the endoplasmic reticulum where the enzyme uridinediphosphate glucuronyl transferase esterifies bilirubin with glucuronic acid to form conjugates. These conjugates are non toxic and are excreted in bile.

The canalicular secretion of conjugated bilirubin is an energy dependent process and for conjugated bilirubin interaction with bile acid micelles appears to be important. Further studies by Ostrow JD et al 1986, suggest that unconjugated bilirubin owes its solubility in bile to bile acids. There is little doubt that bile acids are directly involved in the transport of bilirubin and other organic components of bile and taurocholate has been found to enhance transport of these organic ions. Bilirubin and other organic anions may be incorporated in the bile acid mixed micelles within the canalicular lumen.

3.5 BILE FORMATION

Bile formation begins at the level of the canaliculus which is defined as a narrow 1 micrometre groove of the lateral plasma membrane formed between two adjacent hepatocytes. Bile is formed by the net movement of water and solutes into bile canaliculi. The mechanism of hepatic bile flow involves active transport of bile salts and possibly other solutes to which water transport is coupled. Canalicular bile formation has been divided into two types: ***a)*** the *bile acid-dependent* bile flow and ***b)*** *bile acid-independent* bile flow. These two components of bile flow should be viewed as interrelated rather than independent.

3.5.1.Bile acid dependent bile formation (Erlinger S, 1993).

Bile acid-dependent bile formation bears a linear relation between bile acid secretion rate and bile flow. Bile acids may act by two mechanisms to increase bile flow. ***a)***

Since bile acids are in a micellar form in bile they may provide a limited osmotic driving force for water and electrolyte movement. ***b)*** Secondly they may influence bile flow by alterations in the transport of other solutes in bile; for example ursodeoxycholic acid produces hypercholeresis associated with bicarbonate secretion. Bile acids in general promote electrolyte secretion and in particular chloride and bicarbonate.

3.5.2 Cholehepatic circulation (Dumont M et al 1980, Gurantz D et al, 1991)

Unconjugated bile acids are lipophilic and can be easily absorbed across the epithelial cells in the intestine. It is possible that these bile acids may also be readily absorbed from the biliary canalicular lumen. If protonated following their secretion the process would generate bicarbonate and produce a lipophilic bile acid which can passively cross the biliary epithelium, enter the periductular capillary plexus and return to hepatocyte to be resecreted. This recycling may generate an osmotic bile dependent flow with high bicarbonate content.

3.5.3 Bile Acid Independent Bile Formation (Moseley RH, 1993)

In contrast to information regarding the bile acid-dependent bile formation, less is known about the hepatocellular mechanisms underlying bile acid independent bile flow. The inhibition of sodium, potassium, *ATPase* activity does not affect bile acid independent bile flow. It is possible that bicarbonate transport, which plays a primary role in this component of bile formation, is associated with a functional coupling of sinusoidal sodium hydrogen exchange and canalicular chloride bicarbonate exchange. The two systems are coupled via carbonic anhydrase to generate net biliary bicarbonate secretion. Estradiol which causes cholestasis and decrease in bile acid independent bile flow has been found to inhibit sodium hydrogen exchange activity, while ursodeoxycholic acid has the opposite effect.

Acetazolamide which is an inhibitor of carbonic anhydrase produce an inhibition of ursodeoxycholic acid stimulated bile flow. Inorganic electrolytes may not provide the adequate force for bile acid independent bile flow and it has been proposed that organic anions such as glutathione *(GSH)* may provide a major driving force for canalicular bile acid independent bile flow. For example, concentrations that exceed free (non micelle-associated bile acids) *GSH* may generate important osmotic driving for canalicular bile formation. Although the biliary tract is impermeable to glutathione and therefore retains it, the hydrolytic products may well be reabsorbed and resecreted into bile further enhancing the choleresis.

CHAPTER 4
PATHOPHYSIOLOGY OF GALLSTONE FORMATION

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PATHOPHYSIOLOGY OF GALLSTONE FORMATION

4.1 INTRODUCTION

Gallstones represent a major health problem in Western societies where the prevalence of gallstone disease is around 10%. the frequency of gallstones increases progressively with age and is greater in women than in men. It is believed that about 15 or 20 million people have gallstones in the United States; there are 500,000 cholecystectomies performed each year. In the United Kingdom 6 million people have gallbladder stones. In most patients (60-80%) the gallstones are asymptomatic. Fortunately gallstone disease has a fairly low mortality; however, the overall mortality of open cholecystectomy is around 0.5% in U.S.A. and this would mean about 2,500 patients die each year as a result of gallstones. Furthermore, often cholecystectomy is associated with significant post-operative morbidity particularly in less well equipped hospitals. For all these reasons gallstone disease is a quite significant cause of morbidity and has considerable socio-economic importance in the general population.

4.2 HISTORICAL ASPECTS

Although cholelithiasis is a common disease with an increased incidence in the Western world it is hardly a new problem. A priest of Amey from the twenty first dynasty around *1000 BC* is the first recorded patient to suffer from gallstone disease. Examination of her mummy revealed multiple gallbladder stones (Beal S, 1984). However, the physicians started to recognise this, as a disease around the fifth century *AD* and the Greek physician Alexander *(525-605 AD)* described what appears to be a common bile duct stone (Thudichum, 1863). In the fourteenth century when the study of human anatomy in post mortem examinations became common, the Italian Physician Gentile da Folingo demonstrated the presence of gallstones in a post mortem dissection (Glenn and Grafe, 1966). Around *1500 AD* the interest in gallstone increased and the first speculation about the cause of gallstones started; Paracelsus expressed the opinion that chemical imbalances in the biliary system might promote precipitation of solids in bile (Duane WC, 1992). Frances Glisson (1597-1677) described in detail a case of biliary colic followed by icterus and passage of stones per rectum (Walter and Snell, 1940).

Later in the nineteenth century more speculations about the pathophysiology of gallstones were developed and the interest in biliary tract surgery was increased. In 1867 John S Bobs performed a cholecystostomy (Cutter, 1928) and in the same year other surgeons in the United States and Europe undertook similar procedures. Since then, the exponential increase in medical knowledge has led to exciting developments not only in the pathophysiology of gallstone formation but also in the treatment of gallstone disease with advanced techniques such as lithotripsy or percutaneous laparoscopic cholecystectomy.

4.3 NATURAL HISTORY OF GALLSTONES

Once the gallstones form they do not usually disappear. 85-90% of gallstones remain asymptomatic and of the 10% which are symptomatic only one third cause severe symptoms. However, once symptoms occur about 35% of such patients will develop significant symptoms over a period of eleven years (Wenckert A and Robertson B, 1966). Other studies suggest that 50% or more of subjects with symptomatic gallstones will continue to have symptoms usually at intervals of less than one year (Thistle JC et al, 1982).

Biliary colic is the commonest presenting symptom, but more severe complications include cholangitis, pancreatitis, gangrenous cholecystitis, perforation of the gallbladder or gallstone ileus. Because of the likelihood of progression to more severe symptoms once the stones become asymptomatic, it is recommended that they should be treated definitely, usually with surgery. It should be noted that gallstone disease is a risk factor for carcinoma of the gallbladder but this is a relatively unusual tumour and treatment for gallstones is not recommendedfor that reason alone (Duane WC, 1992).

Once nucleation, which is the primary event in the process of gallstone formation, has occurred the annual rate of growth of the gallstone is around 2-3 mm per year. The mean period of growth is around 8 years and the stones are liable to cause symptoms at around 8-12 years from when the stone was initiated. Cholecystectomy for symptomatic gallstone disease is usually performed 15 years from nucleation (Bouchier IAD and Neoptolemos J, 1993).

4.4 COMPOSITION AND TYPE OF GALLSTONES

A simple and useful classification of gallstones is into cholesterol and pigment stone (Trotman BD et al 1974; Soloway RD et al 1977). Cholesterol stones account for around 80% of gallstones in the Western world.

Cholesterol stones can be divided into two subgroups: *a) pure* cholesterol and *b) mixed* cholesterol stones. The former contain almost exclusively cholesterol with little or no pigments, calcium or protein. A cross section of the stone shows a more radiant than laminated appearance and this represents cholesterol crystals radiating out from the centre of the stone. Commonly these stones are single but they may be multiple.

The other type of cholesterol stone, which is the mixed type, by definition should contain more than 50% cholesterol but often such stones contain over 80% of cholesterol. The other constituents are usually calcium, pigments, carbonate, phosphate and protein. These stones have a tendency to grow in the laminar configuration and they are most often small and multiple. The cholesterol in gallstones is mainly in the form of cholesterol monohydrate crystals and hydroxy-cholesterol (Sutor DJ and Wooley SE, 1969). Calcium carbonate may be found as vaterite, calcite and aragonite; for calcium phosphate there is apatite and whitlockite (Bean JM et al, 1979). Often the centre of the mixed cholesterol stones contains pigmented material which is mainly calcium bilirubinate, which is often trapped in an organic matrix which is possibly glycoprotein. As the stone grows the cholesterol crystals accumulate in a layered fashion giving a laminated appearance.

Pigment stones can also be divided into two types (Soloway RD et al 1977; Duane WC, 1992). The common irregular mulberry pigment stones which are composed mainly of calcium, bilirubinate and to a lesser extent by calcium carbonate, phosphate and protein. These stones are usually small, multiple and 50% of these stones are radiopaque. On a cross section they amorphous or merely crystalline in appearance. These stones can be simply termed as *black stones.* Black stones are sometimes sub-divided according to the presence of calcium carbonate as black carbonate and black non carbonate pigment stones.

The second type of pigment stone is the *brown stone* which tends to form in the bile ducts and it is often associated with infection of the biliary tree. Like the black stones they are made up mainly of calcium and bilirubinate, but this is in crystalline form. In addition they contain around 20-30% free fatty acids, mainly in the form of palmitate and stearate. These compounds are thought to form due to the action of bacterial phospholipases on biliary lecithin and reinforce the hypothesis that these stones are infectious in aetiology. They are almost always radiolucent.

Cholesterol and pigment stones appear to follow different pathophysiological mechanisms in their formation and because cholesterol stones are more common than the pigment ones, most of the research on the pathogenesis of gallstone disease has been focused on this type of stone.

4.5 PATHOPHYSIOLOGY OF CHOLESTEROL GALLSTONES

4.5.1 Supersaturated bile

There is extensive knowledge on the pathogenesis of cholesterol gallstones and many of the principles of pathogenesis for cholesterol stones are common to all types of stones. The first step in cholesterol gallstone formation is the secretion by the liver of supersaturated bile in cholesterol. Cholesterol is virtually insoluble in water and is held in solution in bile, by its association with bile salts and phospholipids in the form mixed micelles. This complex physico-chemical relationship has been defined by Small and his colleagues in 1966 and 1968. Bile can become supersaturated with cholesterol either because there is too much cholesterol or too little bile acid or phospholipid or both to maintain the cholesterol in aqueous solution. These relationships are well demonstrated by the triangular co-ordinate plot (Figure 4). Cholesterol saturation in bile and the potential for precipitation occurs when cholesterol constitutes more than 10% of the total lipids in bile. The triangular co-ordinate plot (diagram) however fails to demonstrate a definite distinction between normal and stone-forming bile.

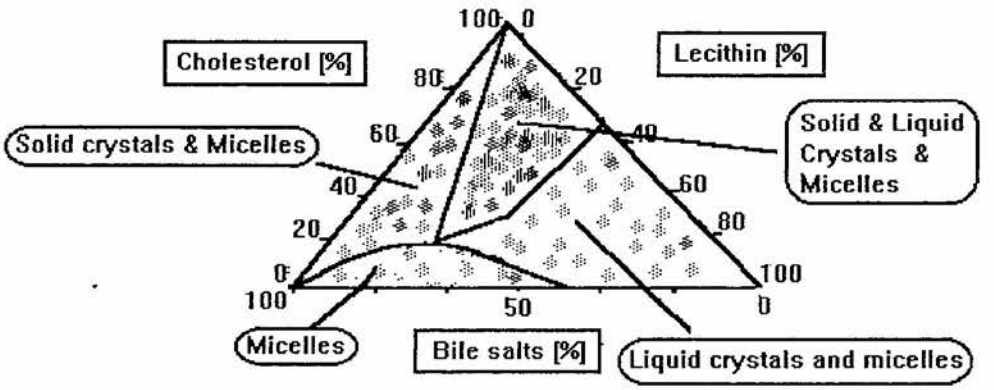


Figure 4: Phase diagram of cholesterol solubility in bile (Small DM & Admirand WH, 1968).

Subsequent investigators have used other indices, such as the solubility index according to the equation of Thomas PJ and Hofmann AF in 1973.

The saturation of bile with cholesterol varies during the day and with food intake. Total cholesterol and phospholipid secretion is decreased during interdigestive periods, but due to the relatively greater decrease of bile acids secretion, the degree of cholesterol saturation in hepatic bile is high. In contrast, in the postprandial period there is a relative increase in bile acid secretion compared to cholesterol and phospholipids and the result is better solubilisation of cholesterol (Afdhal NH and Smith BF, 1991).

The events which lead to cholesterol solubilisation are much more complex than those appreciated initially (Donovan JM and Carey MC, 1993) and are diagrammatically represented in figure 5.

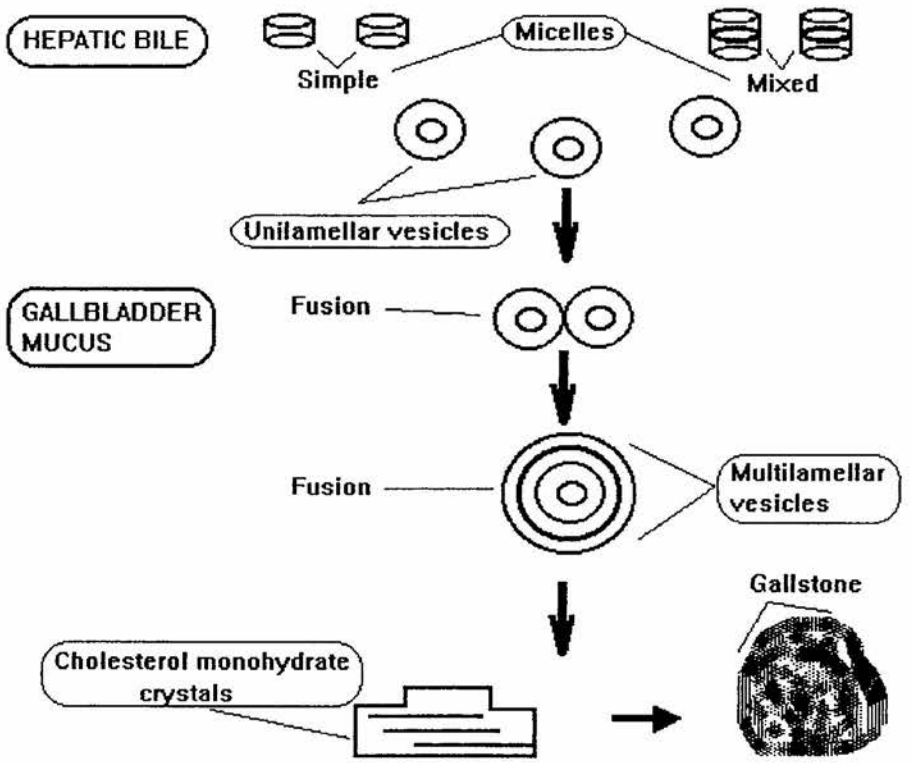


Figure 5:Cholesterol is transported in bile as micelles and vesicles, which fuse into multilamellar vesicles within mucus and form crystals which serve as the nidus of gallstones.

In unsaturated bile, the cholesterol/phospholipid vesicles are secreted by the hepatocyte and solubilised by bile acids into mixed lipid micelles which are thermodynamically stable . However, if during the storage of bile in the gallbladder, the cholesterol concentration exceeds that which can be transported in micelles the excess is transported in unilamellar phospholipid vesicles.These vesicles are an aqueous

suspension of laminan liquid crystals and are thermodynamically labile *(metastable zone).* Fusion and aggregation of these unilamellar vesicles in cholesterol supersaturated bile leads to formation of multilamellar vesicles which is considered a critical first step in the precipitation of cholesterol monohydrate crystals and subsequent gallstone formation There are however gaps in our understanding and the identification of the factors which would influence aggregation and fusion of these vesicles. A high total lipid concentration and a molar ratio of cholesterol to phospholipids in vesicles greater than 1, would accelerate *in vitro* aggregation of those vesicles in supersaturated bile.

4.5.2 Nucleation

Supersaturated gallbladder bile is found in 40-80% of normal persons, many of whom will never develop gallstones (Holzbach RT, 1973). Data from the National Cooperative Gallstone Study (Hofmann AF et al 1982) supports the fact that the difference in the biliary lipid composition between normal and gallstone patients is astonishingly small. An explanation may rest with the stability of the metastable zone which, as already mentioned, is formed by unilamellar phospholipid vesicles. These vesicles can precipitate given enough time and the right change in conditions. Gallbladder contraction is often enough to evacuate from the gallbladder without the vesicles ever precipitating (Duane WC, 1992).

For precipitation to occur nucleation is necessary and on the basis of the above observations the emphasis has shifted from documenting bile chemistry to attempting to define and understand the phenomenon of nucleation in bile. Holan KR et al 1979, studied the *nucleation time* which represents the time taken for a stone-free bile to form crystals. They found that although the mean nucleation time for normal bile was 15 days, it was only 3 days for bile from cholesterol gallstone patients. It has therefore been hypothesised that rapid nucleation can occur in gallstone patients due to either excess of promoters, or a deficiency of inhibitors of crystalisation, or both. *In vitro* comparison of the bile from patients without gallstones with lithogenic biles with an identical cholesterol saturation index, revealed a prolongation of the nucleation time of the native bile when lithogenic bile was added. This would suggest the presence of nucleating factor(s) in the bile of patients with gallstones. Low molecular weight proteins delay the nucleation time and this includes the apolipoproteins A_1 and A_2 (Sewell RB et al 1983). In contrast high molecular weight proteins may act as nucleation promoting agents (Burnstein MJ et al 1983). In the gallbladder, mucin which is a major secretory product of the epithelium is a high molecular weight protein which plays a central role in

gallstone formation. Hypersecretion of mucin causes a concentration dependent acceleration of cholesterol crystal nucleation. Calcium bilirubinate has also been suggested to promote nucleation (Burnstein MJ et al 1983). Recently De Bruijn MAC et al 1992 and Abei M et al 1993, presented evidence of *42 KD* cholesterol crystalisation promoter protein (former) and of *130 KD* heterodimer inhibitor of cholesterol nucleation (latter) in human bile.

The above concepts support the hypothesis of so-called *heterogeneous* nucleation as the principle mechanism of nucleation in bile. Heterogeneous nucleation is defined as a pathway in which an exogenous molecule facilitates aggregation and crystal formation at a relatively low degree of cholesterol supersaturation. *Homogeneous* nucleation can also occur but this is rare and would require, in the absence of nucleating factors, excessive amounts of cholesterol in bile.

4.5.3 Other Factors which Influence Nucleation

The variation in the saturation of bile during the 24 hours and the degree of mixing within the gallbladder are important determinants of cholesterol crystal formation. Bile in the gallbladder may form layers of non-homogenuous bile (Campbell BA and Burton AC 1949) and this stratification of bile may be of importance in precipitation of cholesterol, in particular if it is associated with diminished gallbladder emptying (Fisher RS et al 1982).

Abnormalities in the metabolic function of the gallbladder mucosa may also influence the solubility in bile. Active water and electrolyte absorption leads to high concentrations of cholesterol-rich vesicles and simultaneous hypersecretion of mucus, which is postaglandin mediated, promotes the formation of a viscous gel from the surface of the gallbladder favouring vesicles fusion within this gel. In addition, the release of mucosal enzymes into the lumen may influence biliary lipids and their solubility. Lysolecithin which is present in human gallbladder epithelial lysosomes can adversely influence cholesterol solubility in bile and also a number of esterases and hydrolases can influence biliary composition (Kouroumalis E et al, 1982 and 1984). Very little is known about the influence of bile pH on vesicle aggregation and crystal formation. It is, however, known that in the case of mixed cholesterol stones significant reduction in bile pH in particular very near the gallbladder epithelium may cause denaturation of bilirubinate and precipitation of this pigment (Alvaro D et al 1990).

4.5.4 Gallbladder Motility and Stasis

Biliary stasis was an early hypothesis to explain gallstone formation (Fisher RS et al 1982). Although there is little doubt that impaired gallbladder motility would fail to empty the gallbladder completely so that the likelihood of crystal formation and precipitation in the gallbladder bile would increase, the general role of stasis and altered gallbladder motility in the pathogenesis of cholesterol stones has yet to be clarified. The main controversy is whether the abnormal gallbladder emptying precedes gallstone formation or whether the presence of stones alters emptying. A recent ultrasonographic study by Festi D et al 1990, showed that patients with gallstones had larger fasting gallbladder volumes and increased residual volumes after gallbladder emptying; however the amount of bile and the rate of emptying were similar to normal controls. Van der Werk SDJ et al. 1987, studied 14 patients without gallstones, and found a significant positive correlation between gallbladder emptying and lithogenicity in bile. From those studies , as well as others, it is increasingly recognised that gallbladder hypomotility is an early feature of human cholelithiasis and contributes to the evolution of stones. It has been proposed that cholesterol transfer from supersaturated bile to the gallbladder wall may alter the lipid composition of gallbladder smooth muscle cell membranes , which in turn might affect gene expression for the apocrine signals that influence the contractile state of the smooth muscle (LaMorte MM, 1993).

Patients on long term parenteral nutrition develop biliary sludge and in about 50% of cases progress to formation of stones. In addition patients with somatostatinomas show reduced gallbladder motility and 65% of the patients have gallstones. It is believed that somatostatin inhibits release of cholecystokinin which greatly reduces gallbladder contraction. Prairie dogs on lithogenic diet are much less likely to form stones if they are periodically injected with cholecystokinin or after sphincterotomy. Oral contraceptives may predispose the development of cholesterol stones by slowing gallbladder emptying although they may have an additional effect by increasing cholesterol supersaturation in bile.

4.5.5 Biliary Sludge

Biliary sludge is identified by ultrasonography as an echogenic material which migrates to the dependent portion of the gallbladder, but lacks the typical acoustic shadowing seen with stones. Sludge represents a tenacious mucus gel in which precipitates of cholesterol monohydrate crystals and calcium bilirubinate are found. The pigmented matrix of cholesterol stones in biliary sludge has an identical composition

suggesting that sludge probably forms the pigmented matrix which subsequently binds cholesterol crystals into gallstones. It is possible that this gel also interacts with calcium bile salts and cholesterol to reduce further the solubility of bilirubin and cholesterol and therefore more of this material becomes trapped into the gel. Studies on patients on long term parenteral nutrition (Messing B et al 1983) suggest that sludge is a reversible state which may disappear if normal feeding is restored.

4.6 PATHOPHYSIOLOGY OF PIGMENT STONES

Pigment stones represent the other major type of gallstones. By definition they must contain less than 50% cholesterol and 20% of stones in the Western Society fall into this group where in mainly Asian countries much higher percentages are found. There are two types of pigment stones, black and brown. They have different chemical composition pathophysiology and pathogenesis as well in risk factors.

4.6.1 Black Stones

Black stones are the commonest type of pigment stones in the Western world. They form almost exclusively in gallbladder, they are small, irregular and often multiple and composed primarily of calcium bilirubinate (up to 80%) with variable amounts of calcium carbonate and calcium phosphate (Ostrow JD, 1984). Cholesterol rarely exceeds 10% while mucin glycoprotein constitutes around 30% of the solid matter black stones.

Several risk factors for black stone formation have been identified (Donovan JM and Carey MC, 1993). In chronic haemolytic disorders there is a greatly increased production of bilirubin from haem both in the form of bilirubin monoglucuronide and bilirubin diglycuronide. Non enzymatic hydrolysis and increased glucuronidase activity contribute to increased biliary unconjugated bilirubin concentrations. Patients with chronic liver disease have also increased levels of bilirubin (mono and diglucuronide) secondary to haemolysis; they also secrete less bile acids with reduced solubilisation of unconjugated bilirubin. Patients with impaired ileal absorption of bile salts would also develop pigment stones. The black stones are mostly composed of calcium salts of unconjugated bilirubin deposited in the mucin glycoprotein matrix. Unconjugated bilirubin exists as a dianion and monoanion form and while the monoanion form is soluble at physiological pH the dianion salt is most likely to precipitate. Free radicals may induce polymerisation of calcium bilirubinates to form an insoluble dianion polymer of calcium bilirubinate (Cahalane MJ et al 1988).

An important property of the unconjugated bilirubin is its extreme sensitivity to calcium and even a small concentration of ionised calcium would readily form a calcium bilirubinate salt and precipitate (Gallinger S et al, 1986). Calcium is mostly transported into bile passively and it is in equilibrium with serum ionised calcium.

Mucus hypersecretion also plays an important role and in association with impaired gallbladder emptying may increase the likelihood of pigment stone formation. Inhibitors of calcium phosphate and calcium carbonate precipitation are present in bile but they have not been characterised. The role of pH on the solubility of the inorganic calcium salts has not been adequately addressed and will be investigated in the present thesis.

4.6.2 Brown Pigment Stones (Donovan JM & Carey MC, 1993)

The brown gallstones are again composed of calcium salts of unconjugated bilirubin in a mucin glycoprotein matrix. In contrast to black stones, calcium bilirubinates in the brown variety are not polymerised. Brown pigment stones are crystalline and laminated and contain mucus glycoproteins of ductal origin as well as bacterial glycoproteins. Remnants of bacteria can be seen by scanning electron-microscopy within the brown pigment stones. Bile stasis and bacterial infection precede the development of brown pigment stones. Material which migrates from the gallbladder or bacterial infection may serve as the nidus for brown pigment stones. Bacterial enzymes hydrolyse biliary lipids and form insoluble products. Bacterial action also forms free fatty acids, in particular palmitic and stearic acids as well as lysolecithin (Cetta F 1991) . Ionised calcium binds to free fatty acids forming insoluble calcium salts. Pteryglucuronidase from bacteria, hydrolyses conjugated bilirubin which is more likely to precipitate.

Brown pigment stones tend to form in the biliary tree rather than the gallbladder and once the nidus has been formed the vicious cycle of prolonged biliary stasis and chronic bacterial infection starts. The main pathophysiological difference of brown stones from the other types of gallstones is that these are formed as a result of profound metabolic physico-chemical alteration in bile, while the other types of stones are a result of an excess of normal biliary constituents.

4.7 EPIDEMIOLOGY OF GALLSTONES

Epidemiological studies of gallstone disease have provided important information regarding the prevalence of this disease worldwide. They also give important indicators regarding its aetiology. It is of interest that certain American-Indian communities such as the Pima Indian females have the highest prevalence rates in the world due to production of supersaturated bile (Sampliner RE et al, 1970). In contrast over the last few years the incidence of pigment stones in these subjects has greatly decreased and this is probably related to a reduction in the biliary duct parasites and changing diet. There are also genetic factors which may determine gallstone formation and these would explain geographical and racial differences in the prevalence of gallstones around the world.

Gallstone disease increases with age and in particular there is a sharp increase in prevalence of gallstones after the age of 70, the maximal frequency occurring in patients over 80 (Bateson MC and Bouchier IAD, 1985; Godfrey PJ et al, 1984). There is also consistent evidence that gallbladder disease is more common in females and in particular, the number of pregnancies is associated with an increased risk of gallstones. Bile saturation is increased in pregnant women, particularly during the second and third trimester (Kern F Jr et al, 1981). The use of oral contraceptives induces an increased risk of gallbladder disease. This may be a combination of both alteration of the composition of bile with more supersaturated bile induced by oestrogens and also because of the oestrogen influence on gallbladder motility (Braverman DJ et al, 1980).

Obesity is another independent factor in the development of gallstones. The obese individual produces supersaturated cholesterol bile while bile salt pool size and secretion is normal or increased in obesity (Shaffer EA and Small DM, 1977).

Diabetes mellitus is associated with an increase in the risk of developing gallstones. In a recent study, however, the prevalence of gallstones in diabetes was increased only after the seventh decade (Hayes PC et al, 1991). There is no doubt that in diabetes several metabolic abnormalities in bile have been identified, probably consequent to the disturbed insulin and glucose metabolism. However, it has not been possible so far to isolate specific biochemical defect which would predispose to gallstone formation in diabetic patients. Many of the bile abnormalities in diabetics can be explained because of the associated obesity and high triglycerides. Autonomic neuropathy may affect gallbladder motility and favour gallstone formation. Clofibrate therapy has been found to increase the prevalence of gallstones by increasing biliary cholesterol (Bateson MC et al, 1978).

It is debatable whether diet is important in the development of gallstones. Several studies came up with conflicting results regarding various elements of the diet and in particular the fine carbohydrate or lack of dietary fibre. It has however clearly been

demonstrated that a moderate intake of alcohol would reduce the risk of gallstones (Thornton J et al, 1983). Prolonged fasting periods may also increase the risk of gallstones by prolonging the time which bile stays within the gallbladder. Cirrhosis is associated with an increased prevalence of gallstones (2 or 3 times compared to normal population) mainly of the pigment variety (Bouchier IAD, 1969). The most likely explanation is that pigment stones form as a result of haemolysis secondary to hypersplenism.

Inflammatory bowel disease is associated with four times increased risk of gallstone disease, if there is terminal ileum involvement or resection. Patients with ilial dysfunction have bile supersaturated with cholesterol and this is probably due to bile salt malabsorption resulting in a diminished total bile salt pool (Dowling RH et al, 1972). Gastric surgery, in particular, truncal vagotomy may be associated with increased prevalence of gallstones by affecting gallbladder emptying (Shaffer EA et al, 1982). Total parenteral nutrition is associated with formation of biliary sludge and tends to be reversed once oral feeding is restored (Messing B et al, 1983). Finally patients with chronic haemolytic syndromes tend to form pigment stones due to increased production of haem degradation products with end result an increase in bilirubin concentration bile.

4.8 ROLE OF THE GALLBLADDER EPITHELIUM IN THE PATHOGENESIS OF GALLSTONE FORMATION

The concept of diseased bile supersaturated with cholesterol or pigments has been the focus of intensive research to clarify the mechanism of gallstone formation over the years. However, as the knowledge of the pathophysiological mechanisms of gallstone formation has been increasing it has been realised that the gallbladder mucosa can significantly alter bile composition to either protect or facilitate gallstone formation.

The gallbladder mucosa may play an important role in the regulation of calcium within the gallbladder bile. Most gallstones contain a central core of calcium salts around which layers of cholesterol or calcium bilirubinate are deposited (Bean J M et al, 1979). Lowering of intraluminar calcium concentration reduces the incidence of stone formation in experimental animals (Strichartz SD et al, 1989). In addition biliary calcium ions can reduce solubility of biliary cholesterol making cholesterol nucleation more likely. Biliary calcium can stimulate mucus glycoprotein secretion by the gallbladder mucosa and this effect can be blocked by calcium antagonists (Malet PF et al, 1986). Although the exact mechanisms by which the gallbladder mucosa handles calcium are not known there is little doubt that intraluminal calcium concentration can be influenced indirectly by the concentrating ability of the mucosa or the alteration of bile pH due to either bicarbonate absorption or hydrogen ion secretion by the mucosa.

Inflammation of the gallbladder epithelium can be associated with increased production of prostaglandins which may be of relevance to the formation of gallstones. Prostaglandins are important mucus secretagogues and can also reduce sodium and water absorption by the mucosa producing a more dilute gallbladder bile (Leyssac P et al, 1974). A cholesterol rich diet results to increased synthesis of prostaglandins by the gallbladder mucosa and subsequently mucus secretion into the bile. Treatment with either aspirin or non steroidal anti-inflammatory drugs has clearly been shown to reduce mucous secretion (Wood JR et al, 1988). Prostaglandins also have an effect on motility of the gallbladder.

The lipid content of the bile may also be an important determinant of gallbladder function. During the early stages of cholesterol gallstone formation there is an increase in water and electrolyte absorption by the gallbladder (Conter RL et al, 1986). The gallbladder mucosa of animals and man normally absorbs cholesterol from the bile and this absorption increases when bile contains a large amount of cholesterol. Inflammation of the epithelium, however, may be associated with secretion of cholesterol from the mucosa into the gallbladder lumen (Svanvik J et al, 1986).

When the present work started there was uncertainty whether the gallbladder epithelium was capable of secreting hydrogen ions. The significance of alterations in bile pH had not been adequately investigated. Studies in rabbit (Whitlock RT et al, 1969), guinea-pig (Heintz K et al, 1981) and necturus gallbladders (Weinman SA et al, 1982) had produced evidence for mucosal hydrogen secretion during sodium reabsorption probably representing a sodium hydrogen exchange. More recently it has been suggested that the canine gallbladder has the ability to secrete hydrogen ions in vitro (Rege RV et al, 1987). There are however no studies on the human gallbladder mucosa dealing with the mechanisms of bile acidification and it is not clear whether this is because of hydrogen ion secretion or bicarbonate reabsorption. Acid secretion by the gallbladder has important implications for gallstone formation because the majority of gallstones contain calcium carbonate and changes in the pH of the bile are of significant importance in influencing the calcium solubility in bile. Furthermore, very little is known about acid base regulation across the gallbladder epithelium and the mechanism of hydrogen ion production, in particular, in the human gallbladder and the effect of inflammation of the mucosa on the acidification ability of the gallbladder. The present thesis will address some of these questions and investigate the effect of bile pH changes on inorganic calcium salts solubility. A further clarification of the mucosal factors which influence gallstone formation can lead to therapeutic manipulation of mucosal functions which predispose to gallstone formation and eventually allow the possibility of prevention of gallstones.

EXPERIMENTAL WORK

CHAPTER 5
EXPERIMENTS ON HUMAN GALLBLADDER EPITHELIUM

CHAPTER 5 (PRELIMINARY WORK)
EXPERIMENTS ON HUMAN GALLBLADDER EPITHELIUM

5.1 BACKGROUND AND AIM

In man as well as in other species there is a decline in gallbladder bile pH compared with hepatic bile. Initially this was thought to be the result of bicarbonate reabsorption by the gallbladder mucosa. Studies in rabbit (Whitlock RT et al, 1969), guinea-pig (Heintz K et al, 1981) and necturus gallbladders (Weinman SA et al, 1982) had produced evidence for mucosal hydrogen secretion during sodium reabsorption probably representing a sodium hydrogen exchange. More recently it has been suggested that the canine gallbladder has the ability to secrete hydrogen ions *in vitro* (Rege RV et al, 1987). However, studies on human gallbladder mucosa dealing with bile acidification are rare and it is not clear whether acidification is because of hydrogen ion secretion or bicarbonate reabsorption. The present study is undertaken as preliminary work to specifically answer the following questions:

1.Is the human gallbladder mucosa capable of acidifying luminal solutions in vitro?
2.Is the acidification due to hydrogen ion production?
3.What is the effect of inflammation on acidification?

5.2 MATERIAL.

Studies were undertaken using fresh human gallbladder mucosa obtained at the time of elective open cholecystectomy usually for gallstone disease or as a part of a Whipple's procedure for cancer of the pancreas when the gallbladder was removed as part of the operative procedure. Patient 's sex, age, and type of gallstones , if any, were recorded.

5.3 METHODS.

The gallbladder tissue was retrieved within 15 min of the ligation of the cystic artery. A circular piece of the gallbladder wall *(d=12 mm)* was removed using a tissue punch and placed in Ringer-Krebs glucose bicarbonate solution (table 2) at 4° C and preoxyganated with 95% O_2 and 5% CO_2. To minimise hypoxia , this was done as soon as the gallbladder was removed.

The gallbladder was transfered to the laboratory and within five minutes the mucosa was stripped by blunt dissection from the muscular part of the wall, rinsed with Ringer-Krebs bicarbonate glucose (RKBG) solution to remove debris and bile and mounted in an Ussing chamber (Ussing HH, 1951).

TABLE 2

NaCl	**0.154 M**
$CaCl_2$	**0.11 M**
KCl	**0.154 M**
K_2PO_4	**0.154 M**
Na_2CO_3	**1.3%**
Mg_2SO_4	**0.154 M**
Glucose	**0.3 M**

Composition of Ringer-Krebs bicarbonate glucose solution

5.4 DESCRIPTION OF THE USSING CHAMBER (figure 6).

The Ussing chamber consisted of two 2 ml volume compartments each of which communicated with a glass tube above, through two plastic tubes (inlet and outlet). The gallbladder tissue separated the two compartments creating a 'mucosal compartment' at the mucosal site and a 'serosal' compartment on the opposite side. The term 'serosa' is used throughout to indicate the non-luminal surface of the mucous membrane. Both compartments were filled with 10ml RKBG through the glass tubes (2ml in each compartment of the chamber and 8ml at each glass tube). The mucosal compartment was sealed while 95% O_2 and 5% CO_2 (2l/min) was bubbled through to the serosal compartment only. Preliminary experiments had shown that pO_2 between mucosal and serosal compartments did not differ significantly if 95% O_2 / 5% CO_2 was bubbled into the serosal compartment only and this was preferrred in order to avoid disruption of the 'unstirred water layer' in the mucosal side. The whole system was covered by a thermostatic water jacket to maintain a constant temperature of 37 ° C throughout the experiment. A pair of silver/silver chloride matrix 1mm electrodes *(Clark Electrochemical Instruments, Reading , Berks, U.K.)* monitored the potential difference across the two sites of the tissue and were used as an index of viability of the tissue. Each study lasted for 70 minutes. One millilitre of the solution was removed through a micropipette from each compartment of the Ussing chamber at two minutes , 45

minutes and 70 minutes and was immediately analysed in an 1302 pH / blood gas analyser *(Instrumentation Laboratory system, Lexington, MA, U.S.A.)* for pO_2, pCO_2, hydrogen ion *($[H^+]$)*, and bicarbonate *($[HCO3^-]$)* concentrations. Gallbladders which were macroscopically grossly distorted or damaged were excluded from further study.

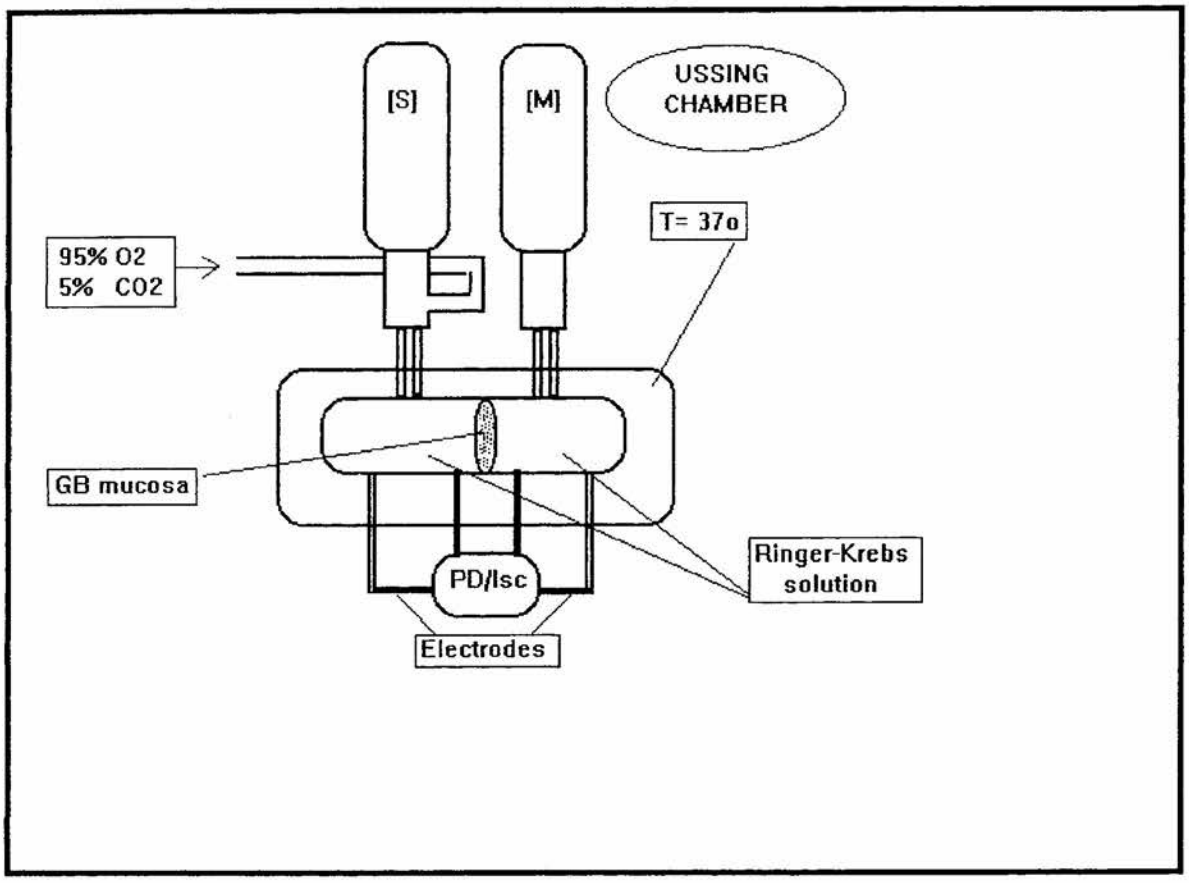


Figure 6. The Ussing Chamber.

5.5 EXPERIMENTS

Preliminary Experiments

Ten preliminary experiments were performed to validate the method and assess the best way of administering O_2 / CO_2. These experiments lasted for 70 min. One ml of the solution was removed through a micropipette from each compartment of the Ussing Chamber every 2 min for 10 min, at 45 min and 70 min and were immediately analysed

in an 1302 pH/blood gas analyzer *(Instrumentation Laboratory System, Lexington MA)* for pO_2 . Gallbladders which were macroscopically grossly distorted or damaged were excluded from further study.

Main Experiments

Forty gallbladders were studied.The experiments were divided into three groups.

First group

Five gallbladders were studied. The effect of oxygen deprivation and immersion in formaldehyde 4% on the resting potential difference was observed. 95% O_2 / 5%CO_2 was bubbled at a steady flow rate of 2l/min from time 0 to 20 min, then stopped for 10 min from time 20 to 30 min and subsequently continued at the same flow rate until the end of the experiment. Thereafter the gallbladder mucosa was exposed to 4% formaldehyde for 2min, rinsed with RKGB solution and re-mounted to the Ussing Chamber and studied for further 10 min. The potential difference *(PD)* was monitored continuously thoughout the experiment.

Second group

Twenty-five gallbladders were studied. Twenty-one were processed according to the standard protocol to maintain viability and four were immersed to formaldehyde 4% for 2min before being mounted on the Ussing Chamber. The hydrogen ion concentration , bicarbonate concentration pCO_2 and pO_2 were measured at the beginning (2min) at 45min and the end of the experiments (70min). The resting transepithelial potential difference was monitored throughout. The aim at this group of experiments was to study the acidification capacity of the viable gallbladder compared with non-viable tissue.

Third group

Ten gallbladders were studied. In the first set of experiments involving 6 gallbladders the mucosal bathing solution was replaced by sodium free Krebs-Ringer solution (NaCl and NaHCO3 removed) and the analytes mentioned above were measured. In the second set of experiments involving 4 gallbladders 2 mM amiloride (a specific Na^+/H^+

inhibitor) was introduced into the mucosal compartment and its effect on acidification was observed after 45 min.

5.6 PATHOLOGY

A small sample of the tissue under study was fixed in 10% buffered formaline before each Ussing Chamber experiment.The rest of the tissue was also fixed in 10% buffered formaline at the end of the experiment. An experienced histopathologist examined each gallbladder blindly on two occasions and reported on the degree of cholecystitis and the viability of the tissue according to cell morphology at the beginning and end of the experiments. The degree of cholecystitis was graded from 1 (mild) to 3 (severe) (Table 3) according to the appearance of the mucosa, muscle layer thickness, presence of Rokitansky-Aschoff sinuses and degree of inflammatory process (Symers W, 1978). The morphology changes were recorded as 0 (healthy looking cells), 1 (mild morphological changes such as cell oedema, presence of granules, vacuolation), 2 (moderate but without evidence of cell necrosis).

	Grade ①	**Grade ②**	**Grade ③**
Mucosa	Folded with columnal cells	Only few folds with columnar cells	Thin flat with ulcerations
Muscle layer thickness	Normal	Thicker than normal	Thick fibrotic
Rokitansky-Aschoff sinuses	Sinuses found frequently between muscle bands	Normal sinuses are still present	Diverticula between the muscle bands
Degree of inflammation	Mild	Mild / moderate	Moderate / severe

TABLE 3: Classification of chronic cholecystitis

The gallbladders used in the above experiments were selected according to the following criteria; (a) satisfactory macroscopic appearance with no obvious ulceration or fibrosis in the beginning of the study and no obvious damage during the study ; (b)

expression of a resting potential difference more than 2mV of serosa positive. Data from mucosa with severe changes in cell morphology were excluded from further analysis .

5.7 STATISTICAL ANALYSIS OF DATA.

Data is expressed as Mean ± standard error of mean *(SEM)* . The data was not normally distributed and was analysed using non- parametric tests; Mann-Whitney test was used for unpaired data and Wilcoxon test for paired data. A 'p' value of 0.05 was taken as significant.

5.8 RESULTS ON THE USSING CHAMBER EXPERIMENTS

5.8.1 Preliminary Studies

In this set of experiments there was satisfactory diffusion of O_2 across the the gallbladder mucosa when O_2 was bubbled in the serosal side. The equilibrium was apparent at 6 min of the experiment. It was therefore unnecessary to bubble O_2 in the mucosal side and therefore to prevent disruption of the unstirred water layer. The results are presented in Table 4.

COMPARTMENT	*2 min*	*6 min*	*45 min*	*70 min*
Serosal pO2 (kPa)	29.1±3.2	27.3±2.1	25.3±2.1	29.1±2.3
Mucosal pO2 (kPa)	14.4±2.6	24.4±4.2	23.5±2.2	26.2±3.3
p-value	<0.01	*NS*	*NS*	*NS*

TABLE 4: pO2 in serosal and mucosal compartments of the Ussing Chamber

5.8.2 First set of Experiments

In the first set of experiments the gallbladders (N=5) documented a mean potential difference of 7.5±3.5 mV serosa positive after 5min in the chamber. This *PD* remained quite stable throughout the ensuing 15 minutes. There was a significant drop in the potential difference when 95% oxygen / 5% CO_2 was discontinued, but when reapplied the potential difference recovered to levels similar to those prior to oxygen deprivation indicating that hypoxia had direct effect on reducing the resting *PD*. However when those gallbladders were immersed to formaldehyde 4% and remounted on the Ussing Chamber

there was a dramatic and irreversible drop in the *PD* indicating non - viability of the tissue *(Figure 7)*.

There was no significant difference in pO2 between the serosal and the mucosal compartment at 10 min. (25.3 ±2.1 kPa *vs* 23.5±2.2 kPa respectively) which shows that the diffusion of oxygen through the gallbladder mucosa was adequate. It was thought therefore unecessary to oxygenate the mucosal compartment directly, as this would disrupt the "unstirred water layer". All gallbladders showed evidence of chronic cholecystitis (three grade ①, two grade ②) without any significant changes in cell morphology at the beginning and end of the experiments.

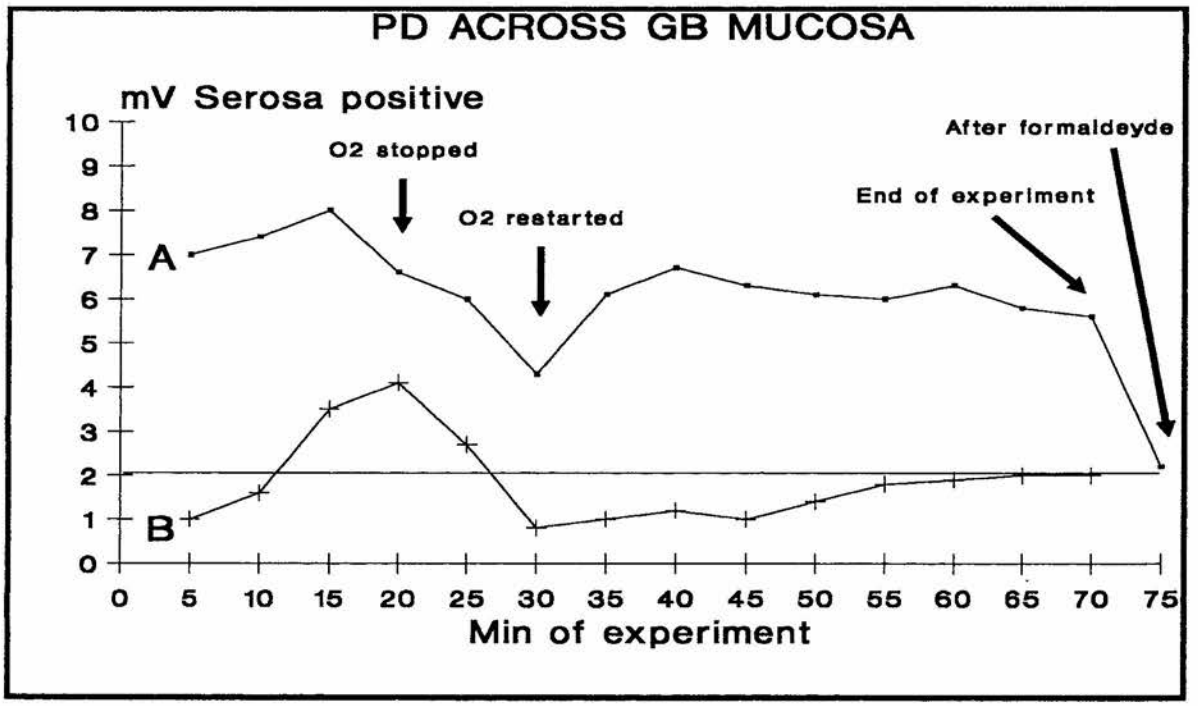


Figure 7: The potential difference across the human gallbladder mucosa; A: represents a viable gallbladder which demonstrated an initial PD of 7 mV. Oxygen deprivation or immersion in formaldehyde resulted in a drop in the potential difference across the epithelium. PD recovered when Oxygen was reintroduced. B represents a gallbladder which demonstrated an initial PD of less than 2mV. Although PD recovered after 15 min , oxygen deprivation resulted in a non-reversible fall in the PD. Gallbladders with initial PD < 2mV were excluded from further study

In the viable gallbladder the mean PD did not significantly change throughout the experiments.However, there was a significant difference in the PD between the viable

and the non -viable gallbladders studied (6.3±2.6 to 1.3±0.9, $p<0.01$) at 5 min. The PD of the non-viable gallbladders rapidly declined to zero.

5.8.3 Second set of experiments

25 gallbladders were studied (18 females, 7 males ; 13 contained cholesterol stones, 9 pigment stones, 3 biliary sludge).Four of those were immersed in formaldehyde and used as controls and from the remaining 21, six showed evidence of grade 3 chronic cholecystitis and were not included in the analysis outlined below.

There was a significant increase in the hydrogen ion concentration from 2 minutes to 45 minutes and to 70 minutes observed in the mucosal compartment ($p<0.01$) while in the serosal compartment there was a significant decrease in hydrogen concentration ($p<0.05$). By contrast in the non-viable gallbladder (those immersed in formaldehyde) there was a significant drop in hydrogen concentration on the mucosal site without any significant change of hydrogen concentration in the serosal side *(figure 8)*.

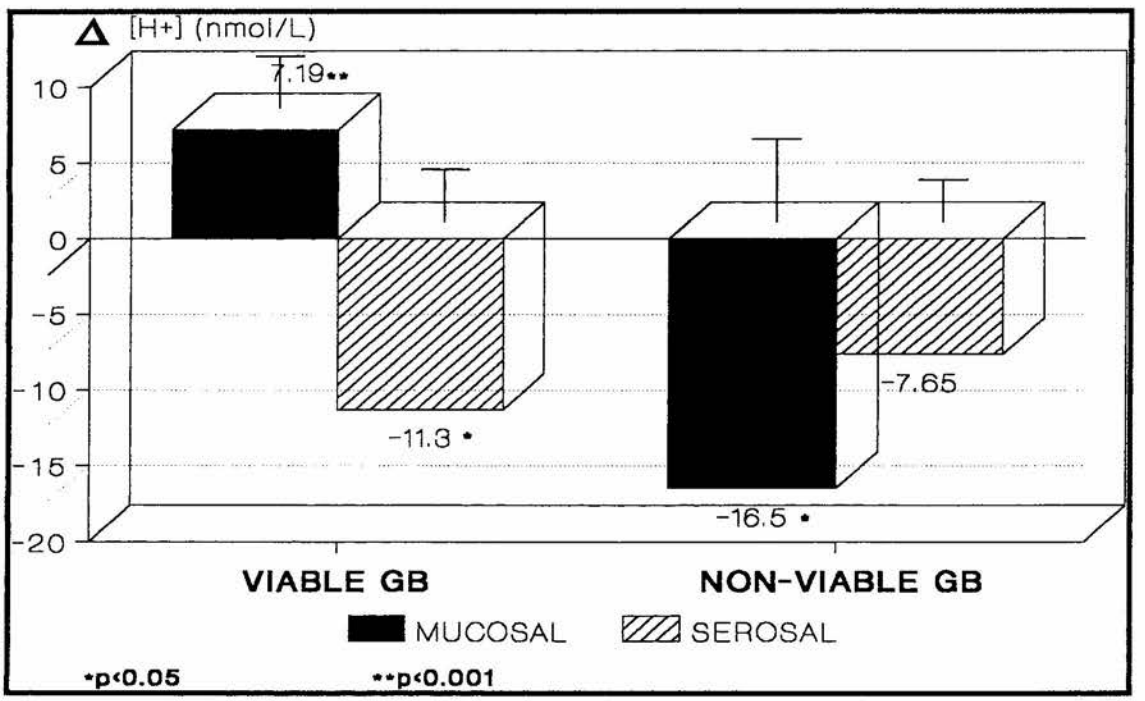


Figure 8: Differences in hydrogen ion concentration between 70 and 2 min in the mucosal and serosal side of the viable and non-viable gallbladders (*p<0.05,**p<0.001).

In the viable gallbladder bicarbonate concentration significantly decreased in the mucosal compartment from 2 minutes to 45 and 70 minutes ($p<0.02$) while on the serosal

side there was a significant increase of bicarbonate concentration from 2 minutes to 70 minutes. In the non viable gallbladder an increase of bicarbonate concentration was observed in both compartments *(figure 9)*.

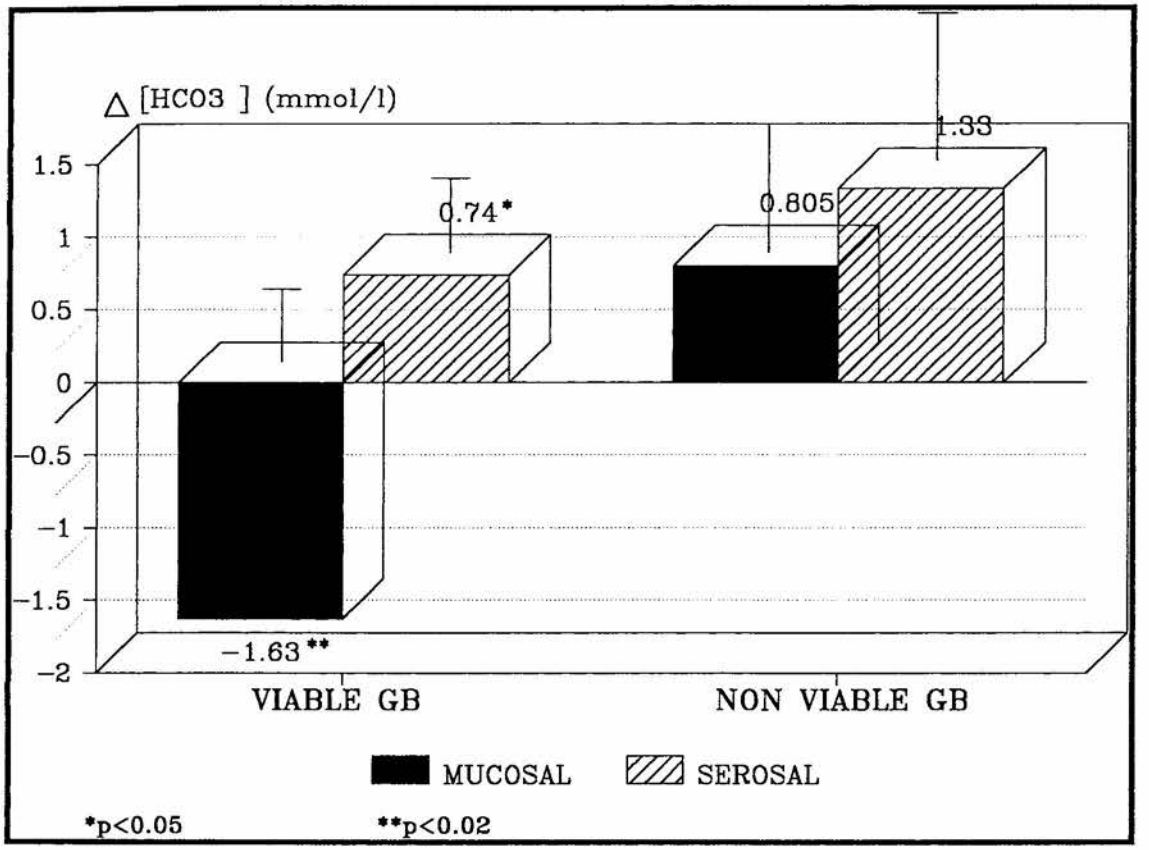


Figure 9:Differences in bicarbonate concentration between 70 and 2 min in the mucosal and serosal side of the viable and non-viable gallbladders (*p <0.05, ** p<0.02).

In the viable gallbladder there was a significant increase in pCO_2 between 2 and 70 minutes on the mucosal site ($p<0.03$) while in the serosal compartment a significant decrease in pCO_2 was observed ($p<0.05$). In the non viable gallbladder there was a drop in pCO_2 in both compartments being significant in the mucosal side ($p<0.01$) *(figure 10)*.

When the acidification ability of the 21 gallbladders was plotted against the degree of chronic cholecystitis there was a progressive and statistically significant decrease of hydrogen ion secretion from grade ① (mild) to grade ③ (severe) cholecystitits *(figure 11)*.

The same was true when hydrogen ion secretion was plotted against the degree of cell morphology changes graded from ⓪ to ② indicating that gallbladder epithelia with impared viability had a tendency to secrete less hydrogen ions *(figure 12).*

There were no differences in the acidification capacity of the gallbladders with sex or type of gallstones, although epithelia from gallbladders with pigment stones had a higher ability to absorb bicarbonate ($\Delta\ [HCO3^{=}] = -1.68 \pm 0.49$ mmol/l (pigment) vs 3.8 ± 0.74 mmol/l (cholesterol) , $p<0.01$).

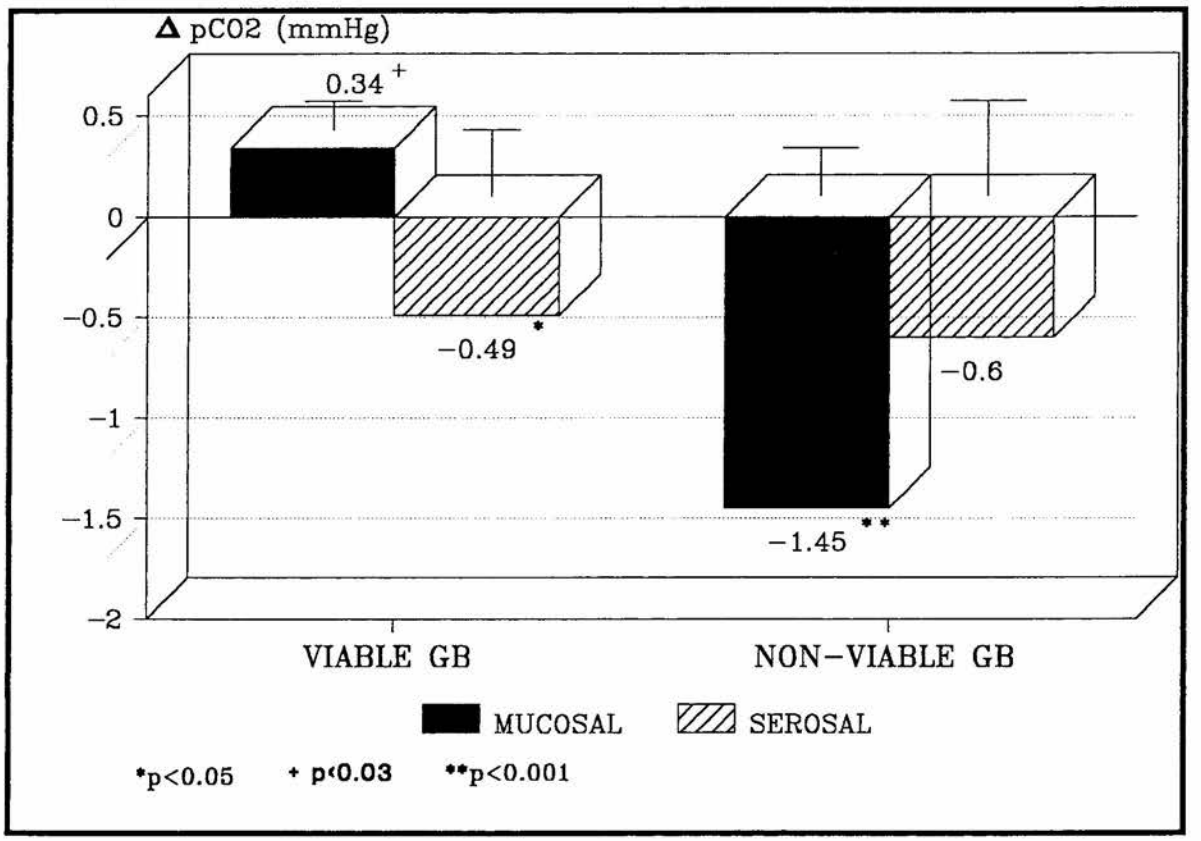


Figure 10: Differences in pCO_2 between 70 and 2 min in the mucosal and serosal side of the viable and non-viable gallbladders (* p <0.05, + p<0.03, ** p<0.001).

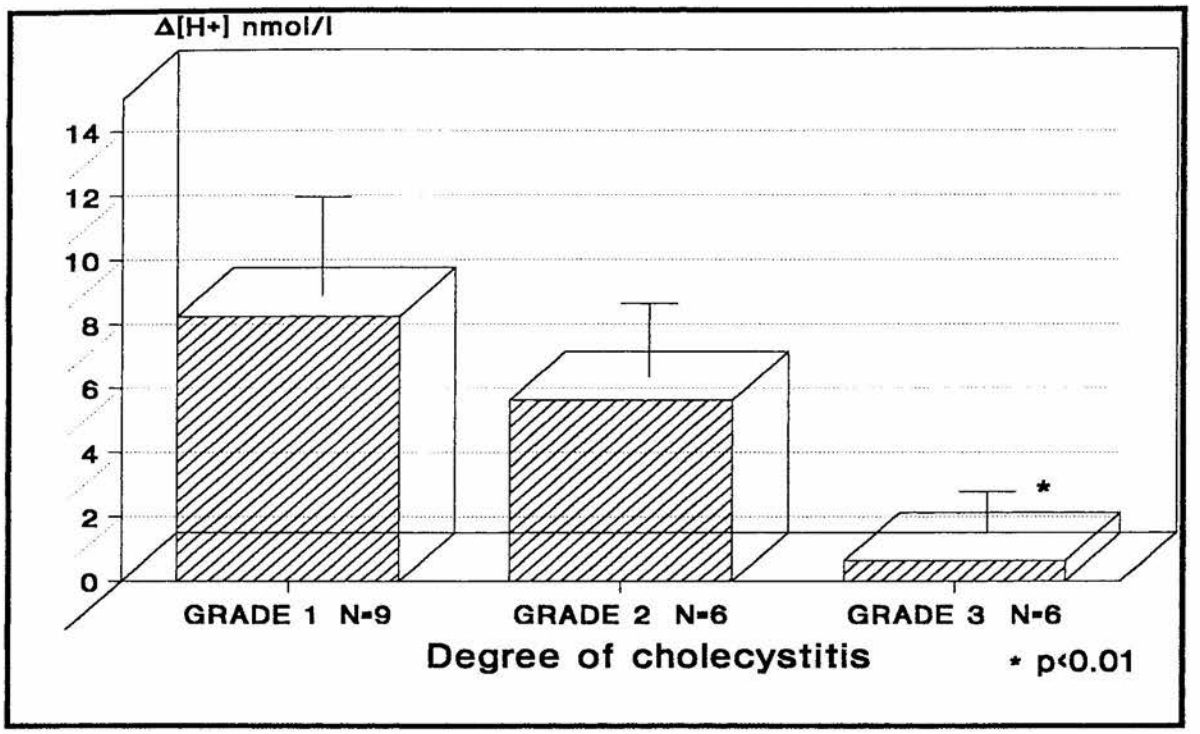


*Figure 11: Differences in hydrogen ion secretion with histology (grade 1 (mild) to grade 3 (severe) cholecystitis) * p<0.01.*

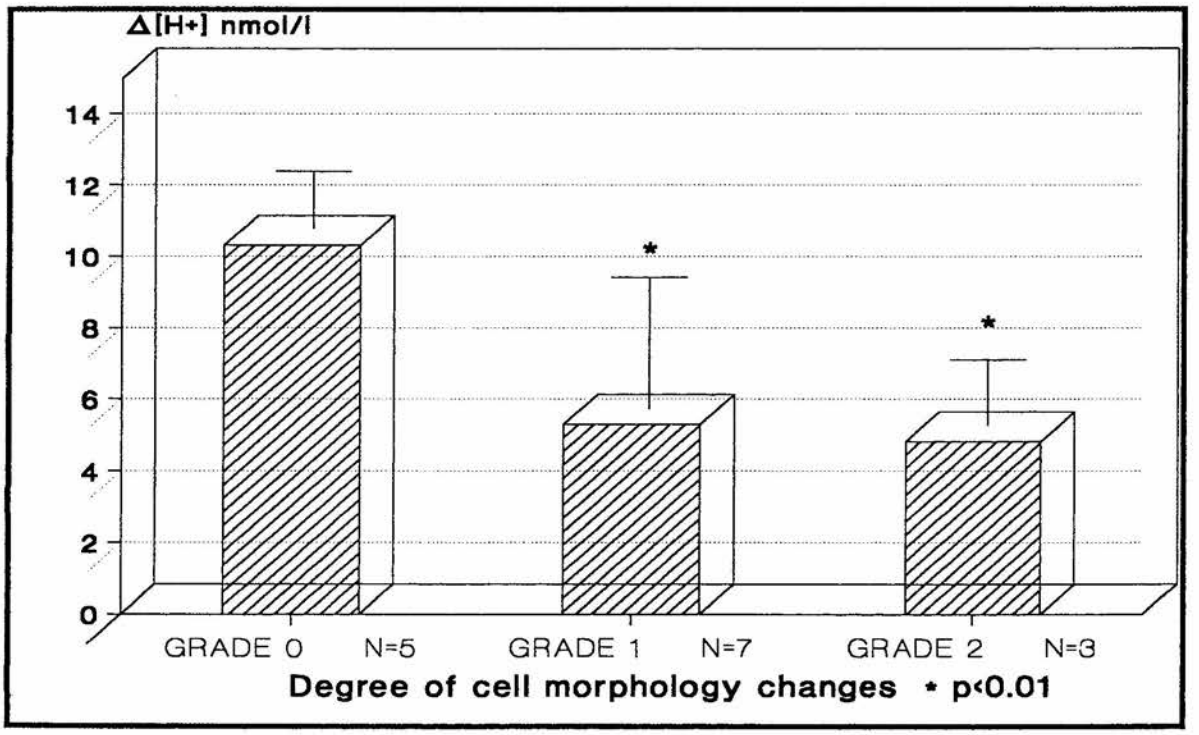


*Figure 12: Differences in hydrogen ion secretion with cell morphology changes.(grade O: healthy cells, 1: mild morphological changes suggesting "sick cells", 2: moderate changes without evidence of necrosis) * p<0.01.*

5.8.4 Third set of experiments

In the third set of experiments (N=10) R-K-G-B solution was replaced with sodium free isotonic solution (containing LiCl) in the mucosal side. No significant differences in the $[H^+]$, $[HCO3^-]$ and pCO_2 were observed in any site of the Ussing chamber, between 2 and 70 min of experiment. Similarly, the use of amiloride (2 mM) in the mucosal side abolished acidification *(Figure 13)*.

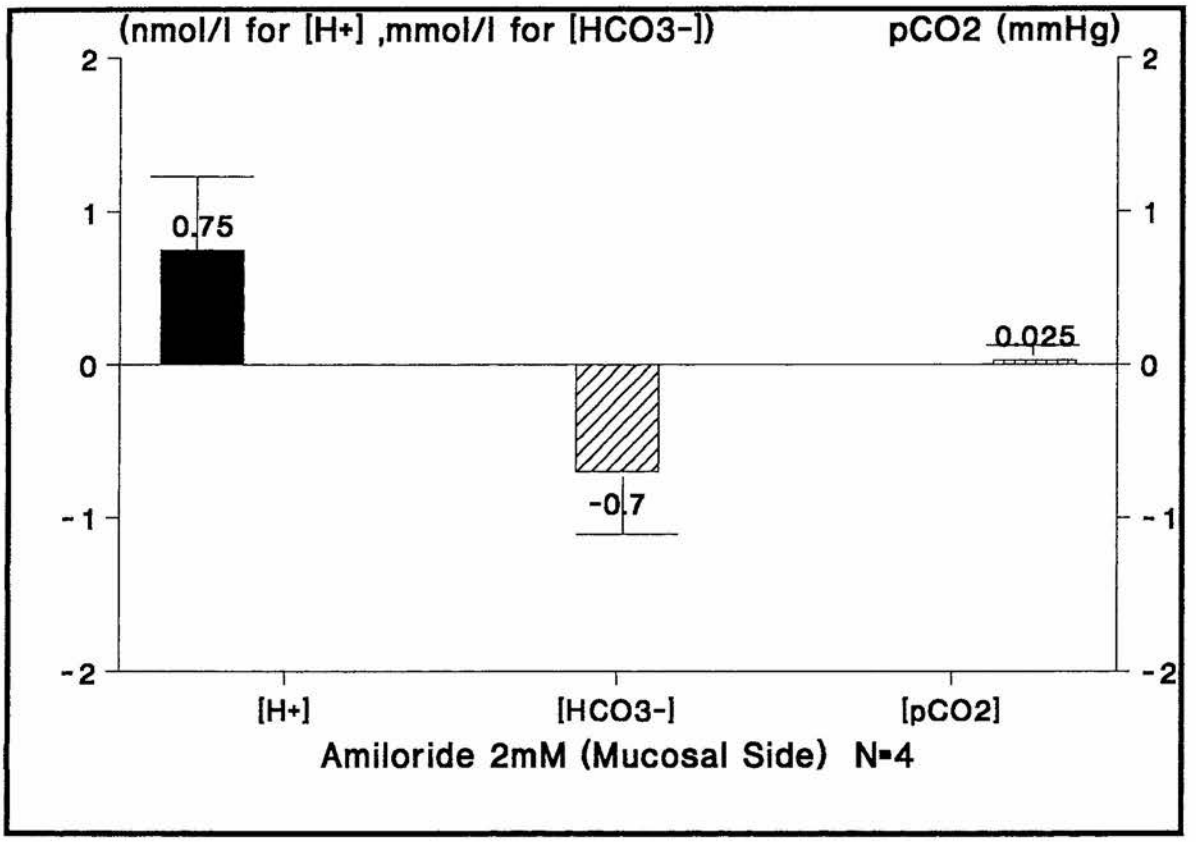


Figure 13: Effect of mucosal application of amiloride (2mM) on hydrogen ion secretion.

5.9 CONCLUSIONS

In this study using the Ussing Chamber technique, epithelia from 40 fresh human gallbladders were studied *in vitro*. Four specific questions were addressed and the results of this study indicate:

a) *Viable human gallbladder epithelium is capable of acidifying mucosal solutions in vitro in contrast to the non-viable gallbladder.* The increase in hydrogen ion concentration in the mucosal site was associated with an increase in pCO_2 and a reduction in bicarbonate concentration in the mucosal site of the viable gallbladder; this provides indirect evidence of active acid secretion from gallbladder epithelium rather than bicarbonate absorption only.

b) *Hydrogen ion secretion from the human gallbladder appears to be affected by inflammation and morphological changes of the cells.* Less inflamed gallbladders and more healthy looking cells appeared to produce more hydrogen ions than compared to gallbladders with significant cholecystitis and morphological changes of the epithelial cells.

c) The results of the third set of experiments suggest that *hydrogen ions are produced by a sodium-dependent process* which is possibly linked to bicarbonate absorption, because when sodium was abolished from the solutions or amiloride was used, acidification was stopped.

The above results provide evidence that the human gallbladder epithelium is capable of secreting hydrogen ions.

CHAPTER 6
EXPERIMENTS ON BOVINE GALLBLADDER

CHAPTER 6
EXPERIMENTS ON BOVINE GALLBLADDER

6.1 AIM

In this set of experiments the aim was to investigate the mechanism of hydrogen ion secretion by the normal gallbladder epithelium and its regulation, as well as the effect of various pharmacological agents on hydrogen ion production.

6.2 METHOD

BACKGROUND

In the previous chapter, the Ussing chamber method has been described and was used to investigate hydrogen ion secretion by the human gallbladder epithelium. However the Ussing chamber has the following limitations:

a) only a very small piece of tissue can be used,

b) the fluid volume used is small,

c) as a result of the above, the amount of hydrogen ions secreted per hour is small.

These limitations made this technique unsuitable for pharmacological manipulations which were necessary to study the regulation of hydrogen ion secretion by the gallbladder epithelium . In order to overcome this , we used a whole gallbladder perfusion system which was modified to enable the study of the electrophysiology and hydrogen ion production of the whole organ. The advantages of such a system are:

a) There is very little manipulation of the tissue and the strucuture of the organ is preserved

b) The magnitude of changes in terms of hydrogen ion production is high and this facilitates pharmacological studies on the regulation of acid secretion.

DESCRIPTION OF THE WHOLE GALLBLADDER PERFUSION SYSTEM

. The whole gallbladder, after retrieval and drainage of bile, was rinsed twice with Ringer-Krebs solution containing N-Acetyl-cysteine as a mucolytic agent (250 mcg/ml) to wash out the rest of bile, mucus, debri and blood. A plastic cannula was then

introduced through the neck into the gallbladder. The neck of the gallbladder was tied around the cannula to ensure no spillage of fluid during the study while permiting the gallbladder to be held in the bath. The gallbladder was mounted on a rigid stand and suspended in the bathing solution of a Pyrex beaker. The whole system was placed into a water bath at a constant temperature of 37° *(figure 14)*. The bathing solution consisted of a buffer solution (usually Ringer-Krebs bicarbonate) continuously oxygenated with 95% O_2 / 5% CO_2. When Ringer-Krebs *without* bicarbonate was used in the serosal side, the bathing solution was oxygenated with 100% O_2. Each of the the bovine gallbladders was filled with 100ml of solution. The term *' mucosal '* was used to indicate the luminal side of the gallbladder , whilst the term *' serosal '* was used to indicate the outside of the organ. The kind of luminal solutions used was dependent upon the type of experiments. The volumes of the mucosal and serosal bathing solutions remained the same throughout the study.

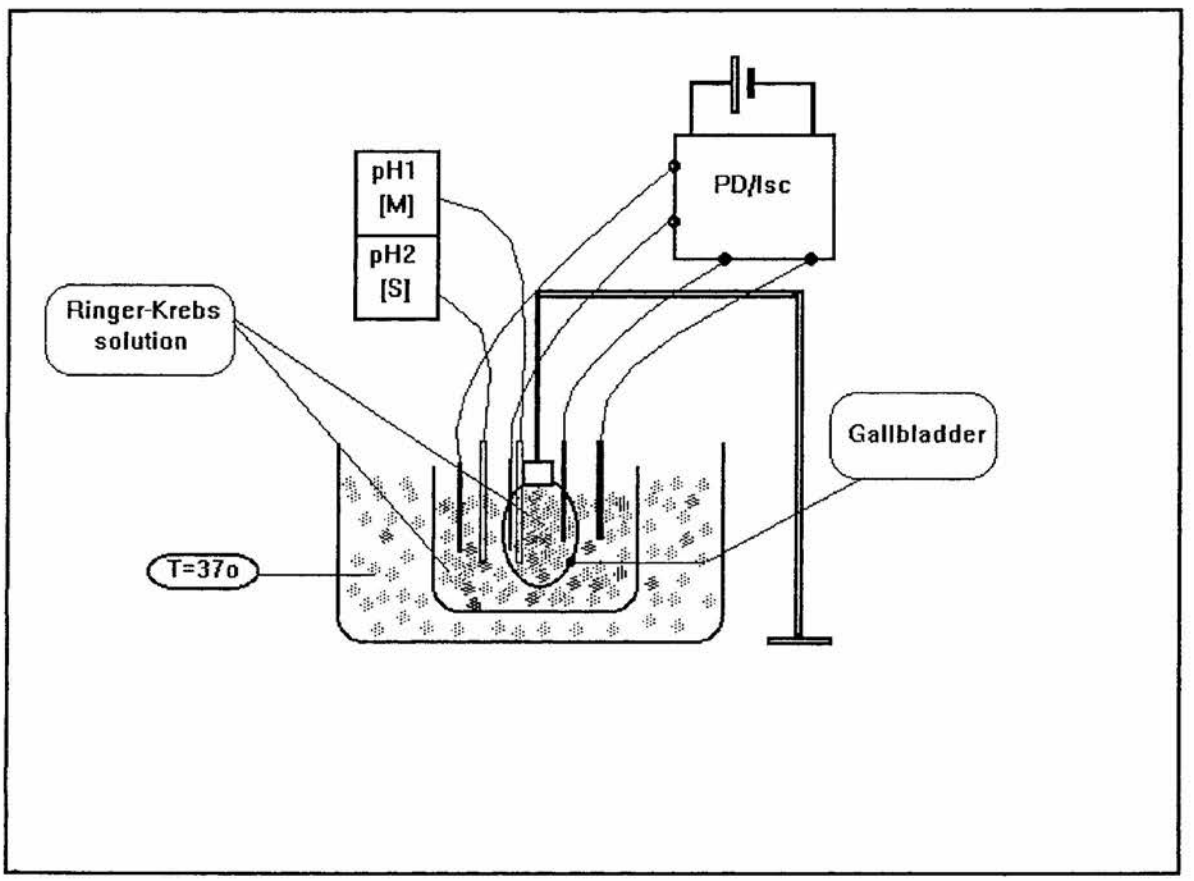


Figure 14: Whole gallbladder perfusion system

The pH of the mucosal and serosal bathing fluid were measured by means of shielded tubular glass electrodes (*Russell pH electrode, type CMAWL/4/5 $HgCl_2$, Auchtermuchty Fife, UK).* These pH electrodes were filled with saturated KCl solution in contact with a silver wire. The electrodes were calibrated at the beginning of each experiment with standard buffer solution at pH 7.0. The electrode for measuring the mucosal pH was passed through the cannula, positioned in the centre of the gallbladder and supported by the cannula. The serosal pH measuring electrode was positioned on the outside of the gallbladder.

This model also permited the study of the electrophysiological parameters of the gallbladder, which were used as an index of viability of the tissue. The electrical potential difference *(PD)* across the gallbladder epithelium was measured by a pair of silver / silver chloride matrix 1mm electrodes *(Clark Electrochemical Instruments, Reading, Berks, U.K.)* through a pair of agar (4%) bridges. One of the agar bridges was positioned, through the cannula, into the lumen and the other on the serosal side of the gallbladder, 2 cm apart. The electrodes were connected through insulated cables to a digital millivoltmeter (M83 Autocal pH Meter, Radiometer Copenhagen) . The transmural *PD* was measured with the serosal surface negative with respect to mucosal surface.

The tissue could be short-circuited by applying external current *(Isc)* through a similar pair of silver / silver chloride electrodes which were connected to agar bridges. Again one of the agar bridges was positioned, through a different hole on the cannula, into the lumen and the other on the serosal side of the gallbladder. Tissue resistance *(R_{tis})* was determined by recording the *PD* deflection in response to *$10\mu A / cm^2$* direct current from the external electrical source.

$$R_{tis} = \Delta (PD) / Isc$$

The electrical asymmetry of the system was measured prior to mounting the tissue and at the end of each experiment to allow for appropriate corrections of the electrical measurements to be made.

6.3 EXPERIMENTAL PROTOCOLS

BOVINE GALLBLADDER MODEL

Intact bovine gallbladders were studied *in vitro* using the above described experimental model. The gallbladders were retrieved from the local abattoir immediately after animal death.and transfered to the Laboratory within 30 min. All animals were at similar fasting state and the time of slaughter was approximately 10 am. The bile was collected anaerobically immediately after retrieval and the gallbladder was placed in a container containing preoxygenated Ringer-Kreb's solution at 4° C and transferred to the laboratory. The isolated gallbladder was mounted on the whole organ perfusion system as described in section *6.2* . The volume of the bathing solution was 200ml and the volume of the luminal solution was 100ml .

Each study lasted for 60 min after an initial 10 min period of stabilization; the mucosal and serosal pH were continuously monitored as well as the electrophysiological parameters *(PD ,R_{tis})* of the epithelium. The acidification ability of the gallbladder epithelium and the effect of several pharmacological agents was studied in a series of *in vitro* experiments. In the results section, pH values were expressed as hydrogen ion concentration *($[H^+]$)* in nmol/l . The following experiments were carried out:

6.3.1 First group: Electrophysiology.

Aim: To study the electrophysiological parameters of the viable bovine gallbladder in relation to mucosal hydrogen ion secretion ability .

Eight (8) bovine gallbladders were studied for 80 min. Both the mucosal and the serosal side contained identical solutions (Ringer-Krebs bicarbonate); only the serosal bathing was oxygenated with 95% O_2 / 5% CO_2. The pH was continuously monitored in both sides together with the electrophysiological parameters ***(PD, R_{tis})*** of the gallbladder throughout the experiments.

N_2 was bubbled in the serosal side from 60-70 min in order to study the effect of anoxia on pH and the electrophysiological parameters of the gallbladder. Oxygen supply was restored between 70-80 min of the experiment.

6.3.2 Second group: amount and rate of acidification

Aim: To calculate the amount of hydrogen ion which is secreted by the mucosal side of the bovine gallbladder and the factors which may influence the rate of acidification.

First set: Fourteen (14) bovine gallbladders were studied for 60 min. The sex of each animal was recorded. The mucosal side contained 100ml of unbuffered solution (NaCl 0.9%) in order to augment pH changes.The serosal side contained Ringer-Krebs *without* bicarbonate, to achieve a comparable pH with that in the mucosal compartment ; only the serosal bathing was oxygenated with 100% O_2. The starting pH of the mucosal solution was between 6.8-7.0 ($[H^+]$=160-100 nmol/l) and of the serosal solution approximately 6.7 ($[H+]$=200 nmol/l). The pH was continuously monitored in both sides. The rate of acidification was calculated between 0-60 min as well as between 0-20 min, 20-40 min and 40-60 min separately. The amount and rate of acidification was studied in relation to the sex of the animal.

Second set: In sixty-four (64) gallbladders, the amount and rate of acidification was studied between 0-20 min. This time-period (0-20 min), was used as a control period prior to use of pharmacological agents to study the mechanisms of acid secretion. The mucosal side contained 100 ml of unbuffered solution (NaCl 0.9%) in order to augment pH changes whilst the serosal side contained Ringer-Krebs *without* bicarbonate; only the serosal bathing was oxygenated. Again the amount and rate of acidification in relation to the sex of the animal and the bile pH were studied. The bovine bile was collected anaerobically prior to each gallbladder experiment and bile pH was measured as it is described in section *7.2.*

Third set: Twenty-two gallbladders (22) were studied. The starting mucosal pH was set from 4.7 to 6.4 by addition of 0.1% N HCl to the mucosal solution whilst the serosal side contained Ringer-Krebs. The number of experiments per starting pH is shown in table 5.

TABLE 5

Starting pH	*Number of experiments*
6.6	2
6.4	8
5.8	4
5.3	5
4.7	3

Only the serosal bathing fluid was oxygenated; pH changes in the mucosal compartment were monitored from 0-30 min .

6.3.3 Third group: Effect of sodium.

Aim: To investigate whether hydrogen ion secretion from the gallbladder epithelium is a sodium-dependant process.

First set: Twelve (12) bovine gallbladders were studied for 60 min. The serosal bathing solution was Ringer-Krebs and was continuously oxygenated with 100% O_2. The luminal (mucosal) side contained an unbuffered solution in order to augment pH changes. In six (6) experiments, *dextrose 5%* was used from 0-30 min followed by *NaCl 0.9%* from 30-60 min in the mucosal (luminal) side. In the next six (6) experiments, the above solutions were used in reverse order *(NaCl 0.9%* from 0-30 min, followed by *dextrose 5%* from 30-60 min). The effect of sodium-free solutions on luminal acidification was studied.

Second set: The effect of *amiloride* (which is a selective *sodium / hydrogen* antiport inhibitor) was studied in five (5) bovine gallbladders. The serosal bathing solution was Ringer-Krebs and was continuously oxygenated with 100% O_2. The luminal (mucosal) side contained NaCl 0.9% (unbuffered) solution in order to augment pH changes.

The experiment was divided into three 20 min periods ; the total experimental period was 60 min. The first 20 min was used as a control period for each gallbladder. At 20 min amiloride was added into the lumen to a final concentration of 100μM (16 mcg/ml) and mucosal hydrogen ion secretion was monitored for 20min. At 40min the luminal solution was replaced by NaCl 0.9% and hydrogen ion secretion was monitored for further 20 min.

6.3.4 Fourth group: Effect of ouabain.

Aim: To investigate whether hydrogen ion secretion is a sodium-dependant, ouabain sensitive process.

Four (4) bovine gallbladders were studied for 80min. The mucosal side contained 100 ml of unbuffered solution (NaCl 0.9%) (to augment pH changes) whilst the serosal side contained Ringer-Krebs *without* bicarbonate; only the serosal bathing was oxygenated. The experiment was divided into four 20 min periods ; the total experimental period was 80 min. The first 20 min was used as a control period for each gallbladder. At 20 min, 1ml of *ouabain* (250 mcg/ml), which is a $Na^+ / K^+ / ATP$-*ase* inhibitor, was added into the lumen to achieve a final concentration of 5 mcg/ml (0.5 mM) and mucosal hydrogen ion secretion was monitored for 20min. At 40min the luminal solution was replaced by fresh NaCl 0.9% and ouabain was added to the serosal compartment (4ml, to achieve a final concentration of 0.5 mM) and hydrogen ion secretion was monitored for further 20 min.At 60 min the serosal solution was replaced with fresh Ringer-Krebs and the mucosal acidification was monitored for further 20min.

6.3.5 Fifth group: Effect of acetazolamide.

Aim: To study the effect of the carbonic anhydrase inhibitor , acetazolamide, on hydrogen ion secretion by the gallbladder mucosa.

Eight bovine gallbladders were studied for 60 min. The mucosal side contained 100 ml of unbuffered solution (NaCl 0.9%) (to augment pH changes) whilst the serosal side contained Ringer-Krebs *without* bicarbonate; only the serosal bathing was oxygenated. The experiment was divided into three 20 min periods ; the total experimental period was 60 min. The first 20 min was used as a control period for each gallbladder. At 20 min, *acetazolamide* was added into the lumen and mucosal hydrogen ion secretion was monitored for 20min. At 40min the luminal solution was replaced by fresh NaCl 0.9% and hydrogen ion secretion was monitored for further 20 min. In four gallbladders, a concentration of 10^{-3} M of acetazolamide was used, whilst in the other four gallbladders 10^{-4} M of acetazolamide was used.

6.3.6 Sixth group: Role of histamine.

Aim: To investigate whether hydrogen ion secretion is an histamine- dependant process.

First set: The effect of a selective H2 antagonist *(famotidine)* and a non-selective histamine receptor antagonist *(diphenhydramine)* on mucosal acidification was studied.

❑ Five (5) gallbladders were studied using *famotidine*. The mucosal side contained 100 ml of unbuffered solution (NaCl 0.9%) (to augment pH changes) whilst the serosal side contained Ringer-Krebs *without* bicarbonate; only the serosal bathing was oxygenated. The experiment was divided into four 20 min periods ; the total experimental period was 80 min. The first 20 min was used as a control period for each gallbladder. At 40 min, famotidine (10^{-5} M) was added into the lumen and mucosal hydrogen ion secretion was monitored for 20min. At 60 min famotidine was added to the luminal solution to a final concentration of 10^{-4} M. At 60 min the luminal solution was replaced by fresh NaCl 0.9% and hydrogen ion secretion was monitored for further 20 min (control period 2).

❑ Four gallbladders were studied using *diphenhydramine*. The mucosal side contained 100 ml of unbuffered solution (NaCl 0.9%) (to augment pH changes) whilst the serosal side contained Ringer-Krebs *without* bicarbonate; only the serosal bathing was oxygenated. The experiment was divided into four 20 min periods ; the total experimental period was 80 min. The first 20 min was used as a control period for each gallbladder. At 20 min, diphenhydramine (10^{-5} M) was added into the lumen and mucosal hydrogen ion secretion was monitored for 15min. At 40 min diphenhydramine was added to the luminal solution to a final concentration of 10^{-4} M. At 60 min the luminal solution was replaced by fresh NaCl 0.9% and hydrogen ion secretion was monitored for further 20 min (control period 2).

Second set: Twelve (12) bovine gallbladders were studied for 60 min. The mucosal side contained 100 ml of unbuffered solution (NaCl 0.9%) (to augment pH changes) whilst the serosal side contained Ringer-Krebs *without* bicarbonate; only the serosal bathing was oxygenated. The experiment was divided into three 20 min periods ; the total experimental period was 60 min. The first 20 min was used as a control period for each gallbladder. At 20 min, buffered *histamine hydrochloride* was added into the lumen and mucosal hydrogen ion secretion was monitored for 20min. At 40min the luminal solution was replaced by fresh NaCl 0.9% and hydrogen ion secretion was monitored for further 20 min. (control period 2). Three different concentrations of histamine were used:

Histamine HCl concentration	*Number of experiments*
10^{-5} M	4
10^{-4} M	4
10^{-3} M	4

Note: The protocol design for the histamine experiments was different from the one used for the inhibition experiments. This was felt necessary because histamine HCl has an acidic pH and, despite the fact that it was buffered to pH 7 before use, some acidification of the initial pH of the bathing solution was observed immediately after the addition of this compound and therefore the use of different doses of histamine during the same experiment might have produced misleading results.

6.3.7 Seventh group: Effect of omeprazole.

Aim: To study whether omeprazole, which is a K^+ /H^+ pump inhibitor, would have any effect on mucosal acidification by the gallbladder epithelium.

Six (6) gallbladders were studied using omeprazole. The mucosal side contained 50 ml of unbuffered solution (NaCl 0.9%) (to augment pH changes) whilst the serosal side contained Ringer-Krebs *without* bicarbonate; only the serosal bathing was oxygenated. The experiment was divided into four 15 min periods ; the total experimental period was 60 min. The first 15 min was used as a control period for each gallbladder. At 15 min, omeprazole (2×10^{-4} M) was added into the lumen and mucosal hydrogen ion secretion was monitored for 15min. At 30 min omeprazole was added to the luminal solution to a final concentration of 2×10^{-3} M. At 45 min the luminal solution was replaced by fresh NaCl 0.9% and hydrogen ion secretion was monitored for further 15 min (control period 2).

6.3.7 Eighth group: Effect of CCK.

Aim: To study whether Cholecystokinin (CCK), would have any effect on mucosal acidification by the gallbladder epithelium.

Five (5) gallbladders were studied using CCK. The mucosal side contained 50 ml of unbuffered solution (NaCl 0.9%) (to augment pH changes) whilst the serosal side contained Ringer-Krebs *without* bicarbonate; only the serosal bathing was oxygenated.

The experiment was divided into four 15 min periods ; the total experimental period was 60 min. The first 15 min was used as a control period for each gallbladder. At 15 min, CCK (0.2 iu/ml) was added into the lumen and mucosal hydrogen ion secretion was monitored for 15min. At 30 min CCK was added to the luminal solution to a final concentration of 0.4 iu/ml . At 45 min the luminal solution was replaced by fresh NaCl 0.9% and hydrogen ion secretion was monitored for further 15 min (control period 2).

6.4 STATISTICAL ANALYSIS OF DATA

The MINITAB V. 7.0 statistics program was used to perform the statistical analysis. Data is expressed as Mean ± standard error of mean (SEM) . When the data was not normally distributed, it was analysed using non-parametric tests; Mann-Whitney test was used for unpaired data and Wilcoxon test for paired data. For normally distributed unpaired data the Student's t-test was used, whilst for normally distributed paired data, the paired t-test was used. One way analysis of variance was used to analyse time-dependant changes in mucosal acidification. A 'p' value of 0.05 was taken as significant.

6.5 RESULTS OF THE EXPERIMENTS ON THE BOVINE GALLBLADDER

6.5.1 First group

In the first set of experiments, the gallbladders (N=8) documented a mean potential difference of 5.5±0.9 mV serosa positive after 5min in the chamber. This PD remained quite stable throughout the ensuing 60 minutes. There was a significant drop in the potential difference towards zero, when oxygen was discontinued and N_2 was bubbled, between 60-70 min. When O_2 was restarted the potential difference recovered to levels similar to those prior to oxygen deprivation indicating that hypoxia had direct effect on reducing the transmural PD *(figure 15)*.

Oxygen was bubbled into the serosal compartment only.; pO_2 in the mucosal compartment had remained between 22-25 *kPa* throughout the experimental period, which shows that the diffusion of oxygen through the gallbladder mucosa was adequate. It was thought therefore unecessary to oxygenate the mucosal compartment directly, as this would disrupt the "unstirred water layer".

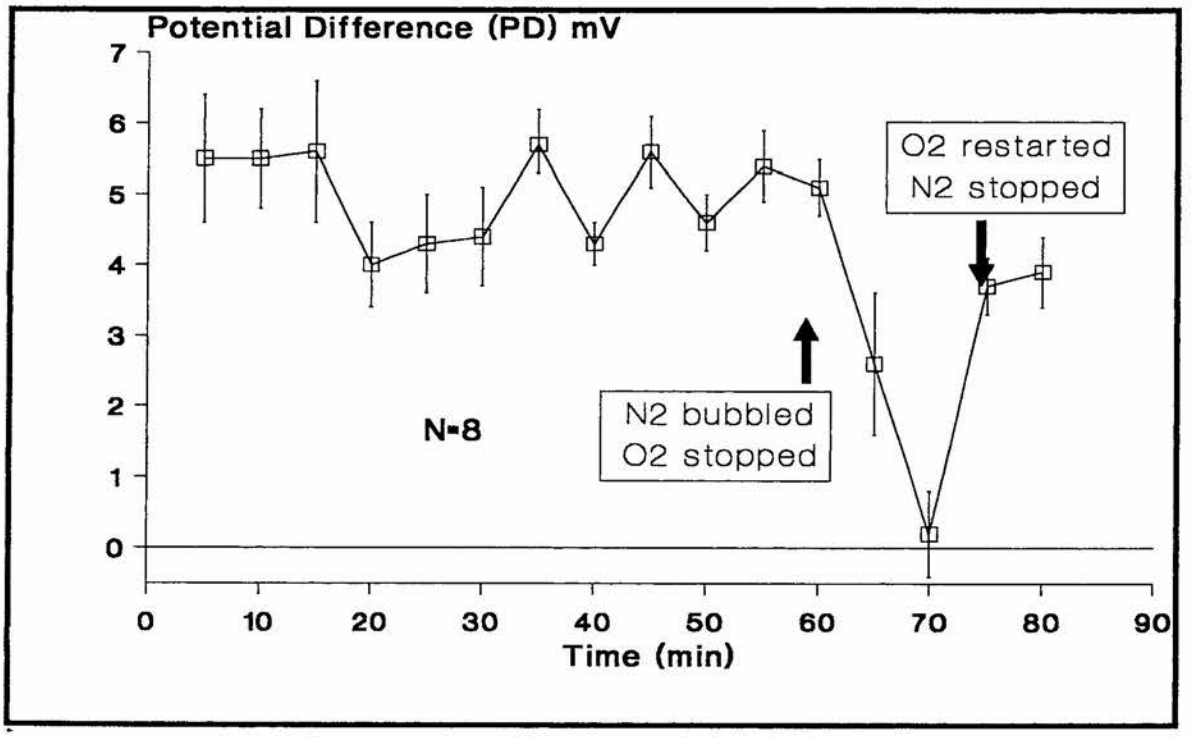


Figure15: Potential difference across the bovine gallbladder mucosa. N_2 administration reduced the PD which was restored to previous levels, when O_2 was restarted.

Tissue resistance (R_{tis}) was calculated by dividing the change in the potential difference [caused by the administration of an external current (I_{SC})= $10\mu A / cm^2$)] by the external current (I_{SC}). The mean surface area for the bovine gallbladder was calculated to 100 cm^2 , therefore the total Isc administered was 1mV.

Tissue resistance had remained quite stable throughout the experimental period but there was a significant drop in tissue resistance, when oxygen was discontinued and N_2 was bubbled, between 60-70 min. When O_2 was restarted the tissue resistance recovered to levels similar to those prior to oxygen deprivation *(figure 16)*.

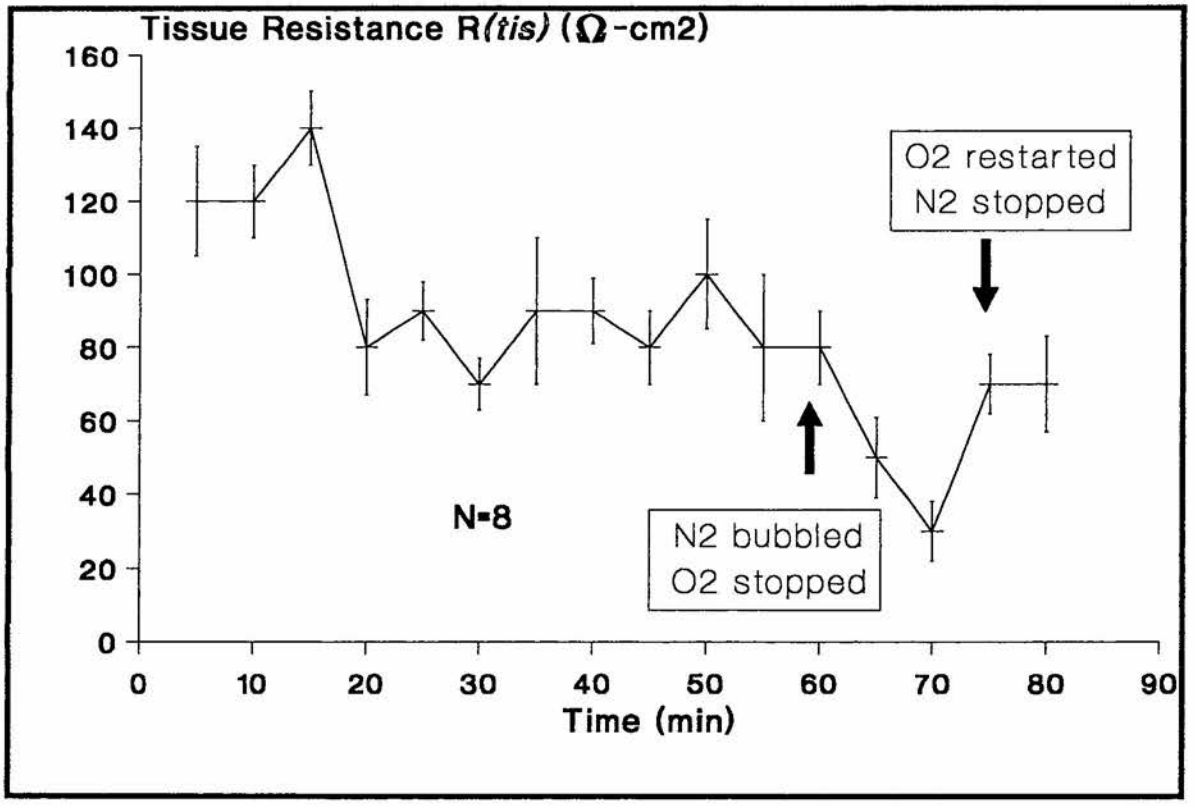


Figure 16: Electrical Resistance of the bovine gallbladder throughout the experimental period. Resistance was reduced during the period of anoxia.

Mucosal acidification was studied for 80 min. There was a significant increase ($p<0.01$) in $[H^+]$ in the mucosal compartment, between 0-60 min of the experiment. The acidification ceased during the anoxic period (70-80 min), but was restored when oxygen was restarted (70-80 min). Mucosal hydrogen ion secretion by the bovine gallbladder was therefore a function of viable tissue and it was impaired during the anoxic period *(figure 17)*.

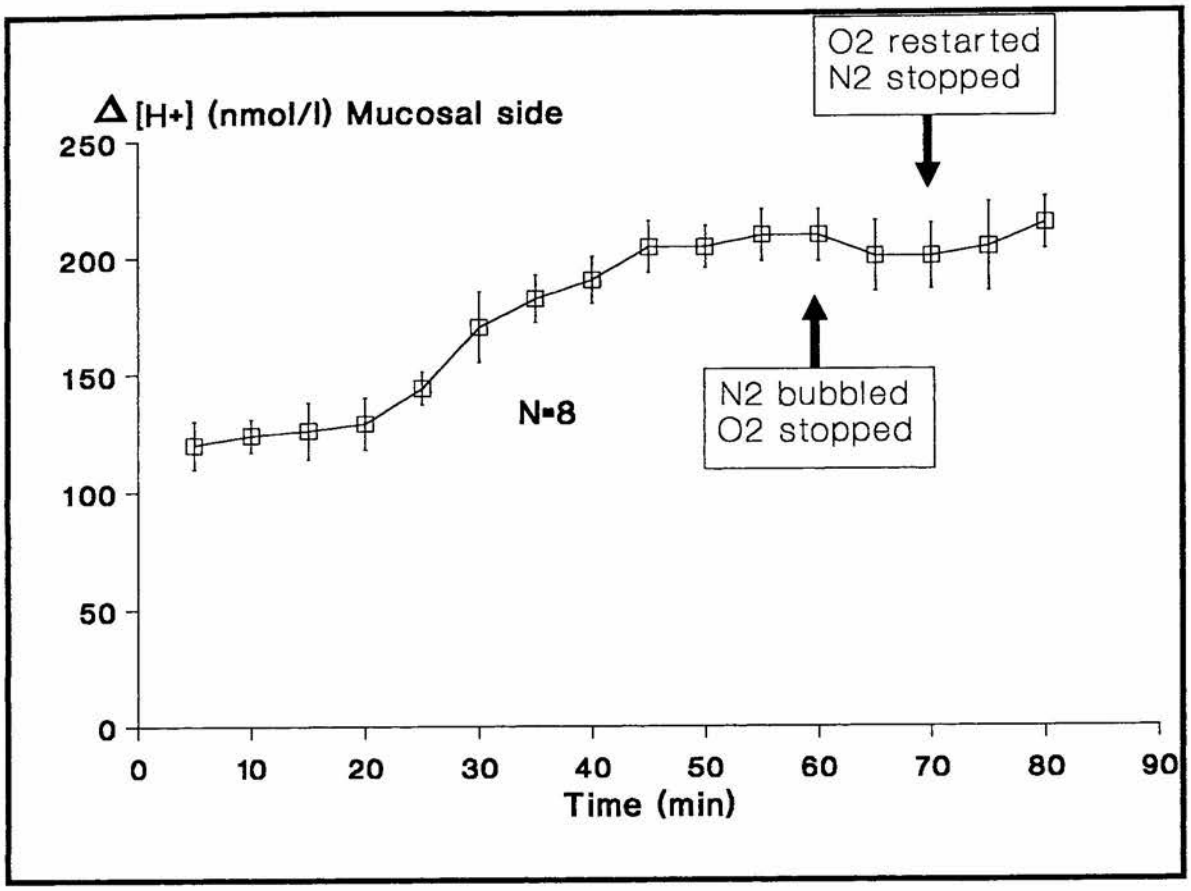


Figure17: Hydrogen ion secretion by the bovine gallbladder epithelium. Hydrogen ion secretion was ceased during the anoxic period.

6.5.2.Second group.

6.5.2.1. First set : Fourteen (14) bovine gallbladders were studied for 60 min. Eight (8) were from male and six (6) from female animals. There was a significant rise in hydrogen ion concentration *($[H^+]$)* in the mucosal side from 0 to 60 min ($p<0.01$), whilst in the serosal side hydrogen ion concentration was reduced during the same period *(figure 18)*. All gallbladders showed satisfactory (PD) at the beggining and end of experiments.

The starting hydrogen ion concentration in the female gallbladders was lower than that of the male gallbladders *(figure 19)*, and although the rate of mucosal acidification was consistenly higher in the male gallbladder did not reach statistical significance within the 60 min experimental period. Serosal alkalisation was similar between male and female subjects .

The degree of acidification in the mucosal side, was 30% higher than the degree of alkalisation in the serosal compertment and this is suggestive of net hydrogen ion

production by the gallbladder epithelial cell *(figure 20)*. The rates of mucosal acidification and serosal alkalisation are shown in *table 6.*

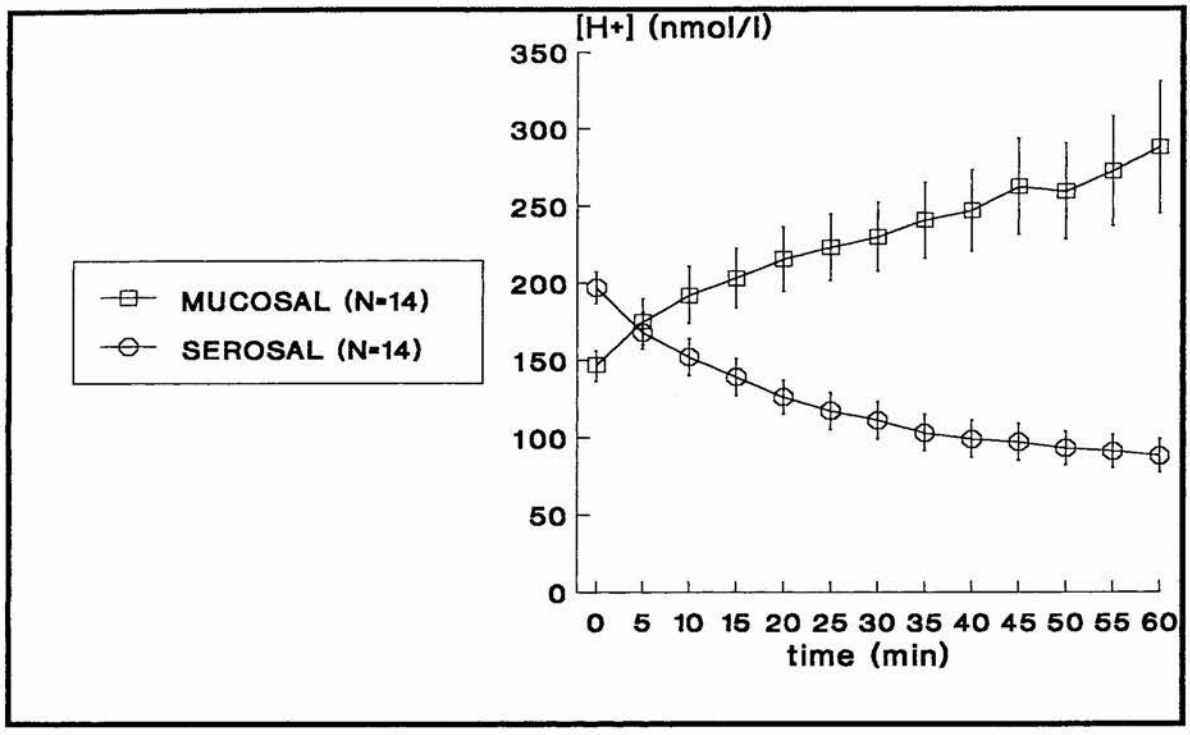


Figure 18: Mucosal acidification and serosal alkalisation in the bovine gallbladder..

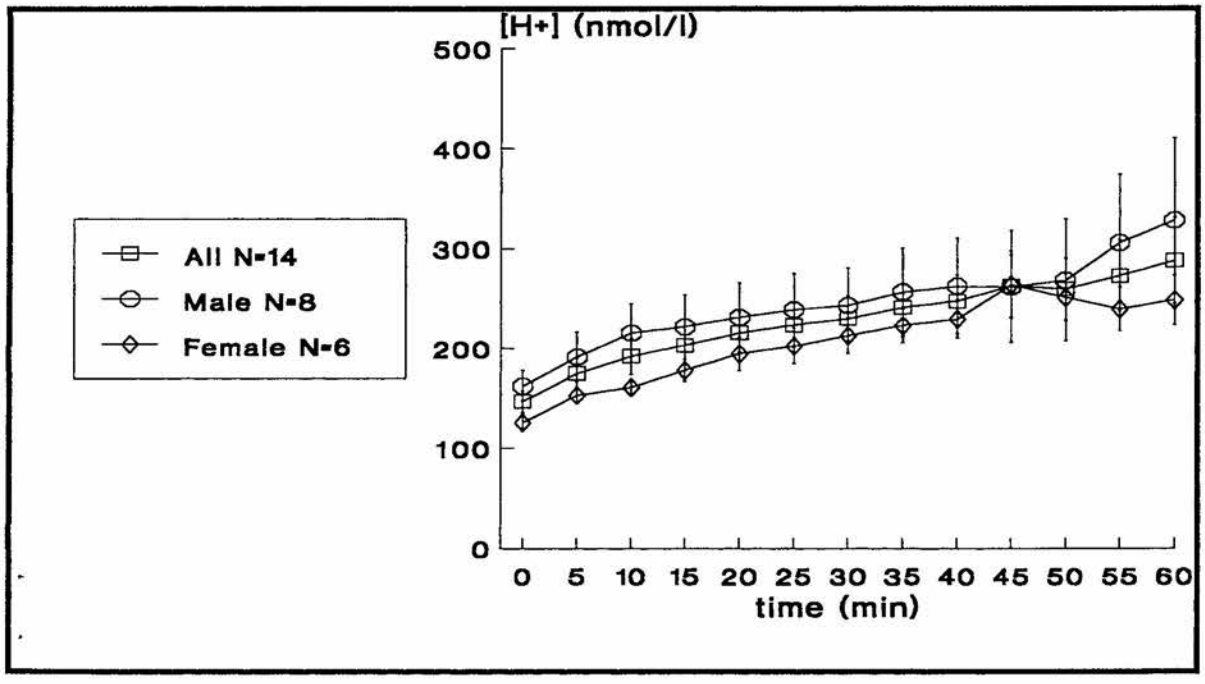


Figure 19: Mucosal acidification in all, in male and in female bovine gallbladders.

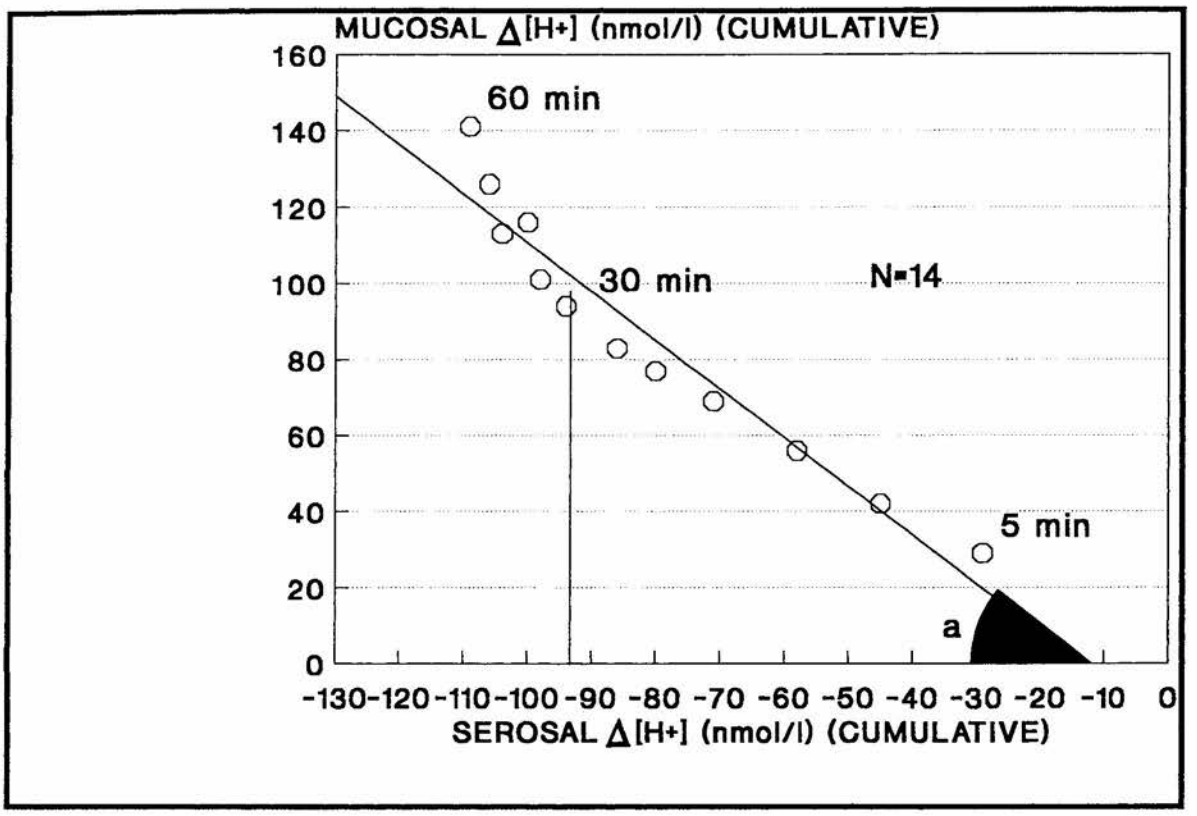


Figure 20: Cumulative mucosal acidification versus serosal alkalisation in the bovine gallbladder; tan a = (Mucosal Δ[H+] / Serosal Δ[H+]) = 1.3 .

TABLE 6

	Total (14)		Male (8)	Female (6)
Δ[H+]	*Mucosal*	*Serosal*	*Mucosal*	*Mucosal*
(nmol / l / hr)	141±25	-109±16	155 ± 47	132 ± 22
(nmol / gallbladder /hr)	14.1±2.5	-10.9±1.6	15.5 ± 4.7	13.2 ± 2.2
p value	p <0.01		Not	Significant

Table 6:Rate of mucosal acidification and serosal alkalisation per bovine gallbladder per hour, overall and in male and female subjects. Minus (-) values indicate alkalisation.

6.5.2.2. Second set

Sixty four gallbladders were studied (26 male, 22 female, 16 sex not known) for 20 min.The rate of mucosal hydrogen ion secretion was higher in the first 20 min of experiment compared to that of the experiments in section 6.5.2.1 where the gallbladders were studied for 60 min. *(Figure 21 and Table 7)* The initial 20 min period was, used as a control period prior to use of pharmacological agents to study the mechanism of acid secretion.

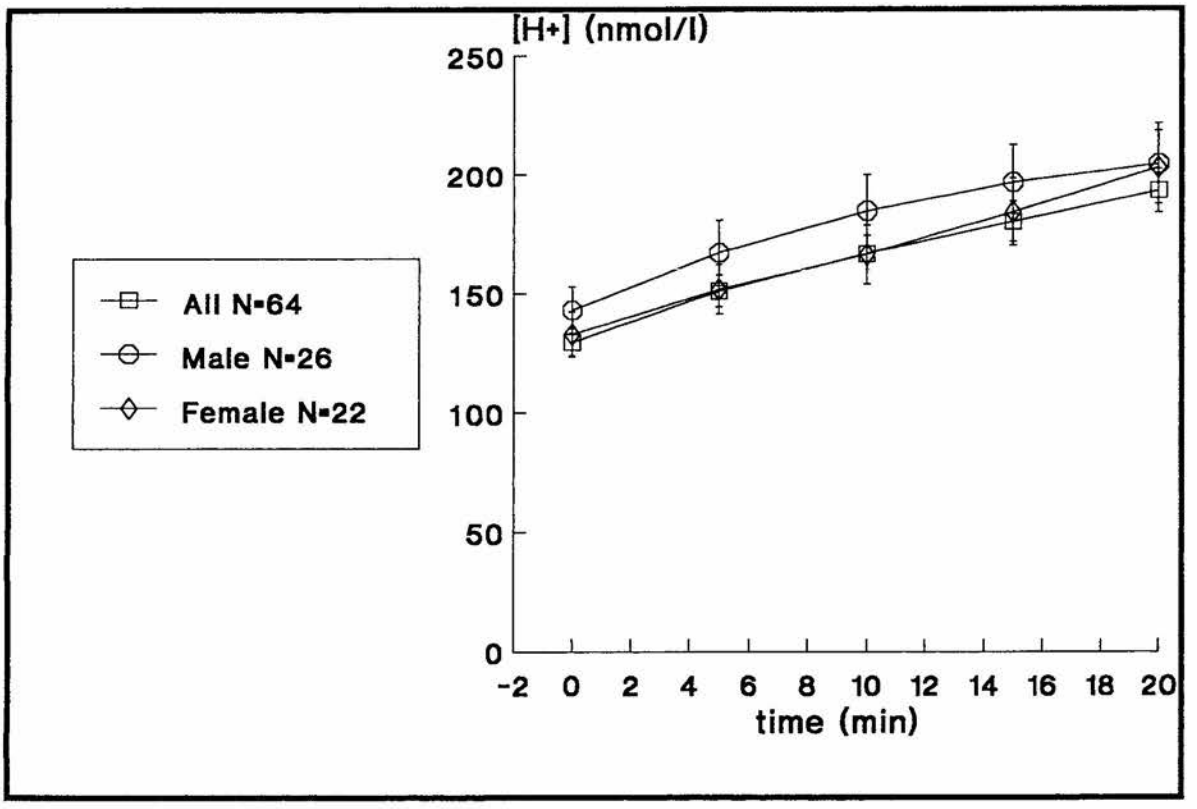


Figure 21: Mucosal hydrogen ion production in all gallbladders; in the male and the female seperately, over a 20 min period.

Hydrogen ion concentration $[H^+]$ measurements on anaerobically collected bile from bovine gallbladders prior to each experiment showed that the starting $[H^+]$ of the mucosal (luminal) solution was significantly higher ($p<0.001$) than the corresponding bile $[H^+]$ in both male and female gallbladders *(Figure 22)*. In addition, both the initial mucosal $[H^+]$ and bile $[H^+]$ were significantly higher in the male gallbladder compared with the female, although the rate of acidification of the mucosal solutions was similar in the two groups *(Figures 20,21 and Table 7)*.

TABLE 7

	All		*Male*		*Female*	
Mucosal acidification	**20 min**	**60 min**	**20 min**	**60 min**	**20 min**	**60 min**
$\Delta[H^+]$ (nmol/l/hr)	189±43	141± 25	183±35	155±47	211±42	132±22
$\Delta[H^+]$ (nmol/gallbladder/hr)	18.9±4.3	14.1±2.5	18.3±3.5	15.5±4.7	21.1±4.2	13.2±2.2
p (male vs female)			Not significant			
p (20 min vs 60 min)	<0.01		<0.01		<0.01	

Table 7: No significant differences in the rate of acidification were found between male and female gallbladder; acidification was more brisk at the first 20 min of experiments.

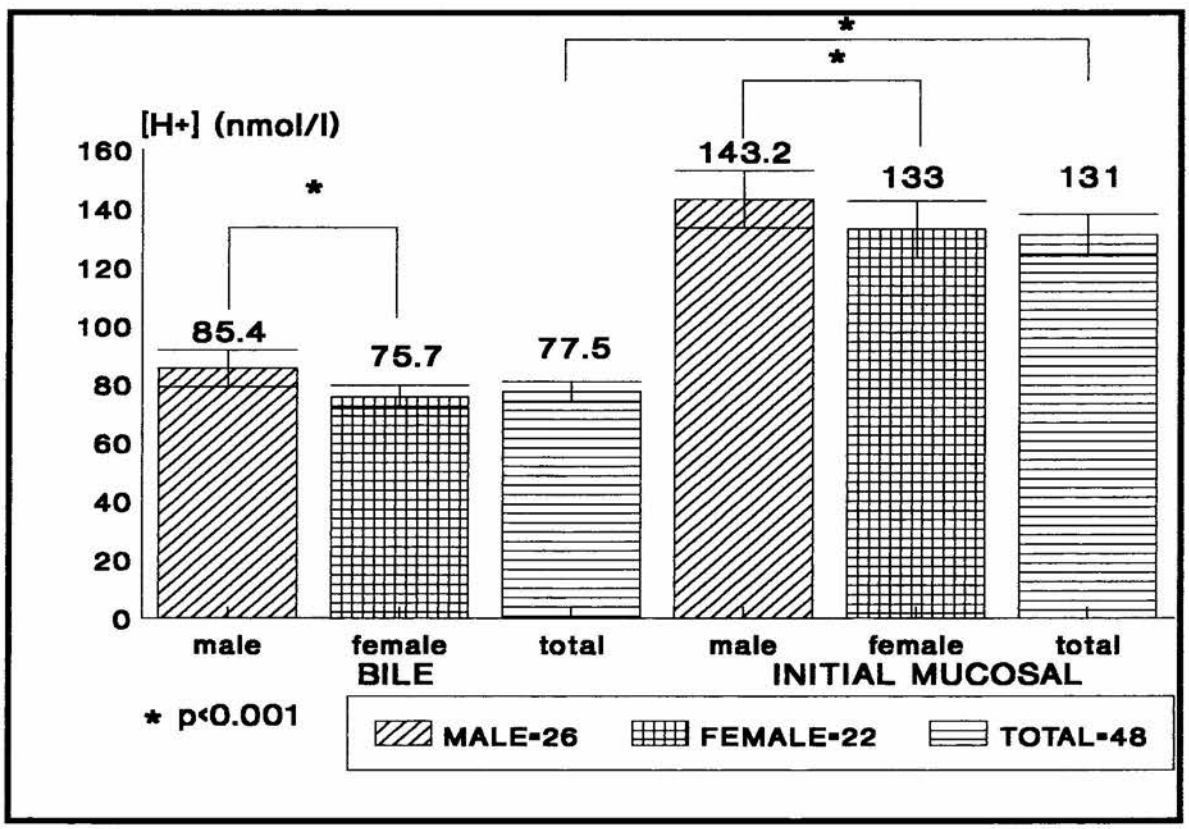


Figure 22: $[H^+]$ of the bovine bile was significantly more alkaline compared with initial mucosal $[H^+]$. Male gallbladders demonstrated significantly higher $[H^+]$ both in bile and initial mucosal solution compared with female ones ($p<0.001$).

6.5.2.3. Third set

Twenty two gallbladders were studied. The initial mucosal $[H^+]$ was varying from 300-20,000 nmol/l (*pH* range 6.6-4.8, *Table 5)*. Mucosal acidification was turned to alkalisation when the starting *pH* was <6.0. This equilibrium point in mucosal $[H^+]$ was independent of the starting *pH (Figure 23).* This indicates that the epithelium is leaky and back diffusion of hydrogen ions occurs when their concentration exceeds 1000 nmol/l (pH=6.0).

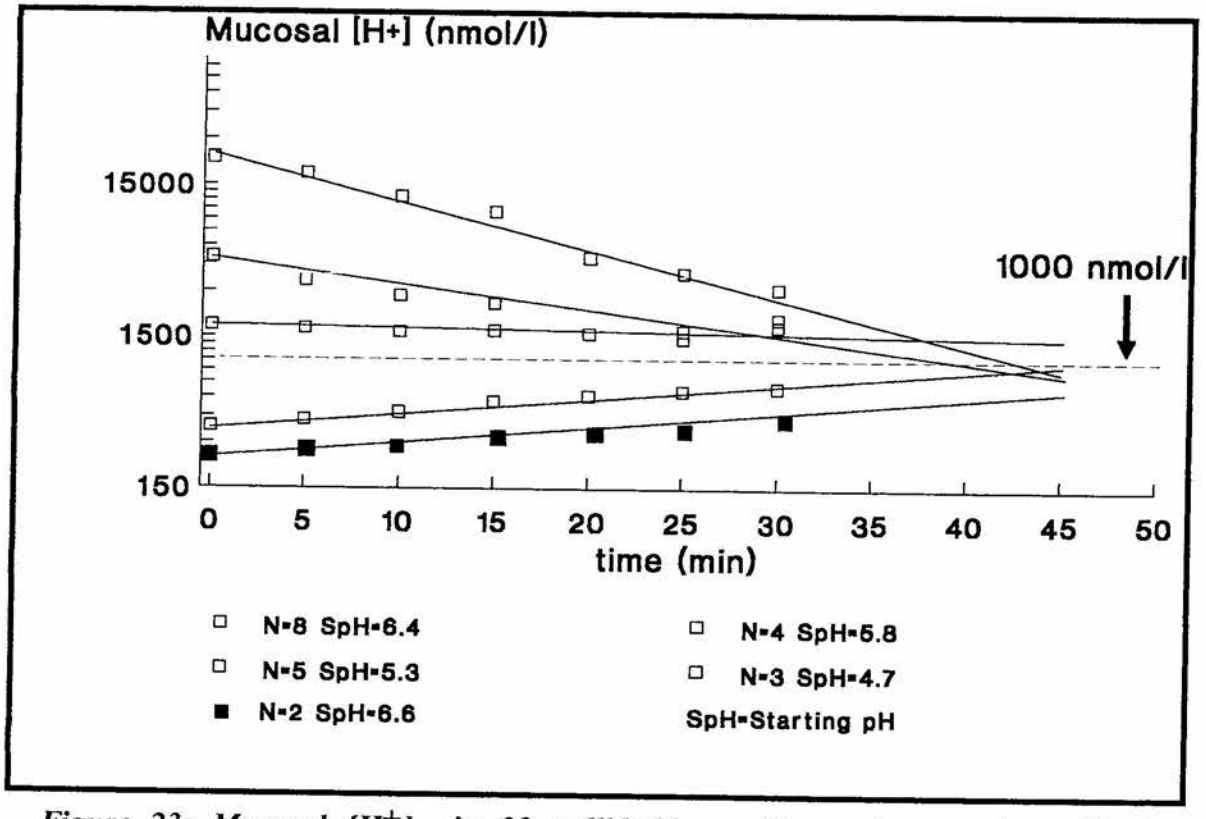


Figure 23: Mucosal $[H^+]$ in 22 gallbladders with varying starting pH. An equilibrium point at mucosal pH=6.0 ([H+]=1000 nmol/l) was found and this was independent of the starting pH. Below this pH, back diffusion of hydrogen ions occur preventing further acidification.

6.5.3..Third group

6.5.3.1 First set.

Ten gallbladders were studied for 60 min. When sodium-free, unbuffered mucosal solutions were used, (either in the first 30 min or the last 30 min of the experiment)

mucosal acidification ceased. Replacement of mucosal solutions with NaCl 0.9% restored acidification *(Figures 24, 25)*.

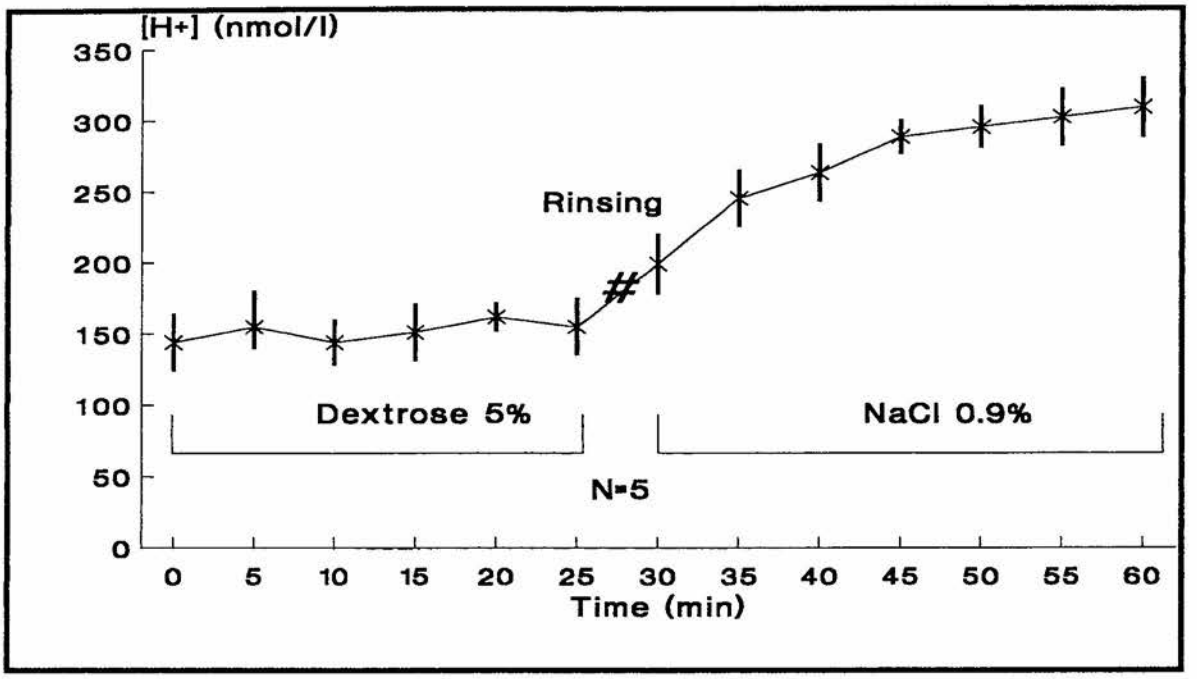


Figure 24: The use of sodium-free mucosal solutions (0-30 min) abolished acidification which was restored when NaCl 0.9% was used.

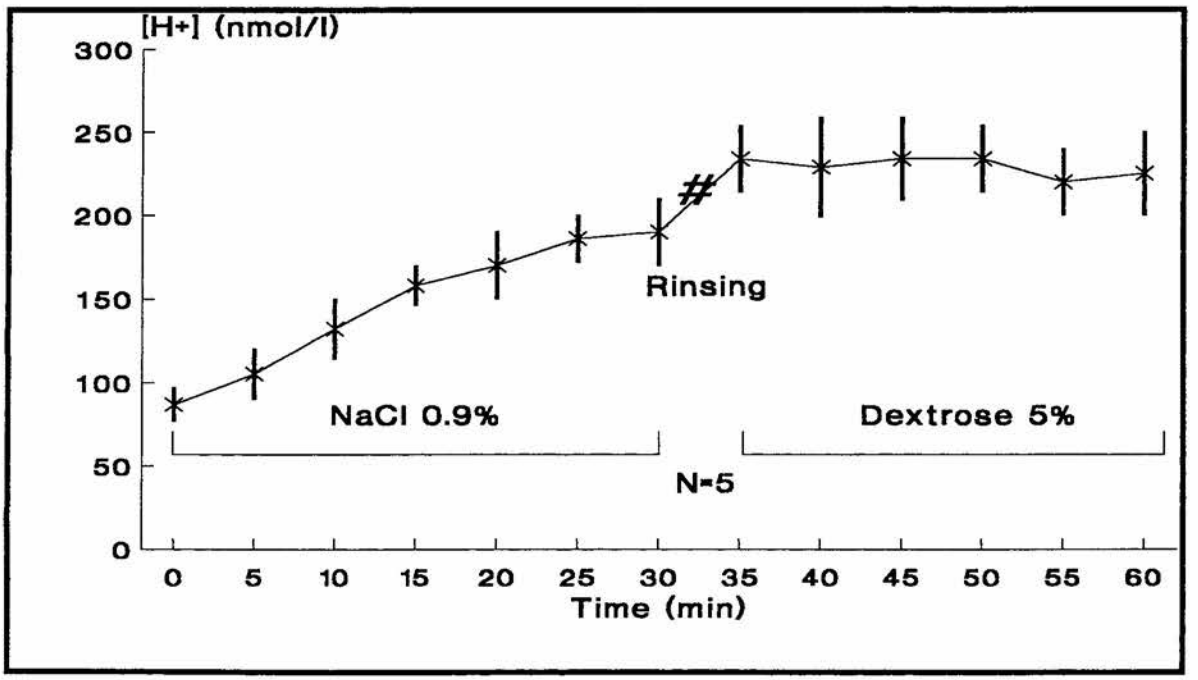


Figure 25: The use of sodium-free mucosal solutions (30-60 min) abolished mucosal acidification.

6.5.3.2 Second set.

The effect of amiloride which is a selective sodium / hydrogen antiport inhibitor was studied in five (5) bovine gallbladders. 100μM of amiloride was added at 20min in the mucosal side and mucosal acidification was monitored for 20 min. Amiloride inhibited mucosal acidification in all five experiments ; when the luminal solution was replaced by NaCl 0.9% acidification was restored *(Figure 26).*

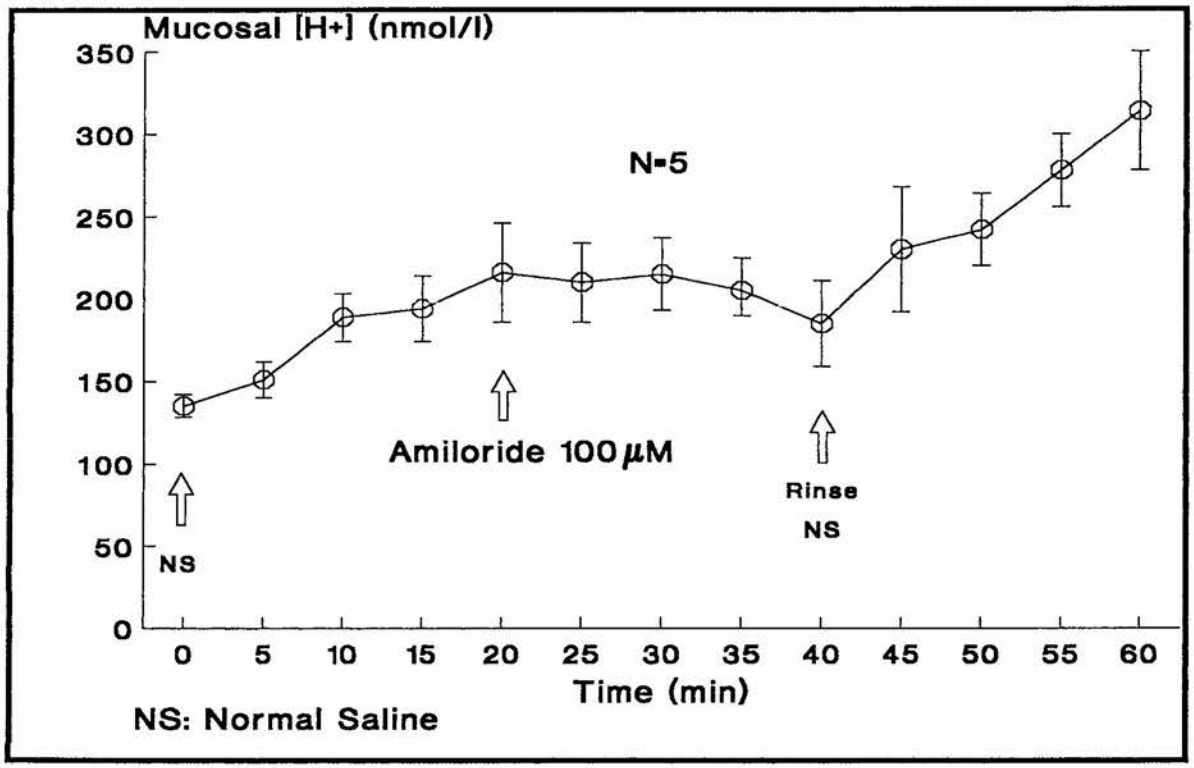


Figure 26: Effect of amiloride on mucosal acidification in the bovine gallbladder.

6.5.4 Fourth group

The effect of ouabaine, which is a sodium-potassium-ATPase pump inhibitor, on mucosal acidification was studied in 4 bovine gallbladders. Each experiment lasted for 80 min and the effect of both mucosal and serosal application of ouabaine was studied. 0.5 mM of ouabaine inhibited mucosal acidification when added to the serosal compartment but not when added to the mucosal. Mucosal acidification was restored when ouabaine was removed *(Figures 27, 28).*

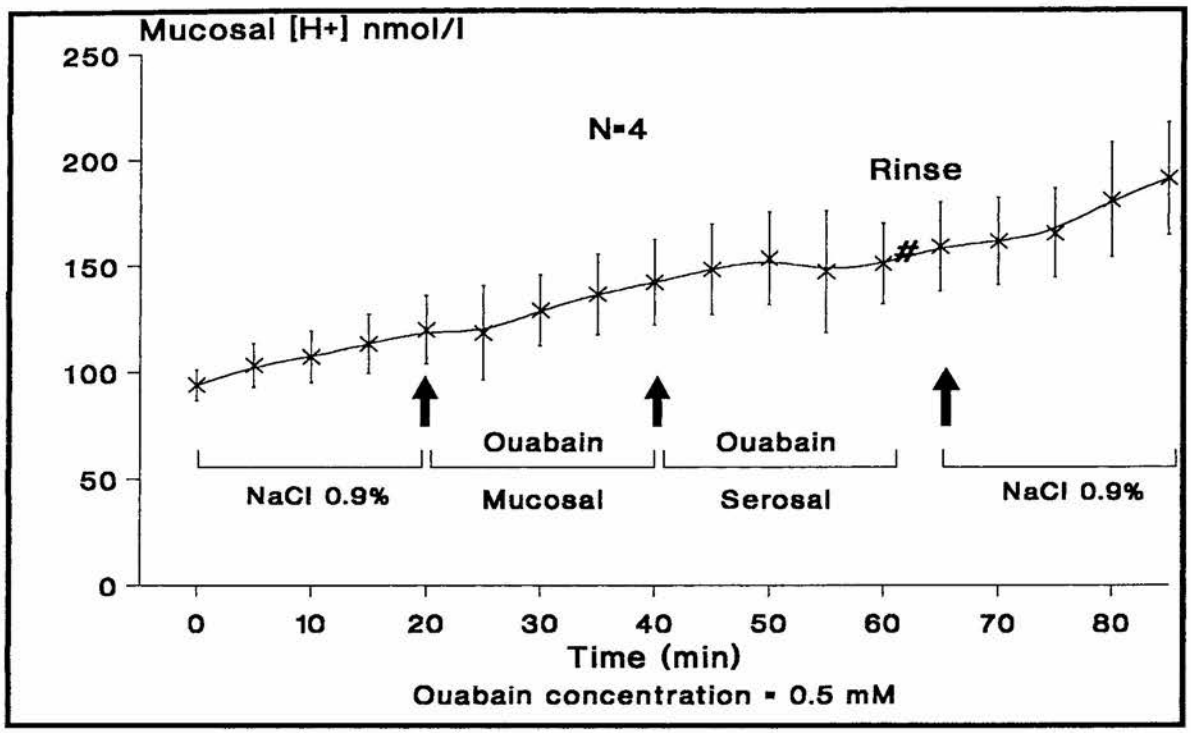


Figure 27: Effect of mucosal and serosal application of ouabain on hydrogen ion secretion by the gallbladder epithelium.

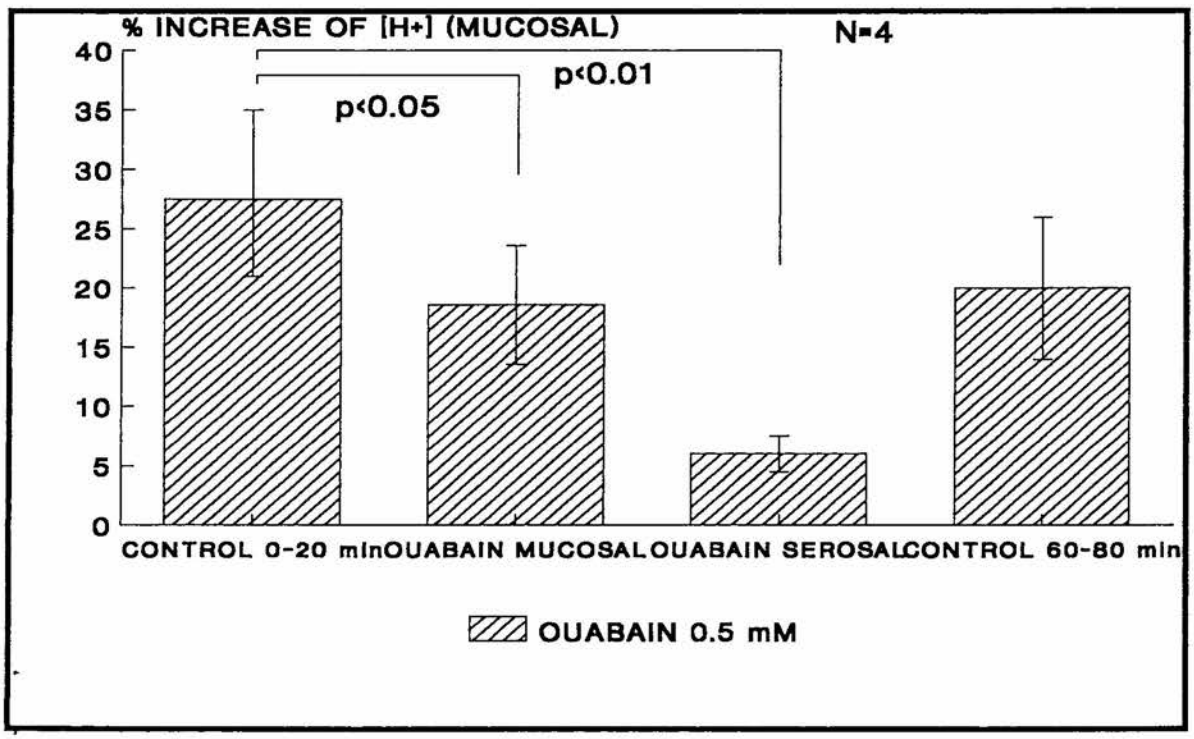


Figure 28: Percentage change in hydrogen ion production by the gallbladder after serosal and mucosal application of ouabain .

6.5.5. Fifth group.

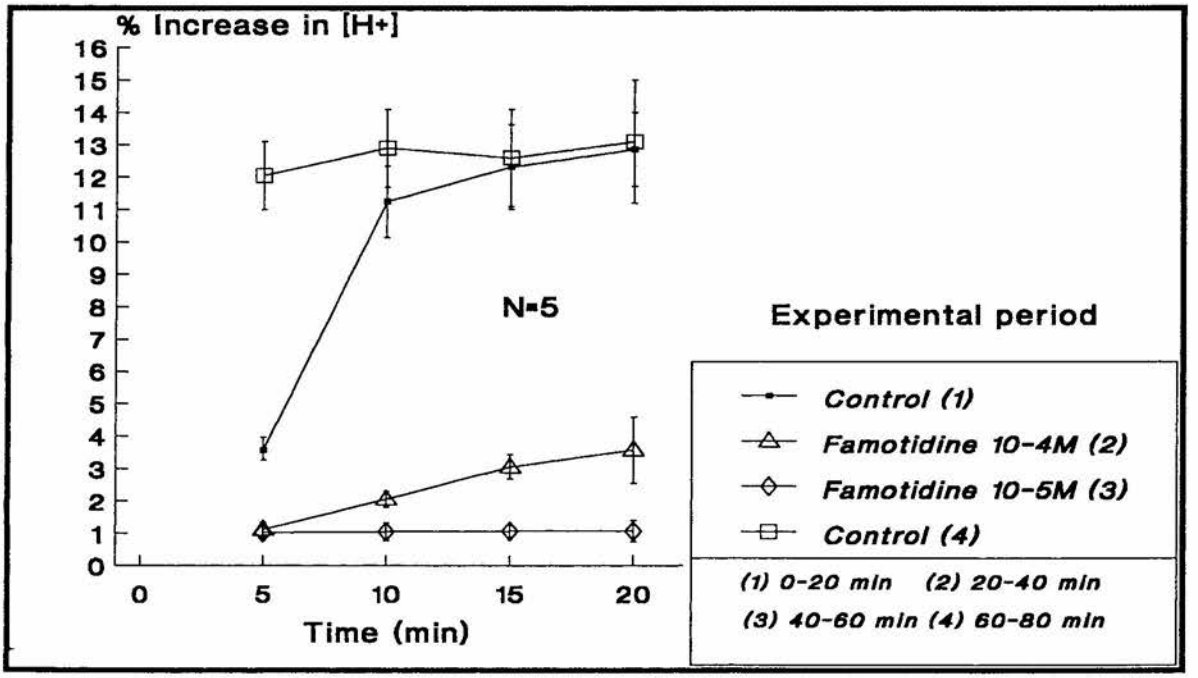
The effect of acetazolamide (carbonic anhydrase inhibitor), was studied in eight gallbladders. Two different concentrations were used; 10^{-4} M and 10^{-3} M. Acetazolamide application in either the serosal or the mucosal side had no effect on mucosal acidification .

6.5.6 Sixth group.

First set: Effect of histamine receptor antagonists.

The effect of famotidine (an H_2 receptor antagonist) and diphenylhydramine (non-specific Histamine receptor antagonist) were studied.

Famotidine (10^{-4} and 10^{-5} M) significantly inhibited hydrogen ion production by the gallladder epithelium (N=5, $p<0.001$). Inhibition was maximum when famotidine was applied to the mucosal side at a concentration of 10^{-4} M and it was reversible when famotidine was removed (*Figure 29*).



. ***Figure 29: Mucosal Famotidine significantly inhibited hydrogen ion production by the gallbladder epithelium when it was applied in the lumen of the gallbladder (periods 2 &3). Inhibition of acid secretion was reversible (period 4)***

Similarly, diphenhydramine significantly inhibited acidification when it was applied in the mucosal compartment at concentrations 10^{-4} M and 10^{-5} M (N=4, $p<0.001$). This inhibition was reversible when diphenhydramine was replaced by NaCl 0.9% in the gallbladder lumen *(Figure 30)*.

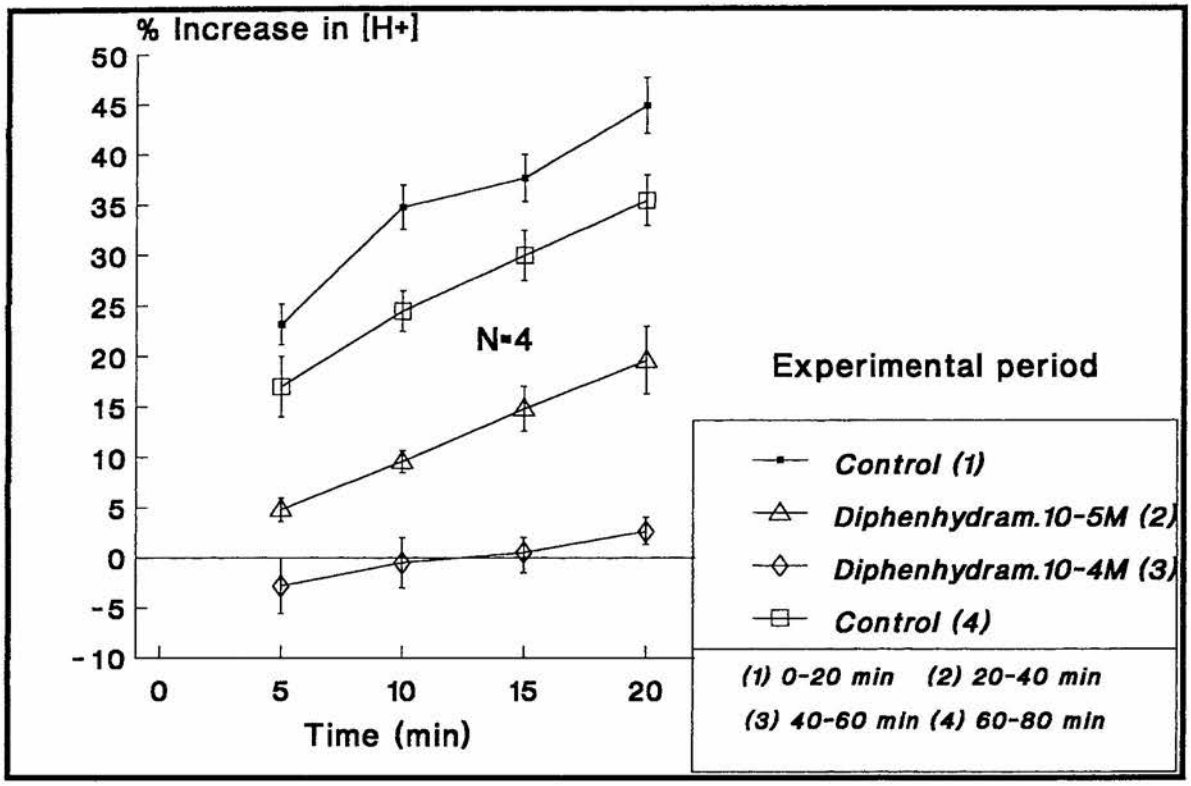


Figure 30: ***Mucosal diphenhydramine significantly inhibited hydrogen ion production by the gallbladder epithelium when it was applied in the lumen of the gallbladder (periods 2 &3). Inhibition of acid secretion was reversible (period 4)***

Second set: Effect of histamine.

The effect of buffered histamine hydrochloride on mucosal acidification was studied in 3 different concentrations (10^{-5} M, 10^{-4} M, 10^{-3} M) in 9 experiments. The mucosal application of 10^{-5} M and 10^{-4} M histamine did not significantly influence mucosal acidification *(Figure 31a,b)*. However 10^{-3} M histamine had a significant stimulatory effect on mucosal acidification *($p<0.01$)(Figures 31c, 32)*.

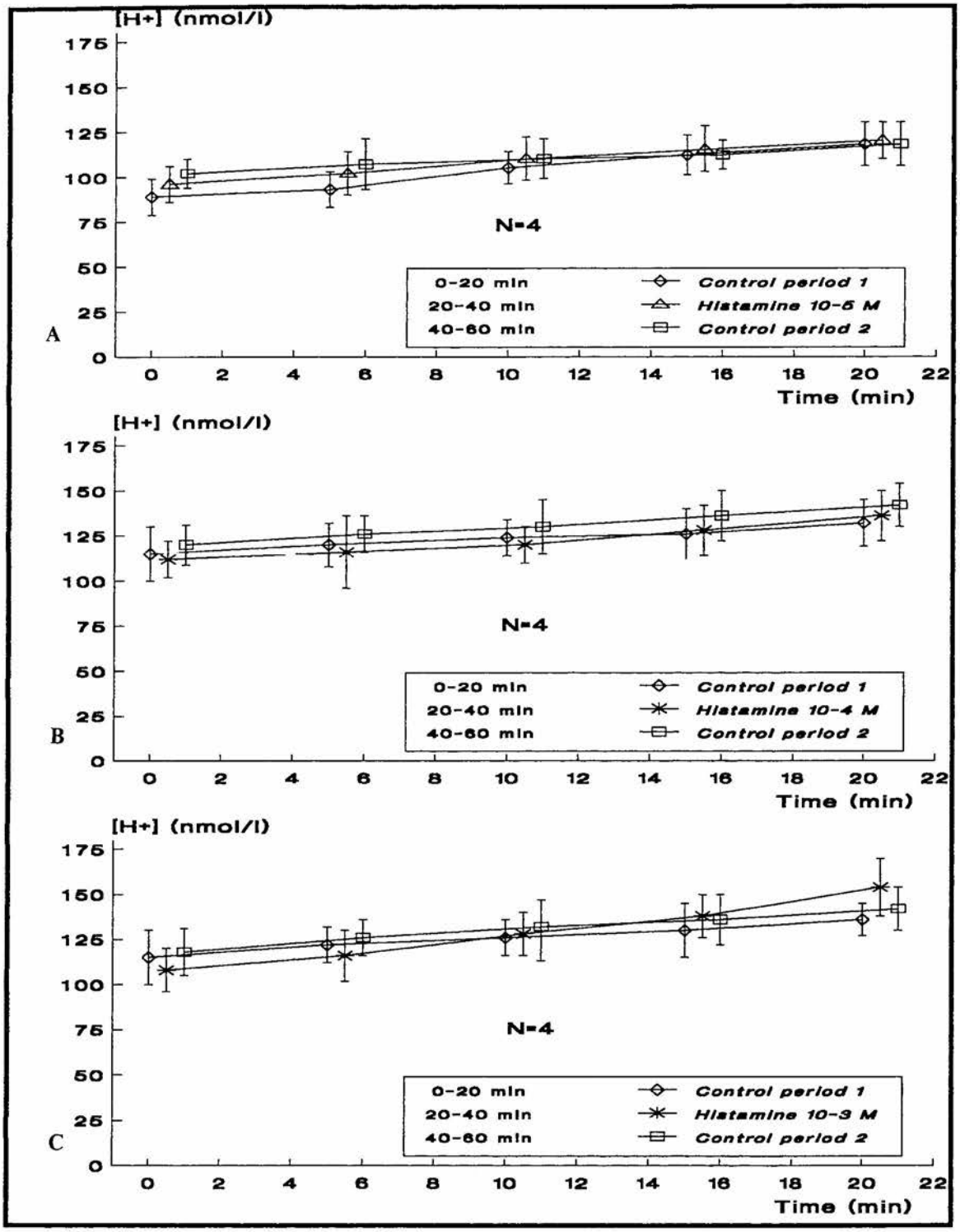


Figure 31a,b,c: Mucosal application of high concentration (10^{-3} M) of Histamine hydrochloride had a stimulatory effect on mucosal acidification, but no effect was observed with lower concentrations.

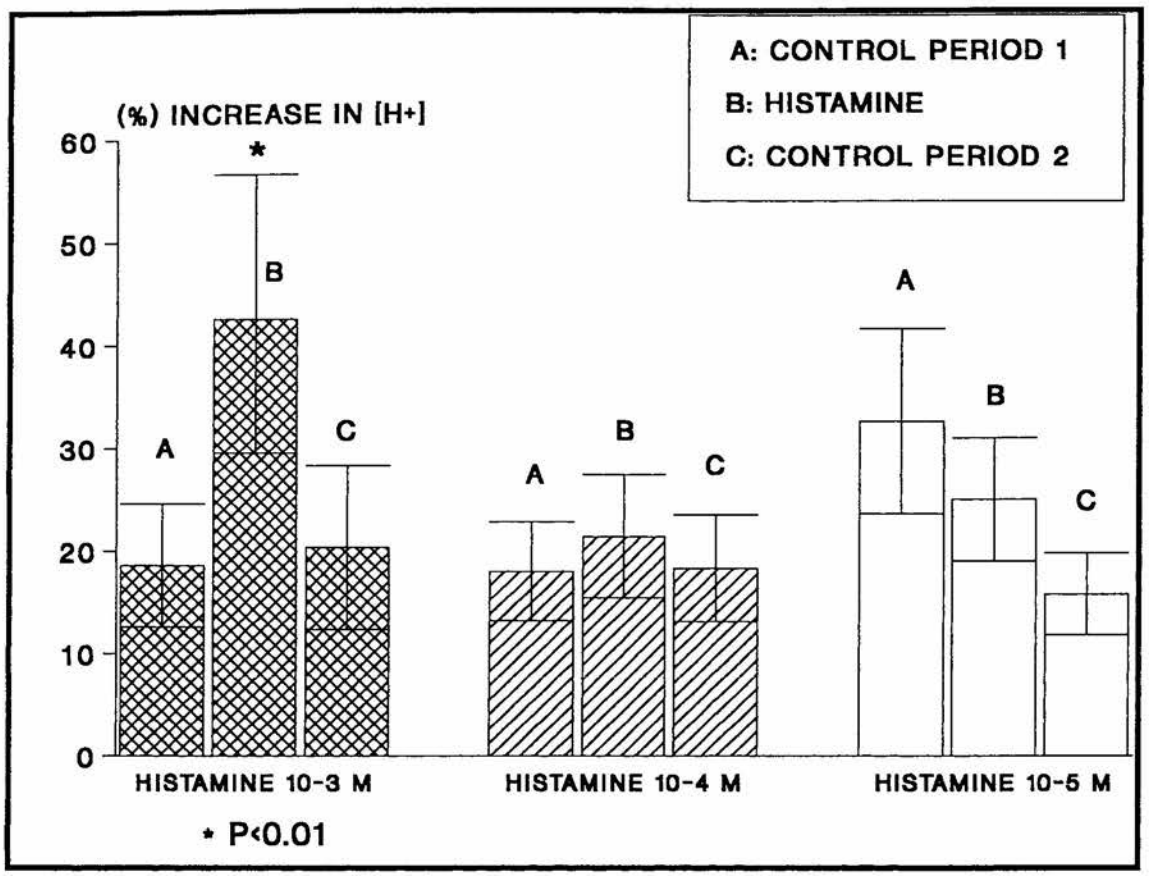


Figure 32: Percentage increase in mucosal hydrogen ion concentration with different concentrations of histamine. 10^{-3} M of histamine stimulated acidification.

6.5.7.Seventh group: Effect of omeprazole

Six gallbladders were studied. Omeprazole was applied into the gallbladder lumen; two different concentrations were used ($2x10^{-4}$ M and $2x10^{-3}$ M). Mucosal acidification was significantly reduced in a dose-dependent fashion, but it was not abolished The inhibitory effect of omeprazole was reversible; however, the rate of acidification following replacement of omeprazole was reduced compared with the control period 1 *(Figure 33).*

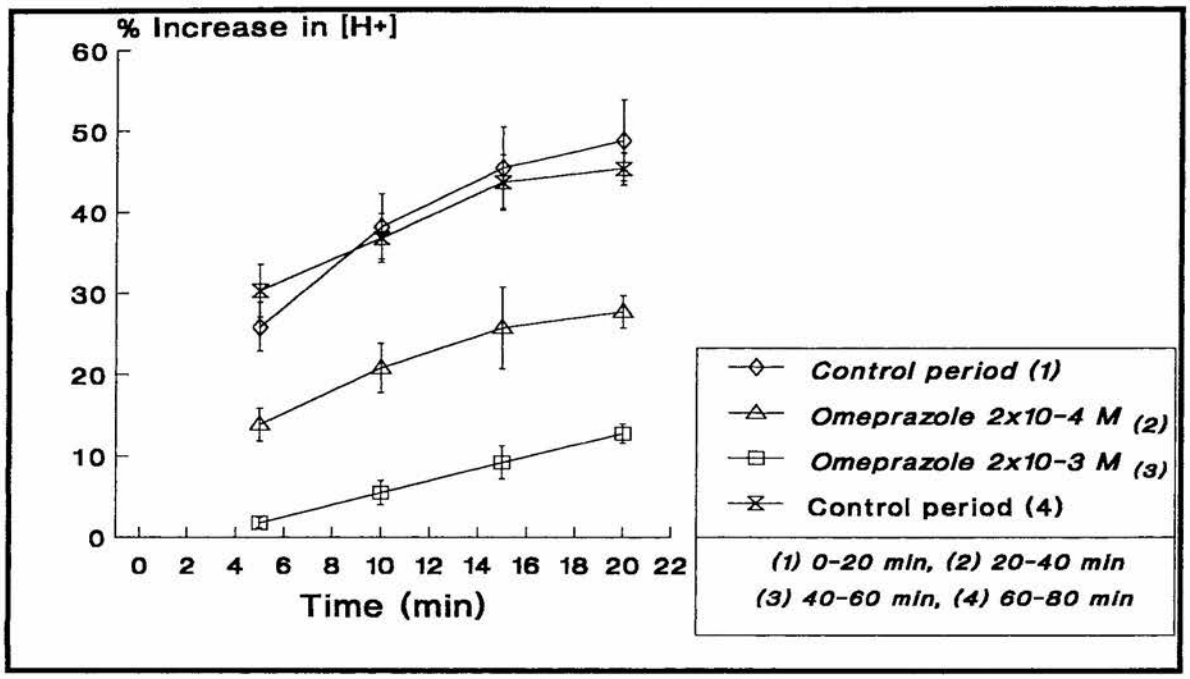


Figure 33: Effect of omeprazole on mucosal acidification by the gallbladder epithelium. Reduction but not abolishment of acidification was observed with high doses of omeprazole.

6.5.8 Eighth group: Effect of CCK

Five gallbladders were studied. Cholecystokinin was applied into the gallbladder lumen; two different concentrations were used (0.2 iu/ml and 0.4 iu/ml). Mucosal acidification was stimulated in a dose-dependent fashion *(Figure 34)*.

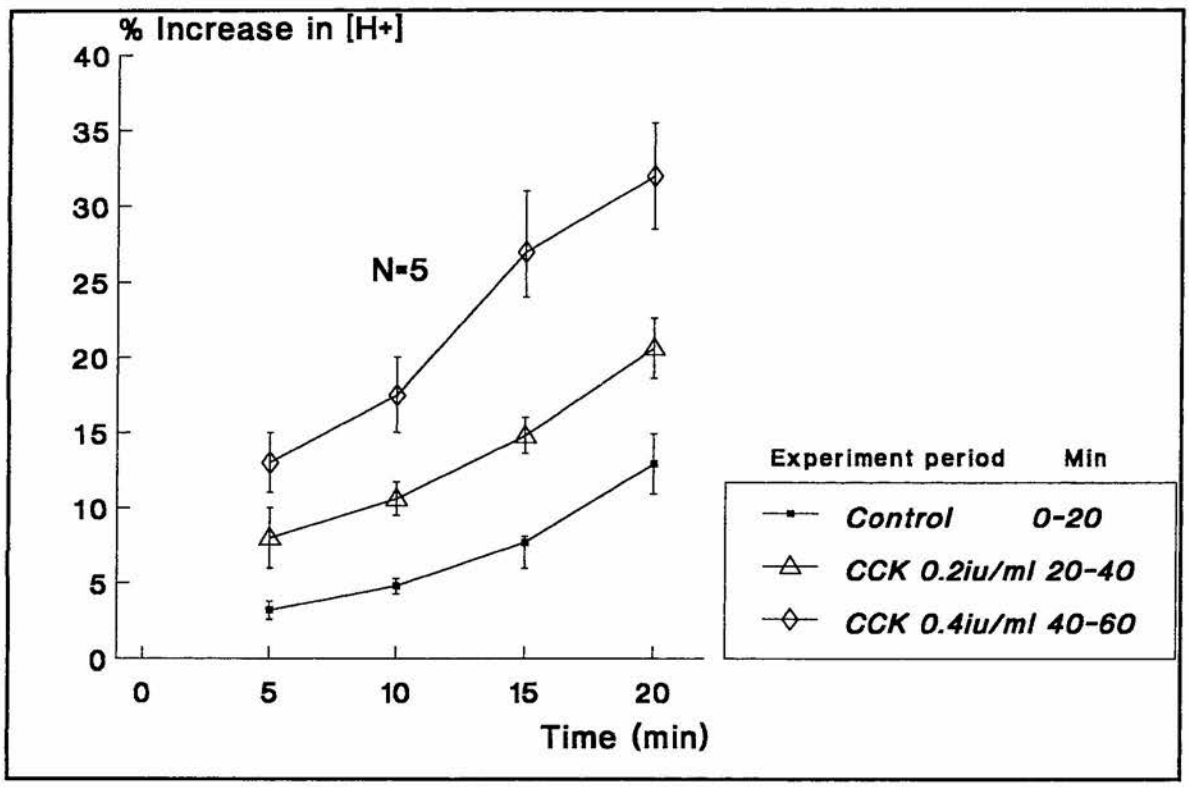


Figure 34: Percentage increase in mucosal hydrogen ion concentration with different concentrations of Cholecystokinin (CCK). CCK stimulated acidification when it was applied in the mucosal side at a concentration 0.2-0.4 iu/ml.

6.6. Conclusions

In this study, using a whole gallbladder perfusion system, a total of 115 fresh bovine gallbladders were studied *in vitro*. The results of this study indicate:

a.) *Hydrogen ion secretion by the gallbladder mucosa is a function of viable tissue.* Hypoxia affected hydrogen ion secretion, as well as the electrophysiological parameters, which were used as markers of viability.

b.) Mucosal acidification was associated with simultaneous serosal alkalisation but the rate of mucosal acidification was higher than that of serosal alkalisation, indicating that *mucosal acidification is not only due to hydrogen ion transfer from serosa to lumen, but also due to production of hydrogen ions by the gallbladder epithelium.*

c.) Although *no differences in the rate of acidification were observed between male and female animals , gallbladder bile from males was significantly more acidic compaired with that from females.*

d.) Acidification reached a steady state around pH 6; when mucosal pH was set below pH 6, acdification was reversed to alkalisation until pH reached 6. This indicates that *the gallbladder epithelium is designed in such a way not to allow bile pH to drop below 6, by becoming leaky to hydrogen ions (back-diffusion) when their concentration exceeds 1000 nmol/l.*

e.) *Hydrogen ion secretion in the bovine gallbladder is a sodium-dependant process* and it was abolished when sodium-free solutions or amiloride , which is a sodium / hydrogen antiport inhibitor, were used. *The most likely system involved is an apical sodium / hydrogen antiport system.*

f.) Hydrogen ion secretion was abolished by serosal but not mucosal ouabain (sodium / potassium / ATP-ase inhibitor) ; *this indicates that hydrogen ion secretion is dependant upon the energy produced by sodium / potassium / ATP-ase pump, which is present at the basal side of the epithelial cell.*

g.) Hydrogen ion secretion was inhibited by a selective H_2 (Famotidine) and a non-selective (Diphenhydramine) histamine receptor antagonist. In contrast high concentration histamine stimulated acidification; *this indicates that hydrogen ion secretion by the gallbladder epithelium is a histamine-dependant process, but the effect of histamine is much weaker and less specific in terms of receptors compared with the stomach..*

h.) Omeprazole in high doses had a relative weak inhibitory effect on acidification which indicates that *hydrogen / potassium exchange is not the principal mechanism of hydrogen production by the gallbladder epithelium.*

i.) Acetazolamide , which is a carbonic anhydrase inhibitor, did not affect acidification in the pharmacological doses used and this indicates that the principal source of hydrogen ions within the gallbladder epithelial cell is not through splitting of carbonic acid.

j.) CCK stimulated acidification . Whether this is a direct effect of mucosal secretion or a secondary effect is not clear.

CHAPTER 7: PATHOLOGY STUDIES ON HUMAN AND BOVINE GALLBLADDER EPITHELIUM

CHAPTER 7: PATHOLOGY STUDIES ON HUMAN AND BOVINE GALLBLADDER EPITHELIUM

7.1 AIMS

The aims of this set of experiments were:

1. To study the morphology of the bovine gallbladder epithelium in comparison with the human gallbladder epithelium.

2. To study the distribution of carbonic anhydrase, which mediates the acid-base equilibrium within the gallbladder epithelial cell , in the normal and inflammed gallbladder.

7.2 MATERIALS AND METHODS

Human and bovine gallbladders were routinely fixed in 4% buffered formalin. Blocks were sampled from the fundus.and were processed to paraffin wax and routinely stained with haematoxylin and eosin. The morphological characteristics of the bovine gallbladder were studied in relation to the human gallbladder.

The distribution of carbonic anhydrase in the gallbladder epithelium was studied in histologically normal human gallbladders as well as in diseased gallbladders using immunocytochemistry according to standard methods.

Immunohistochemical staining was originally carried out using the peroxidase antiperoxidase (PAP) method of Sternberger et al, 1970, as follows: sections of 3μm were taken from the blocks of fixed tissue, dewaxed and treated with 1% (vol/vol) hydrogen peroxide in methanol for 20 min to block the endogenous peroxidase activity. Sections were washed in Tris buffered saline (TBS pH 7.6) and treated with 20% normal donkey sera (Scottish Antibody Production Unit, Carluke, UK) for 15 min before being incubated with the primary antibody (a polyclonal antibody against carbonic anhydrase type II (Gift of Professor Weller), at a concentration of 1:1,000 in serum), for 30 min. Normal rabbit serum (1/20) was used in place of the primary antibody for the negative controls. Incubations for 30 min with 1/30 donkey antirabbit (Scottish Antibody Production Unit) and 1/100 rabbit peroxidase anti-peroxidase (Scottish Antibody Production Unit) were serially performed with TBS rinses and 10 min incubations with 20% normal donkey sera between antisera incubations. Sections were subsequently

treated with ^{3,3} diaminobenzidine solution (5 mg /10 ml TBS plus 0.1 M imidazole plus 100 μl of 100 vol hydrogen peroxide) for 10 min. After thorough rinsing in tap water , sections were examined by light microscopy . Counterstaining was carried out with Mayer's haematoxylin. Reproducibility was ensured by repeat staining of a sample of cases.

7.3 RESULTS

Morphology of the bovine gallbladder

The bovine gallbladder has several similarities with the human. Macroscopically it is a large gallbladder, as it is expected from the size of the animal, and can accommodate over 100ml and if distended up to 500ml of bile. The supporting structures of the bovine gallbladder are similar with the human , however there is significant amount of fat surrounding the organ. Microscopically numerous microvilli with columnar epithelium are present with a rich capillary network under the epithelial layer. A thick muscular layer and a layer of connective tissue are supporting the epithelial structure. (Figure 35 a,b).

Morphology of the human gallbladder

a. Haematoxylin and eosin staining.

Samples of normal, mildly inflamed, moderately inflamed and severely inflamed gallbladders are presented in Figure 36 (a,b,c,d).

Severely inflamed gallbladders were not included in any physiological experiments.

b.Immunocytochemistry.

Carbonic anhydrase.

Carbonic anhydrase was distributed patchily in all gallbladders. However, no apparent difference in distribution or density of staining was noted between normal and diseased gallbladders (Figure 37 a,b).

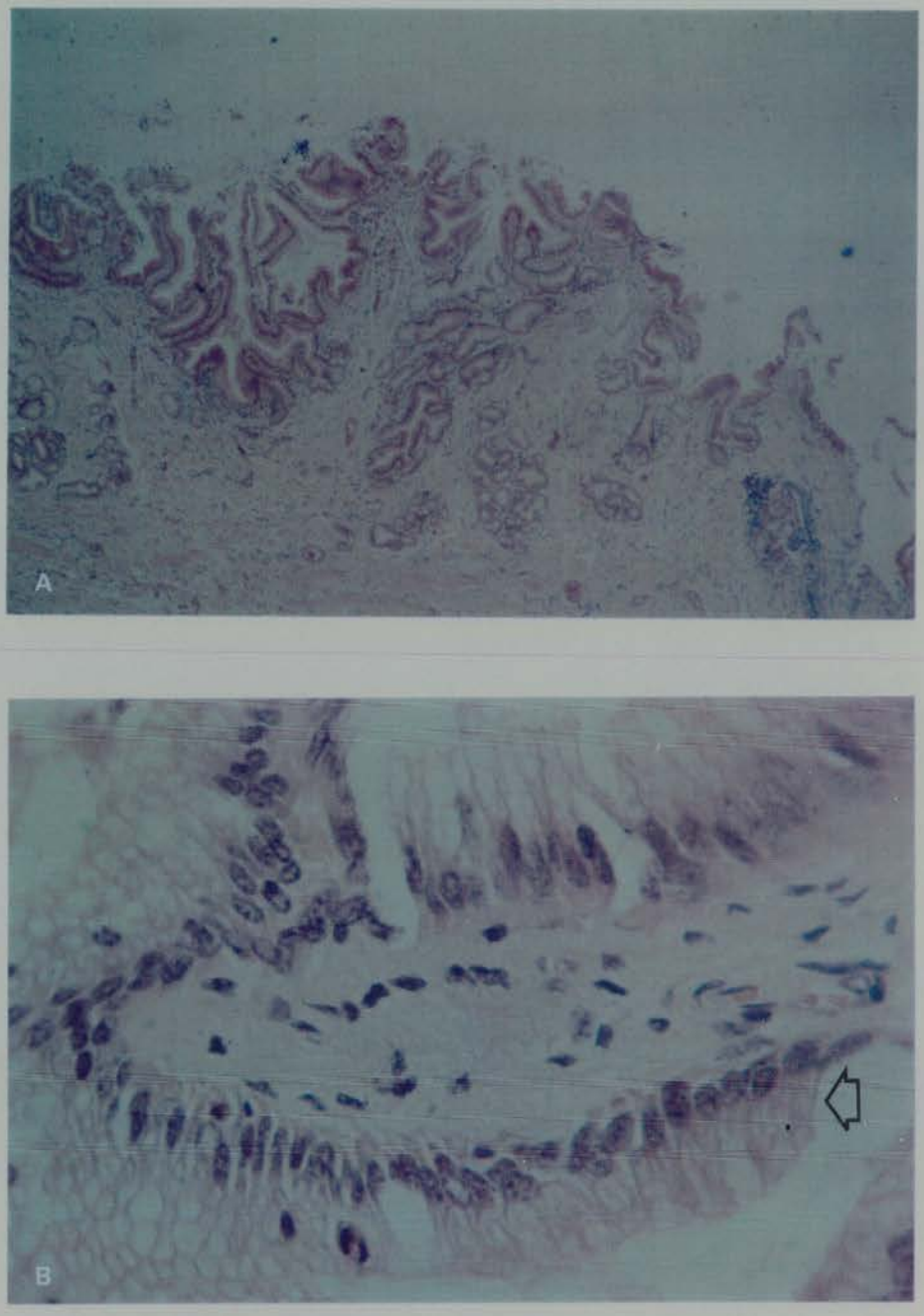
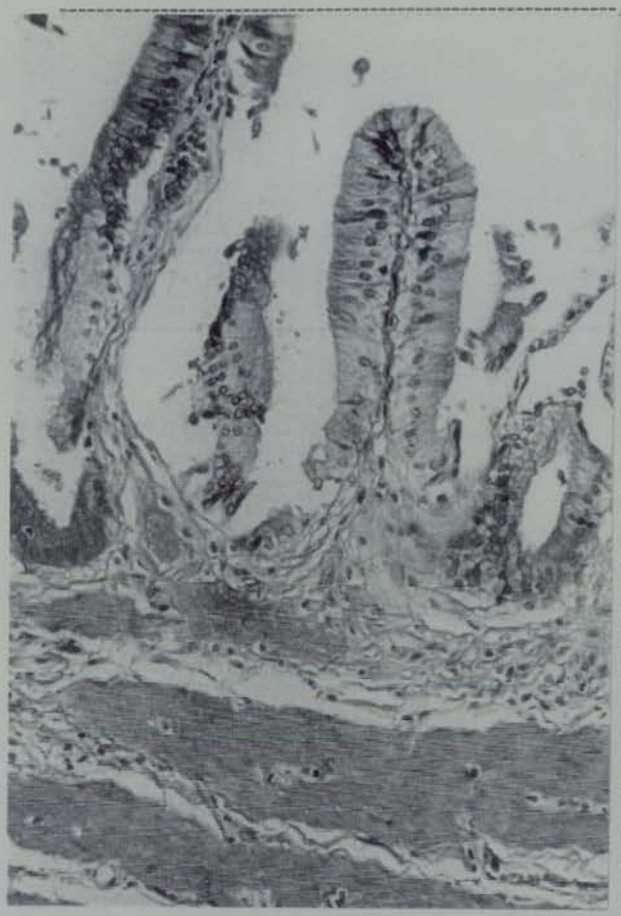


Figure 35: Microscopic anatomy of the bovine gallbladder epithelium.

(a) Bovine gallbladder epithelium showing numerous folds and the supporting structures (x 20).

(b) The normal columnar epithelium (arrow) is seen in higher magnification (x 40).

A



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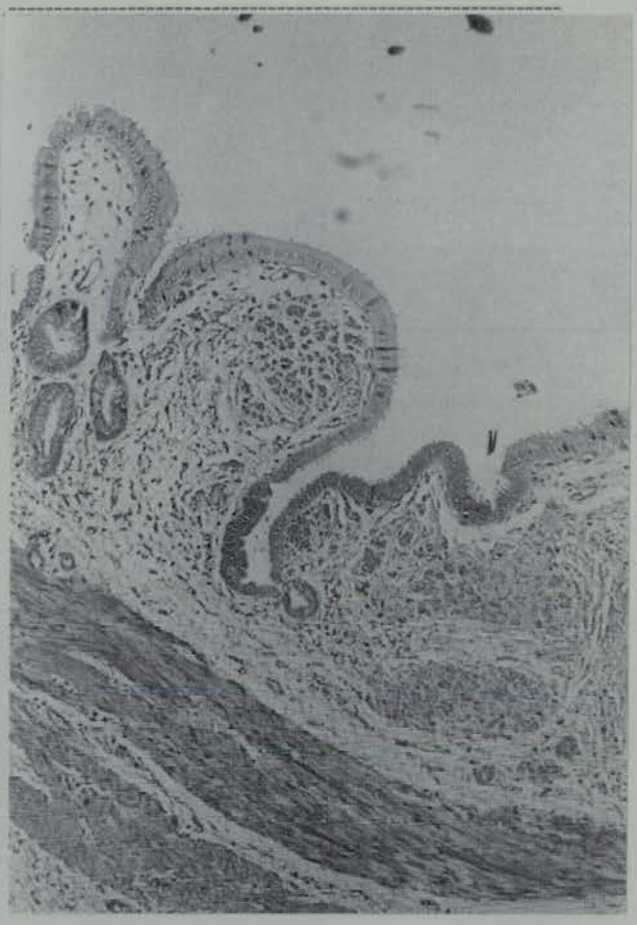

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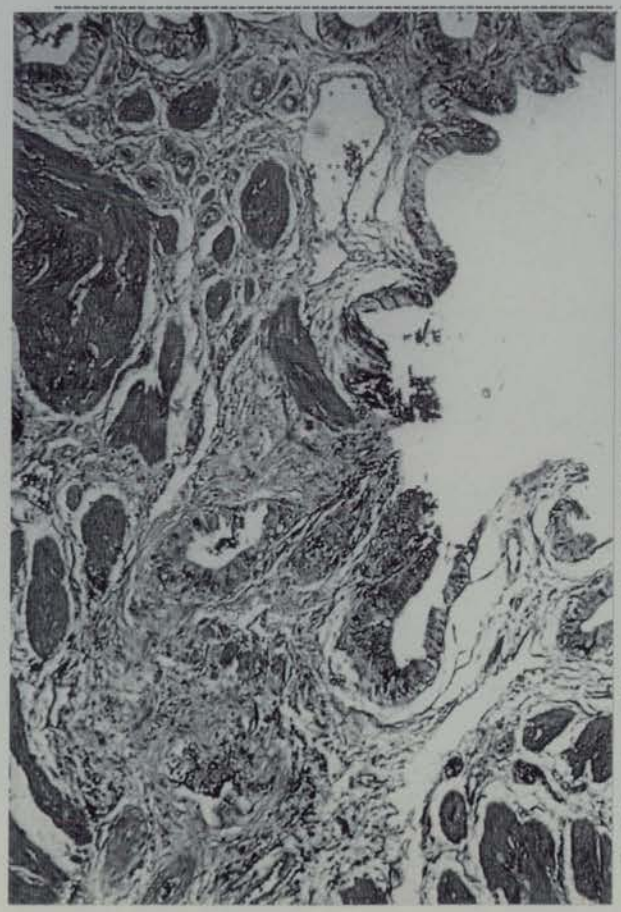

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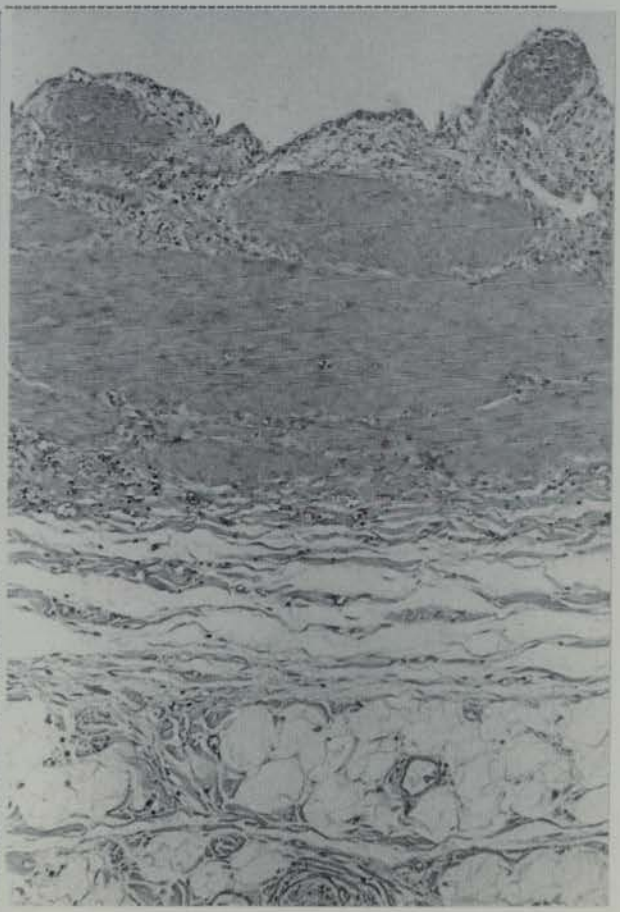

Figure 36:

(A) Gallbladder epithelium showing normal columnar epithelium with microvilli.

(B) Mildly inflammed gallbladder showing flattening of the columnar epithelium; damage of the brush border is seen in some cells.

(C) Moderately inflammed gallbladder with quite extensive epithelial injury, associated focal epithelial ulceration, oedema and fibrosis.

(D) Chronically inflammed gallbladder with mucosal loss, marked chronic inflammatory changes, with hypertrophy and fibrosis of the muscular layer.

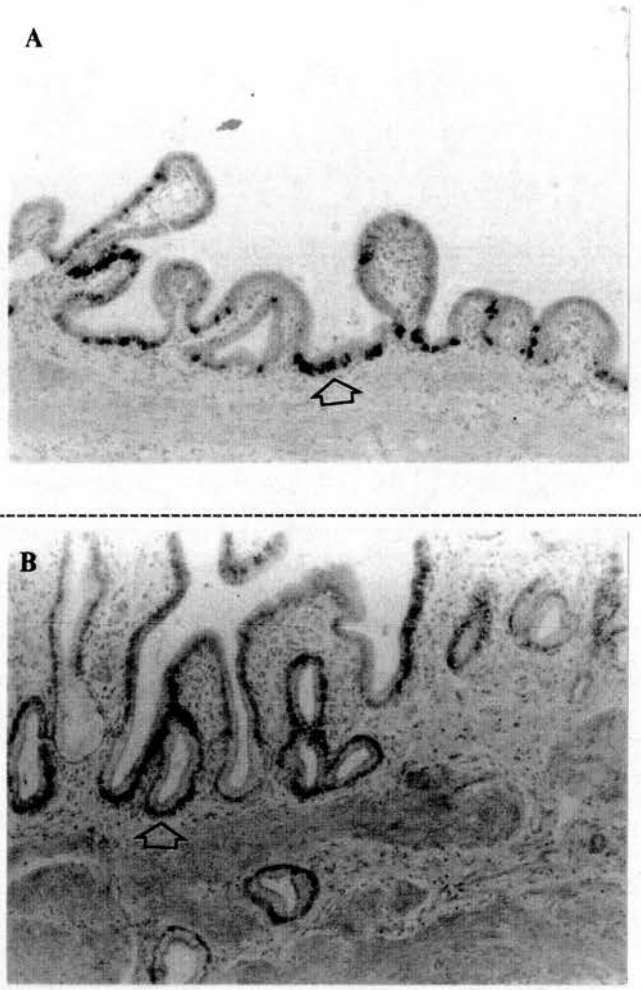

Figure 37: Distribution of carbonic anhydrase in the epithelium of the normal (a) and the abnormal (b) gallbladder (x100).

7.4 CONCLUSIONS

In this study we have demonstrated that:

a.) the bovine gallbladder has striking morphological similarities with the human gallbladder.

b.) samples of human gallbladder tissue were presented to demonstrate the morphological changes caused by inflammation.

c.) the distribution of the enzyme carbonic anhydrase within the gallbladder epithelial cells was not different between normal and inflammed gallbladders.

CHAPTER 8
EXPERIMENTS ON BOVINE GALLBLADDER BILE.

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8.1 BACKGROUND

Over the last few years the importance of bile pH and electrolytes has become appreciated, in particular the role of calcium is considered of relevance in the formation of both cholesterol and pigment stones (Rege RV et al 1985, Moore EW 1990). Calcium salts are the major inorganic components of all pigment stones and are present as the salts of 'calcium - sensitive' anions such as bilirubinates, carbonates, phosphates and fatty acids (Moore EW 1990, Heuman DM 1990). Cholesterol stones also contain calcium in their central nidus and often show peripheral calcification. Bile pH , the concentrating properties of the gallbladder, bicarbonate concentration, and the concentration of other ions present in bile such as sodium , chloridc and phosphate can influence calcium concentration and in particular the free ionised component *(Ca^{++})*. The consequences of bile acidification on calcium carbonate solubility have been studied in dogs (Rege RV et al 1987) and it has been shown that acidification of bile may prevent gallstone formation by reducing the likelihood of the precipitation of calcium carbonate in bile. It is not yet clearly established whether disturbances in bile pH and electrolyte composition are at least partly responsible for gallstone formation. Further studies to enhance our understanding of the normal physiology and pathophysiology of these costituents of bile are justified.

8.2 AIM

There were two aims in this study:

a) to evaluate whether the bovine bile model can be used to study normal gallbladder physiology with relevance to the human situation and

b) to use this model to study bile electrolyte regulation and to evaluate the influence of biliary pH on calcium salt solubility.

8.3 MATERIAL AND METHODS

Gallbladder bile was obtained from the intact bovine gallbladder retrieved immediately after animal slaughter at the local abattoir. The bile was aspirated

anaerobically, transferred to the laboratory in sealed syringes at temperature of 4°C, and aliquoted into 4 separate containers for the subsequent measurement and calculation of the various analytes and derived parameters. The time between animal death and the sample reaching the laboratory, was approximately 30 min. The following measurements were performed;

(a). Bile pH was measured immediately using a pH electrode *(Russel CMAWL/4/5 Hg_2/Cl_2 electrode)* connected to a digital display pH meter *(M83 Autocal pH Meter, Radiometer, Copenhagen)*. The mean of two measurements was calculated for each bile sample.

(b). pCO_2 was measured in a 1302 pH/ blood-gas analyser *(Instrumentation Laboratory system (UK) Ltd, Warrington, UK)* at 37°C .Total CO_2 concentration was determined by the enzymatic method which utilises phosphoenolpyruvate carboxylase (Forrester RL et al 1976) adapted for use on a centrifugal analyser; these values were used to test whether the Henderson-Hasselbach equation was applicable in bile.

(c). Sodium and potassium concentrations were determined by flame photometry *(IL 543 photometer, Instrumentation Laboratory (UK) Ltd, Warrington, UK)*. Chloride concentration was measured by coulometric amperometric titration using a Corning 920 chloride meter *(Corning Ltd., Halstead, Essex, UK)*. Total calcium concentrations were measured using the calcium- cresolphthalein complexone reaction (Connerty HV et al 1966) adapted for use on a centrifugal analyser.

(d). An ionised calcium analyser *(NOVA 2 Biomedical, Newton, U.S.A)* was used to measure the ionised calcium concentration in bile samples. This analyser uses a calcium - selective and a reference (KCl) electrode which are all straight-tube design allowing the sample to flow straight through the electrodes. The calcium electrode uses an immobilized liquid membrane which develops an electricochemical potential for calcium ions in solution, according to the Nerst equation. The reference electrode provides continuous flow of both standards and samples. The potential from the calcium electrode/reference pair is amplified and presented to the microcomputer of NOVA 2 for analytical calculations. Ionised calcium concentrations were measured and corrected for variations in the ionic strength of each bile sample (10): Mixed $CaCl_2$/NaCl standard solutions were prepared ($[Ca^{++}]$ = 1 - 3 mmol/l) at various ionic strengths ($[Na^+]$ = 150 - 300 mmol/l) and standard curves were developed. The corrected $[Ca^{++}]$ of each bile

sample was then calculated from the standard curves and the ionic strength (based on sodium concentration) of each bile sample.

(e). Total inorganic phosphate concentration was determined using the method of Wentz PW et al, 1976.

(f). The saturation index (SI) of $CaHPO_4$, which is the salt most likely to precipitate in bile (Moore EW et al 1989) was calculated using the following formulae (Moore EW, 1989):

$$(A)\ [HPO_4^{=}]=[TPO_4]/(1+10^{pk2'-pH})$$

$$pK2'=6.99 \text{ at } 37°C$$

$$(B)\ SI(CaHPO_4)=[Ca^{++}]x[HPO_4^{=}]/ksp'.$$

$ksp'= 1.65x10^{-6}$ *M* (determined by Moore et al (12)).

(g). The $[CO_3^{=}]$ in each sample was calculated from the Henderson-Hasselbalch equation as modified by Moore EW and Rege RV, 1986:

$$[CO_3^{=}]=[HCO_3^{-}]x[10^{pH-pk'1}]$$

$$pK'1=9.8 \text{ at } 37°C$$

(h). The saturation index (SI) of calcium carbonate was calculated from the formula :

$$SI(CaCO_3)=[Ca^{++}]x[CO_3^{=}]/Ksp'$$

$ksp'=3.76x10^{-8}$ *M (14).*

(i). The total bile acid concentrations and the concentrations of the conjugated bile acids which were present in bovine bile were determined by High Performance Liquid Chromatography (HPLC). The bile salts were separated by reverse-phase ion pair chromatography using a modified method previously described by Wildgrube HJ et al, 1983. HPLC was performed using a *Waters 510 pump, Perkin-Elmer ISS 101 Autosampler, Perkin -Elmer Diode Array Detector and a Kontron Integration pack.* For the analysis, the bile salts were diluted in methanol (1:2) , centrifuged and the supernatant passed through a 0.22 um filter *(Micro Separators Inc.)*.Samples of 10ul were injected onto the stationary phase, Rosil Column (250 x 4.6 mm) *(Capital HPLC)*. A mobile phase of Acetonitrile -water (40-60) containing 0.5 mol/l tetraethylammonium phosphate at a flow rate of 1.1. ml/min was used. The samples were quantitated with reference to bile acid standards by UV absorption at 210 nm.

(j). Cholesterol concentration was determined by enzymatic estimation using cholesterol- oxidase method adapted for use on a centrifugal analyser.

(k). The osmolalities of the bile samples were measured on the Advanced Micro-osmometer Model 3MO *(Kontron Instruments, Cumbernauld, UK)*.

8.4 STATISTICAL ANALYSIS OF DATA

Data analysis was performed using the MINITAB V 7.0 statistics program . The data were expressed as mean±SE. Regression analysis was carried out to identify correlations between the various variables and the Pearson coefficient was used to measure the degree of correlation. Values of $p<0.05$ were considered statistically significant.

8.5 RESULTS OF THE STUDIES ON BOVINE GALLBLADDER BILE

8.5.1 Profile of bovine bile

A total of 47 gallbladder bile samples were studied. The composition of human and bovine gallbladder bile is presented in *Table 8.*

	HUMAN BILE	*BOVINE BILE*
pH	7.18±0.026*	7.11±0.02
HCO_3=(mmol/l)	18.2±0.8*	21.1±2.1
Na (mmol/l)	186.4±3.4*	200.7±3.5
K (mmol/l)	10.3±0.4*	8.6±0.2
TCa (mmol/l)	3.7±0.8**	4.24±0.16
Ca++ (mmol/l)	1.14±0.04**	0.97±0.05
pCO_2 (mmHg)	47.1±1.2*	65.7±1.6
Osmol(mOsm/l)	280.4±1.4*	284.7±1.2
Chol (mmol/l)	7.2±0.4**	1.12±0.04
TBA (umol/l)	119±9.2**	139.5±3.6
GC "	37.89±4.79(31.6%)**	60.7±2.6(43%)
GCDC "	43.16±4.54(36.3%)**	5.47±0.23(3.6%)
GDC "	16.87±2.12(14.18%)**	15.75±1.3(11.5%)
TC "	6.13±0.88(5.15%)**	37.6±2.7(27%)
TCDC "	12.89±1.85(10.8%)**	4.11±0.26(2.9%)
TDC "	2.93±0.6(2.46%)**	15.64±1.1(11.5%)

TCa=Total calcium	*GC=Glucocholic acid*
Ca++=Ionised calcium	*GCDC=Glucochenodeoxycholic acid*
Osmol=Osmolality	*GDC=Glucodeoxycholic acid*
Chol=Cholesterol	*TC=Taurocholic acid*
TBA=Total Bile Acids	*TCDC=Taurochenodeoxycholic acid*
pCO_2=partial pressure CO_2	*TDC=Taurodeoxycholic acid*

All values are expressed as MEAN±SE.

TABLE 8 : Composition of human and bovine gallbladder bile; (**) represents measurements on human bile performed in our laboratory (N=15, unpublished data); (*) represents measurements from other studies (Aronchick CA et al. 1985).

Comparison was made with the data for human bile produced from measurements in our laboratory (unpublished data) and from other published studies (Aronchick CA et al 1985). Bovine gallbladder bile composition was similar to human regarding pH and electrolytes concentration; however the cholesterol concentration was significantly lower in bovine bile (6-fold) and there was a significantly higher percentage of tauro - conjugated bile acids compared with human gallbladder bile. Sodium concentration has been used as a marker of the degree of concentration of the gallbladder bile because it was linearly correlated with total bile acid concentration *(Figure 38)*.

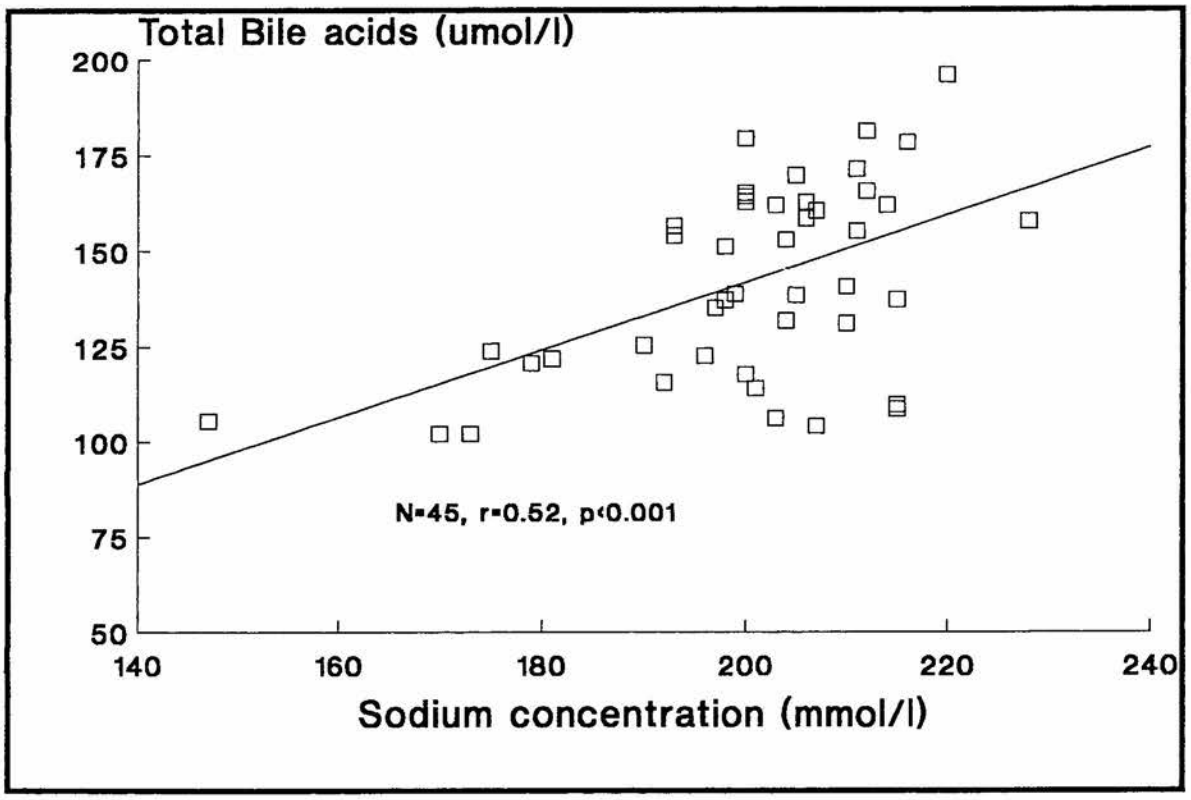


Figure 38: Bile acids concentration as a function of sodium concentration in bovine gallbladder bile.

The osmolality of bovine gallbladder bile remained unchanged over a wide range of sodium concentrations and bile pH values *(Figure 39)*.

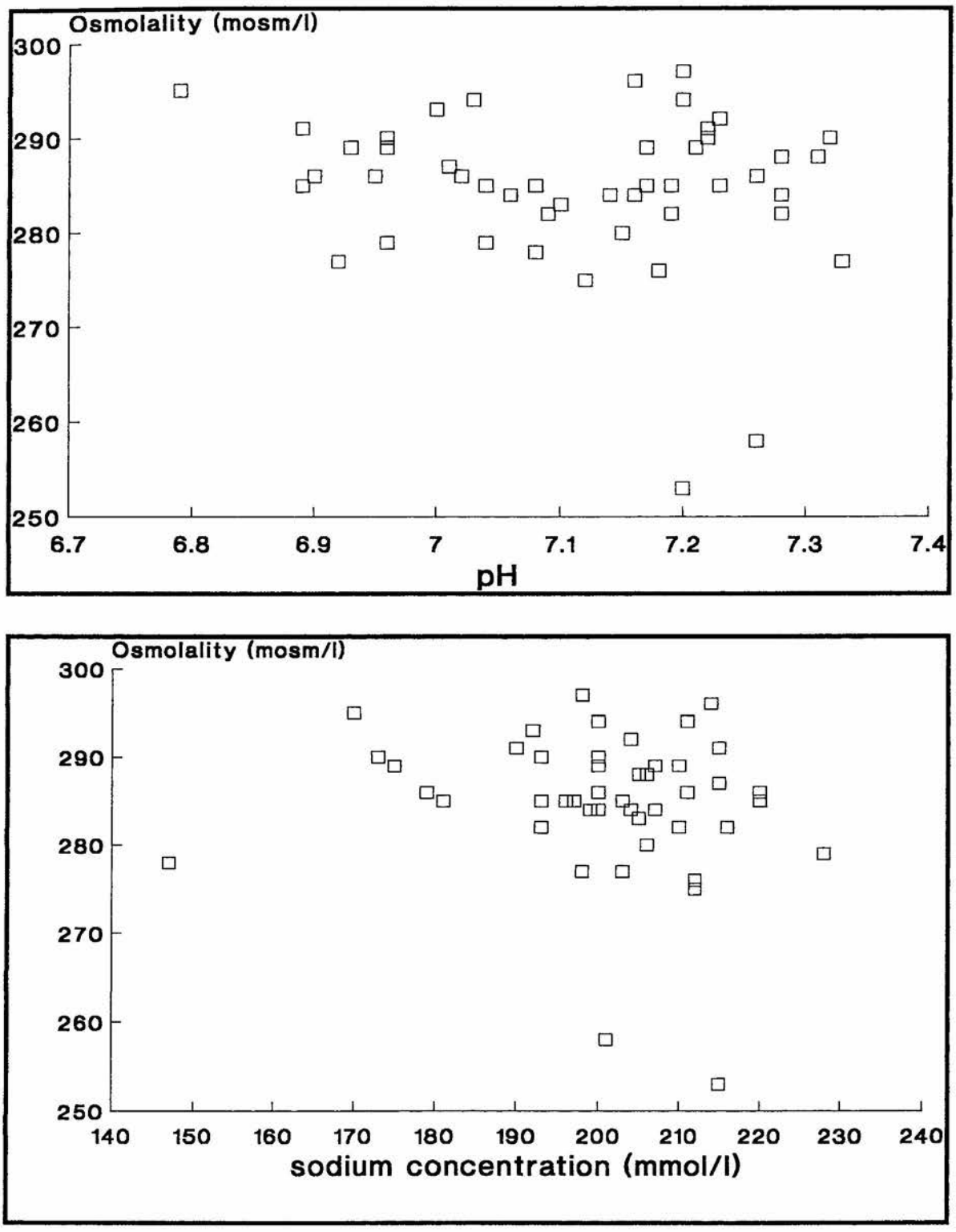


Figure 39: Bile osmolality as a function of pH and sodium concentration. No change in osmolality was present over a wide range of values.

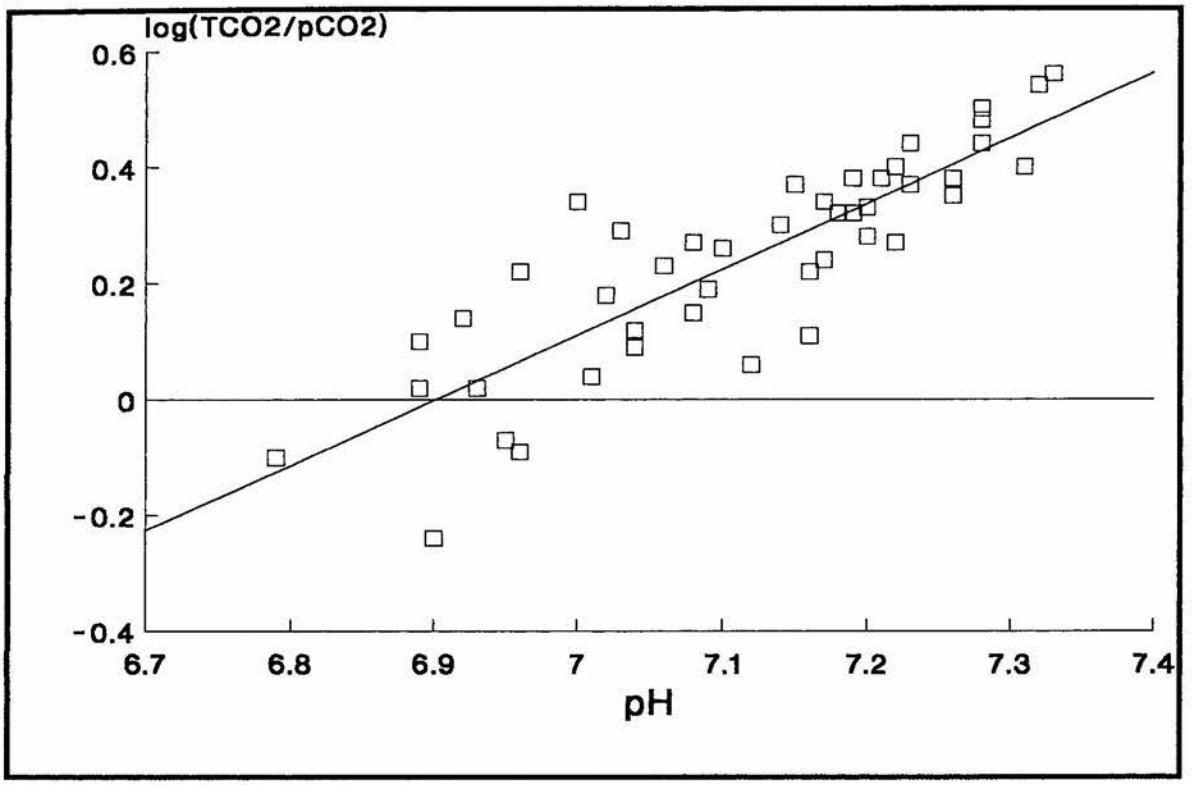


Figure 40: Log (TCO_2/pCO_2) as a function of pH for 47 samples of bovine gallbladder bile. The data demonstrated that the Henderson-Hasselbach equation is applicable to the bovine gallbladder bile ($r=0.9$, $p<0.001$). TCO_2=Total bicarbonate concentration. pCO_2=Partial pressure of carbon dioxide.

8.5.2 Acidification of the gallbladder bile

A linear correlation between bile pH and log(TCO_2/pCO_2) was present. TCO_2 represented the total bicarbonate concentration measured enzymatically and the linear correlation indicates that the Henderson-Hasselbalch equation is applicable in bovine bile and that bicarbonate is the primary buffer in gallbladder bile *(Figure 40)*. The partial pressure of CO_2 (pCO_2) was linearly increased as bile pH became more acidic *(Figure 41)*. There was a linear correlation between bile pH and sodium concentration in those bile samples with a sodium concentration greater than 200mM *(Figure 42)*. Bile pH showed a positive linear correlation with chloride *(Figure 43a)* and a negative

correlation with potassium *(Figure 43b)* and calcium *(Figure 44a)*. Bile chloride correlated linearly with the bicarbonate concentration *(Figure 44b)*.

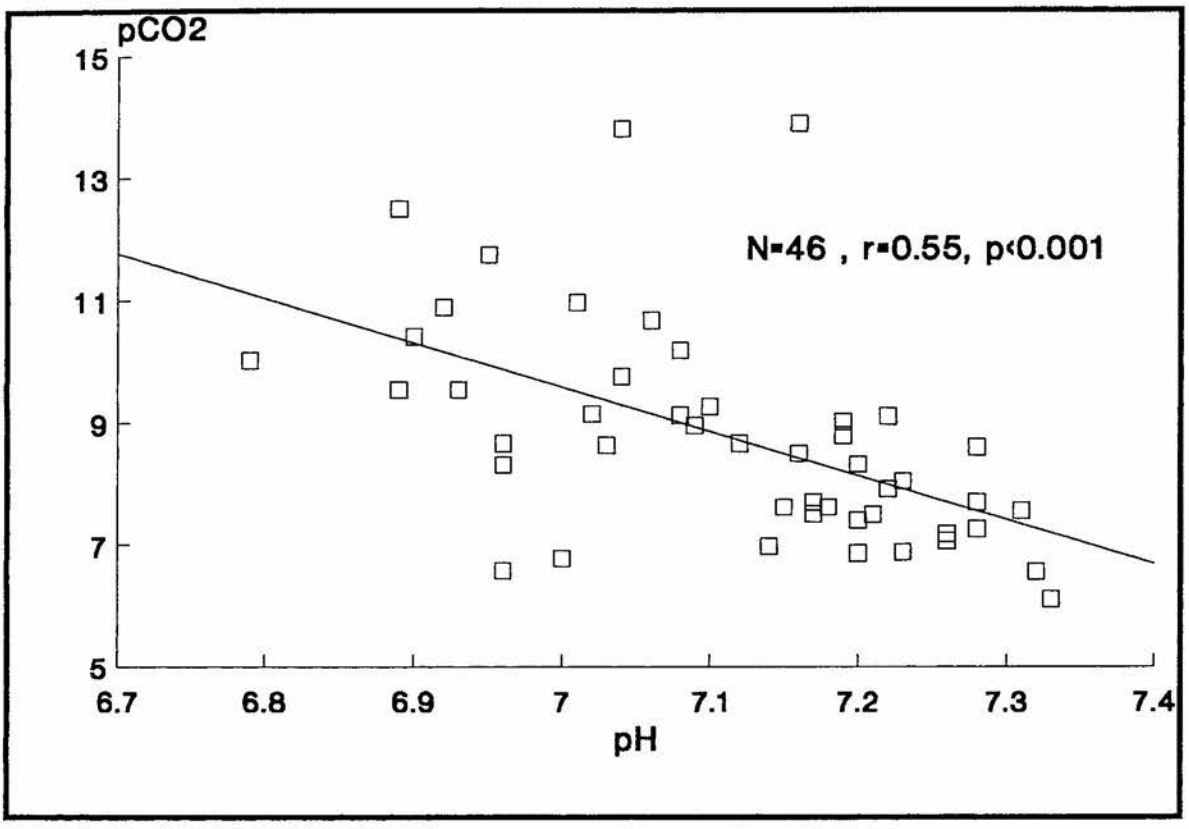
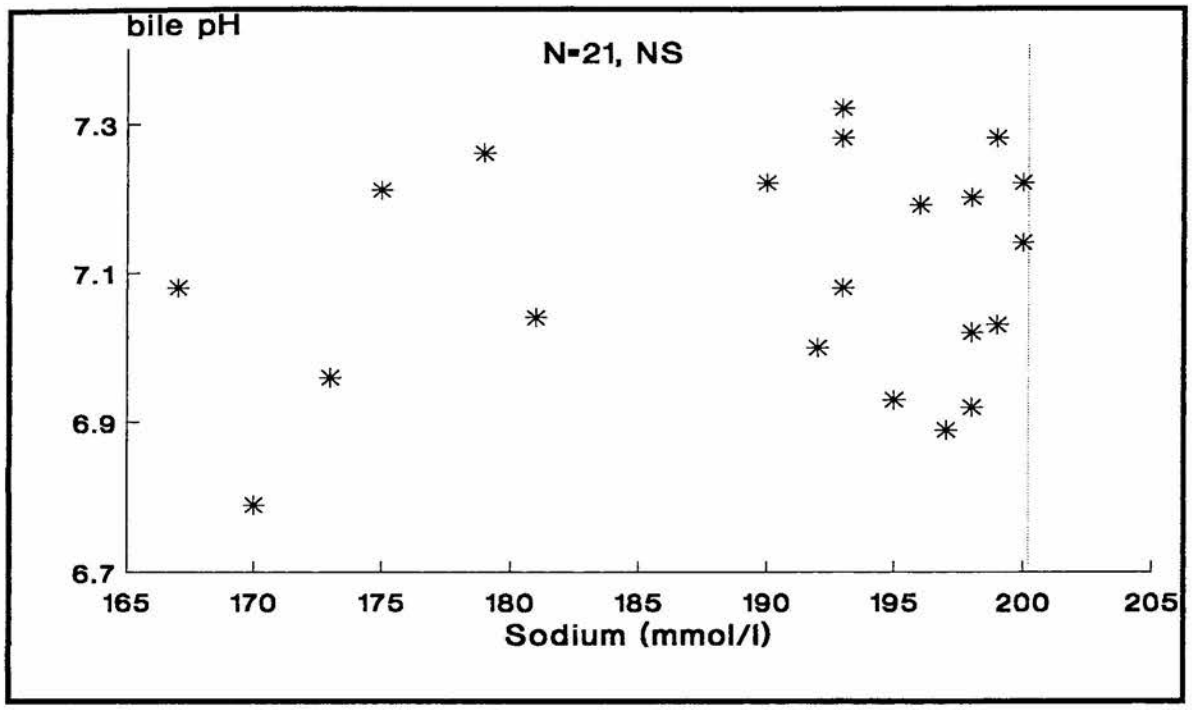


Figure 41: The partial pressure of CO_2 was linearly increased with bile pH.



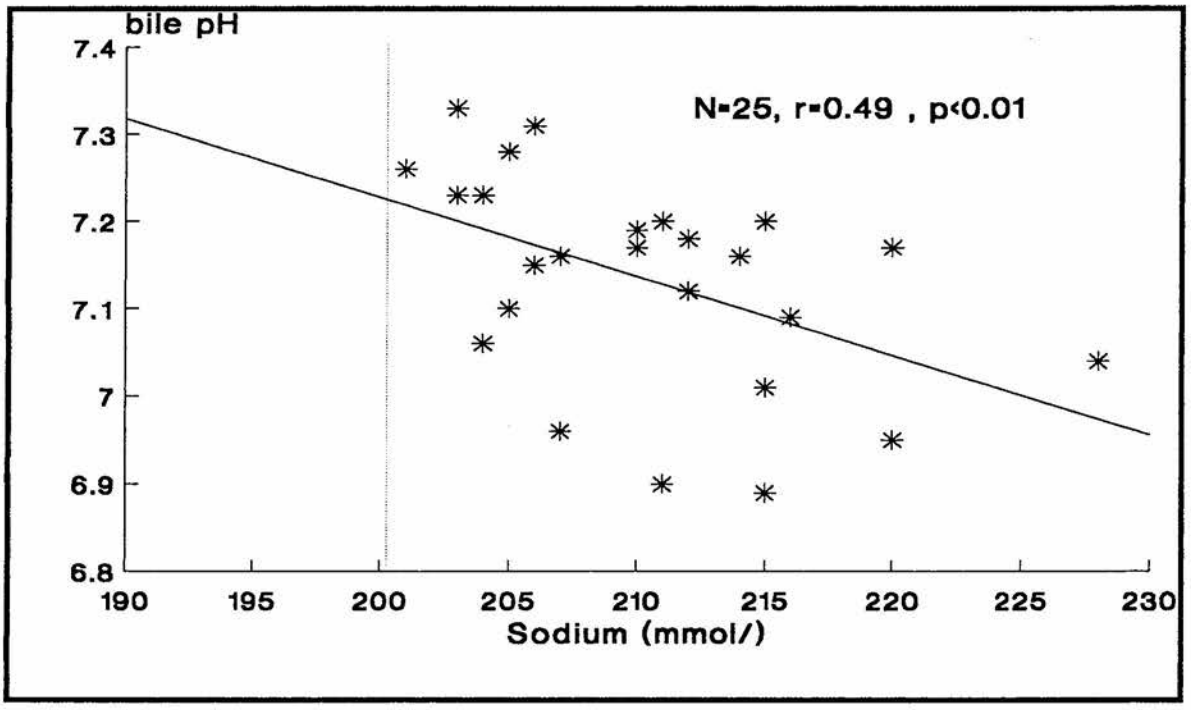
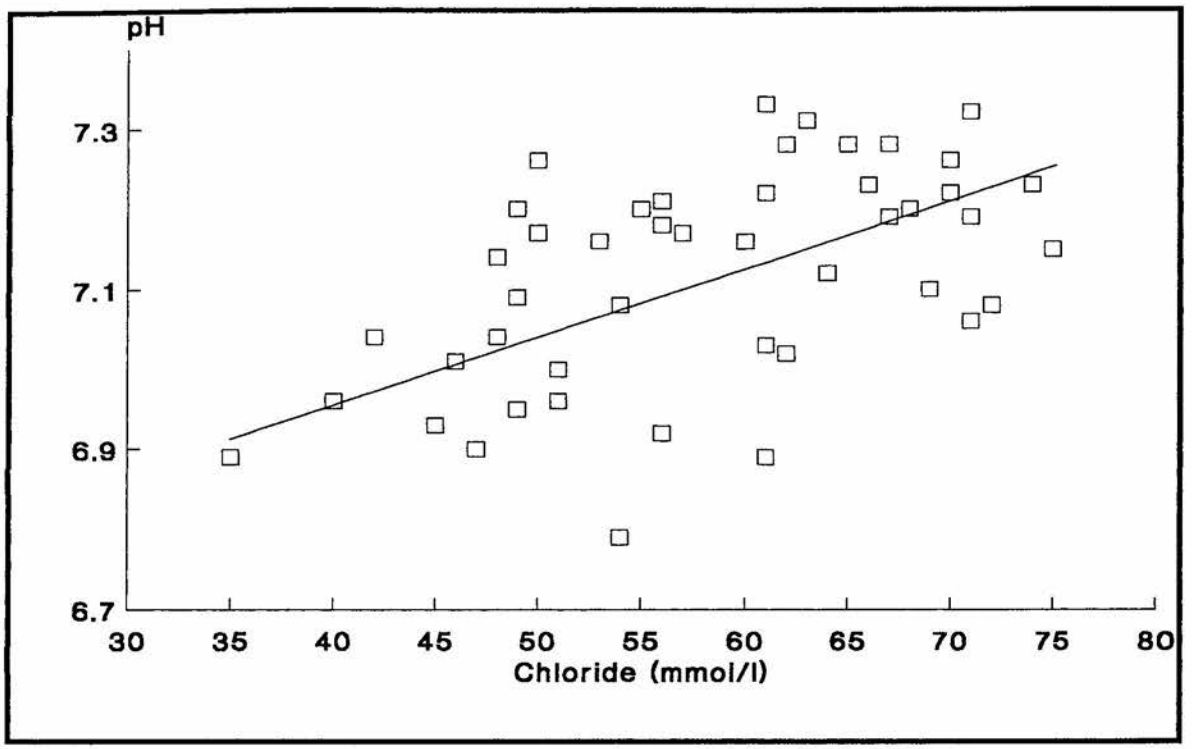


Figure 42: Bile pH expressed as a function of sodium concentration in 47 samples of bovine gallbladder bile. A linear correlation between bile pH and sodium concentration was present in bile samples with sodium concentration >200mmol/l (r= 0.49 , p<0.01).



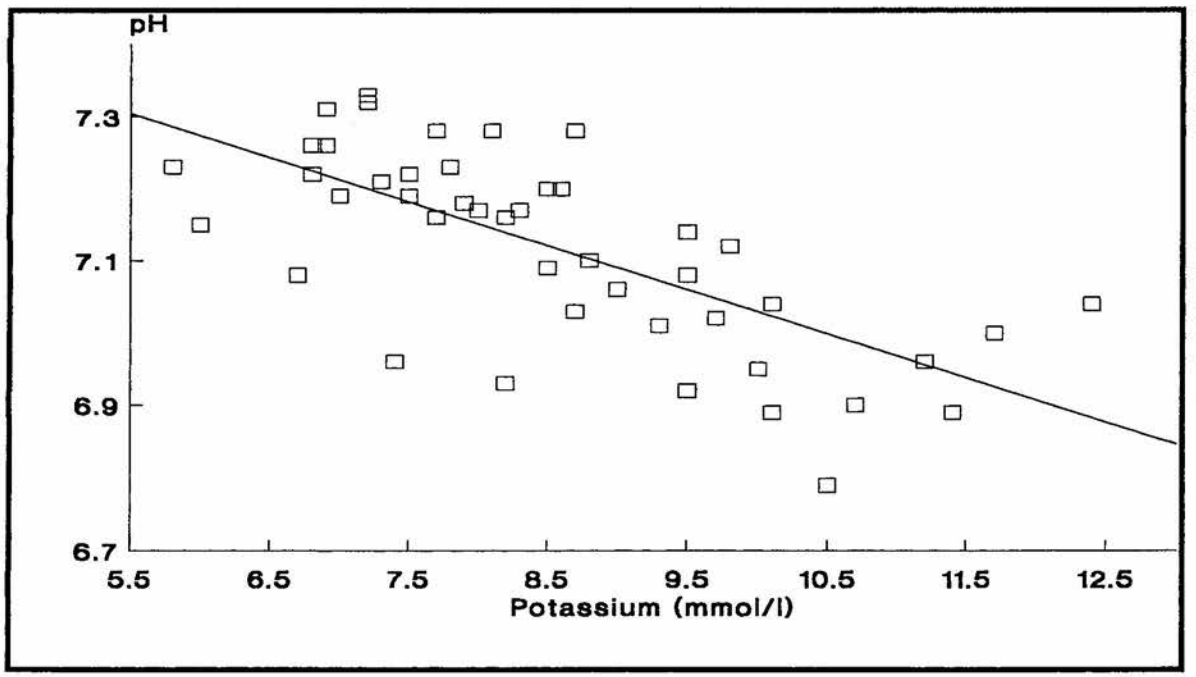
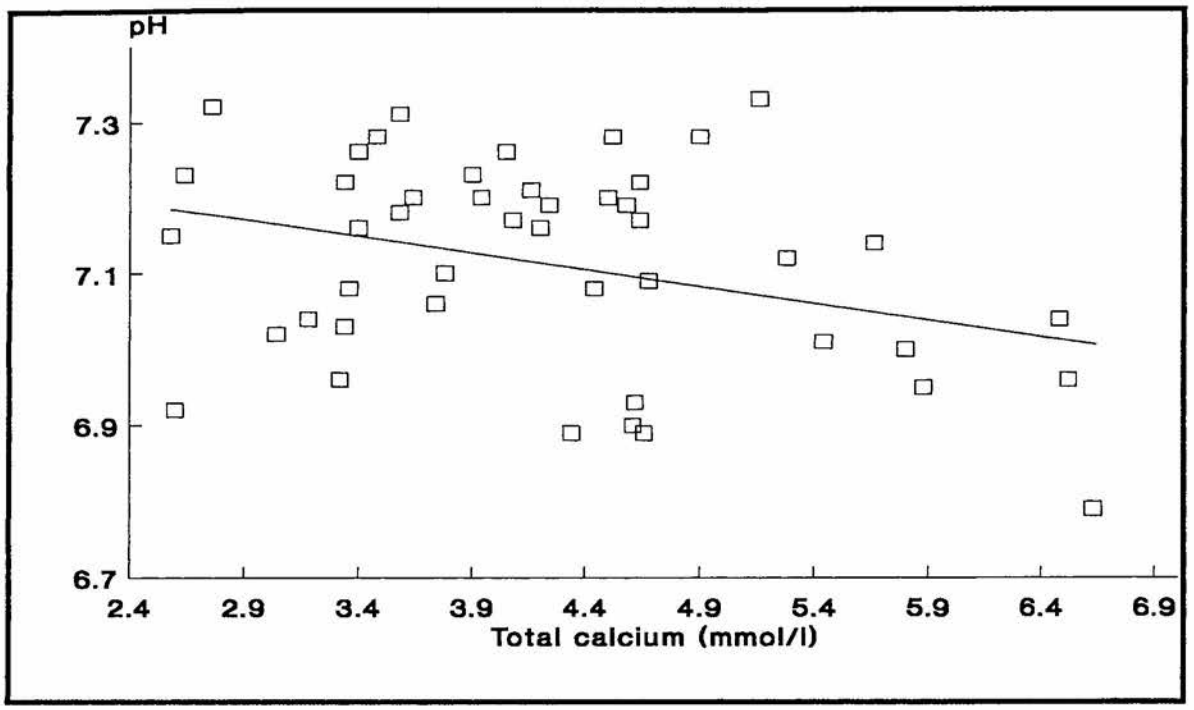


Figure 43: Total chloride (a) ,(r= 0.53 , p<0.01), and potassium (b) (r=0.48 , p<0.01) concentrations expressed as a function of bile pH in 47 gallbladder bile samples.



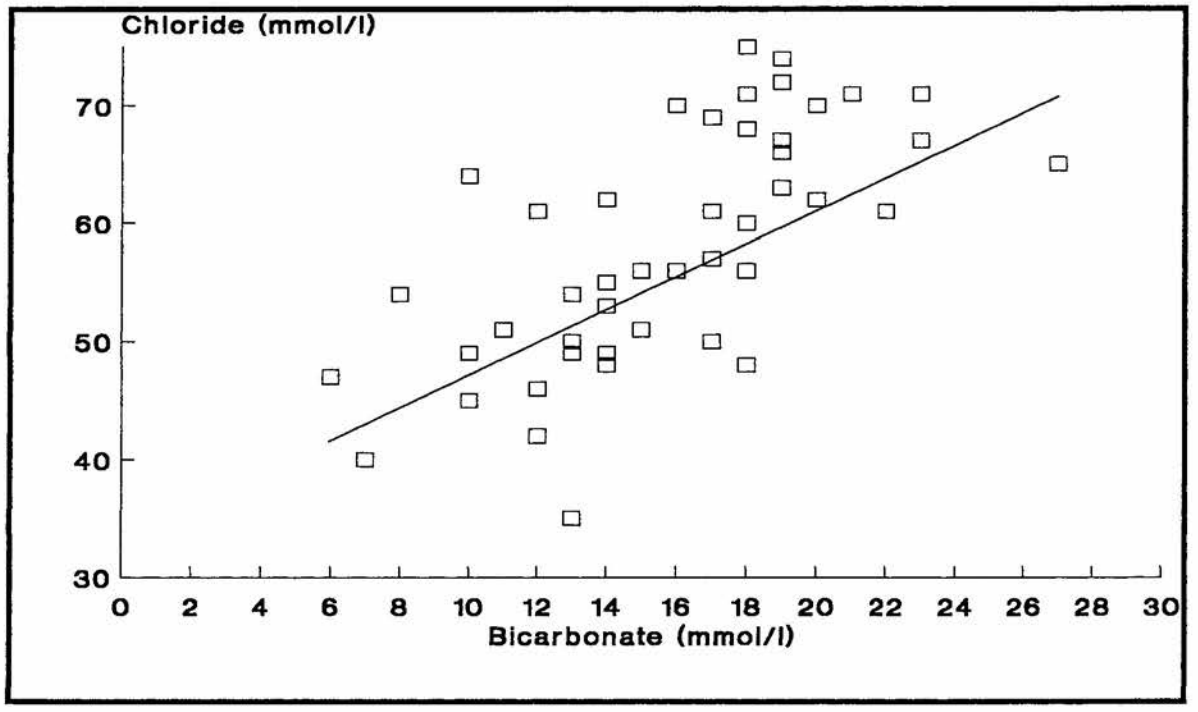


Figure 44(a): Negative linear correlation between bile pH and total calcium concentration (N=46, r=0.7, p<0.01); (b) Positive linear correlation between bile chloride and bicarbonate concentration (N=46, r=0.41, p<0.02)

8.5.3. Solubility of calcium salts

The ionised calcium concentration of each bile sample, measured by the Ca^{++} analyser, was corrected for bile sodium concentration according to the graph in *Figure 45*. The saturation indexes (SI) of calcium carbonate against calcium phosphate were subsequently calculated. Both were influenced by bile pH and acidification of bile had a favourable effect on the solubility of those salts *(Figure 46)*. Within the pH range of the bile samples observed in this study , all but one bile sample were unsaturated with calcium phosphate. In contrast, 60% of the samples with pH above 7.10 (the mean bile pH in this study) were supersaturated with calcium carbonate *(Figure 46)*.

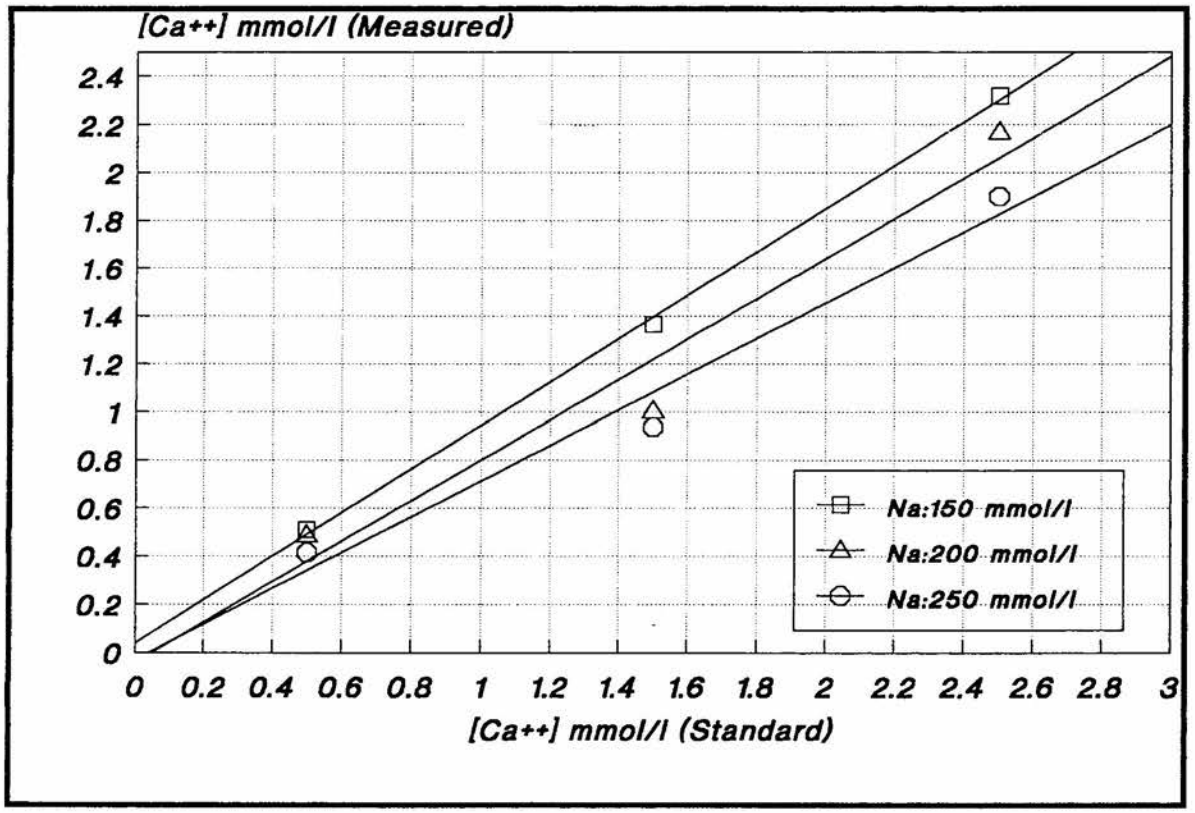


Figure 45: Measured $[Ca^{++}]$ versus actual $[Ca^{++}]$ of standard samples prepared at 3 different Na^{+} concentrations (150, 200 and 250 mmol/l).

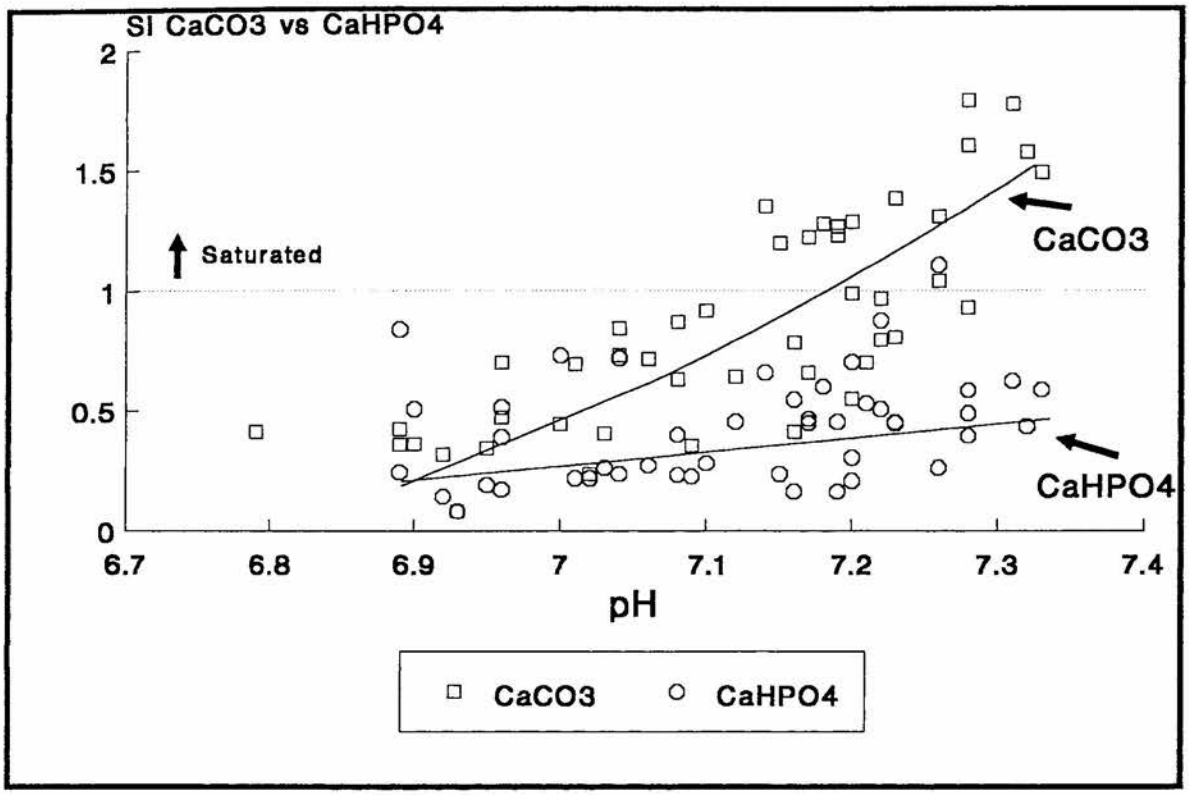


Figure 46: Saturation indexes for $CaCO_3$ and $CaHPO_4$ as a function of bile pH ($r=0.8$, $p<0.001$ for $CaCO_3$ and $r=0.38$, $p<0.03$ for $CaHPO_4$)in 47 bile samples.Both saturation indexes were increased with bile pH.

8.6.CONCLUSIONS

In this study we have shown that:

a) *bovine bile is a good model to study bile physiology* particularly in relation to pigment stone formation, because the concentrations of most of its components are strikingly similar to human , with the exception of the lower cholesterol concentration.

b) *the Henderson-Hasselbalch equation is applicable to bovine bile* and that *bicarbonate appears to be the main buffering system in bile* because the calculated bicarbonate concentrations from the Henderson-Hasselbalch equation matches those measured enzymatically.

c) *the reduction in bile pH linearly correlated with sodium concentration in samples with sodium concentration above 200 mM.*

d) *the concentration of potassium and calcium ions was found increased in the concentrated bile,* whilst *chloride concentration was reduced* as bile became more concentrated presumably due to its active absorption and distribution according to electrochemical forces .

e) *the osmolality of the concentrated bile remained remarkably stable through a wide range of sodium and pH values.*

f) relatively *more alkaline gallbladder bile was associated with a higher saturation index for calcium carbonate*; in our study when bile pH exceeded 7.10, 60% of the bile samples were supersaturated with calcium carbonate and the likelihood of precipitation of this salt was thermodynamically increased. *In contrast with calcium carbonate, $CaHPO_4$ solubility remained high within the pH values encountered in the bovine gallbladder* in all but one of the bile samples ; however, the effect of acidification was still to enhance the solubility of this salt.

CHAPTER 9
EXPERIMENTS USING ISOLATED BOVINE AND HUMAN GALLBLADDER EPITHELIAL CELLS.

9.1 PRIMARY CULTURE OF BOVINE GALLBLADDER EPITHELIAL CELLS

9.1.1 AIM:

9.1.2. MATERIAL AND METHODS

CELL ISOLATION

TRYPAN BLUE EXCLUSION METHOD

CELL CULTURE

MONITORING OF CELL GROWTH AND CHARACTERISATION OF CELLS.

9.1.3. RESULTS

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9.2 VISUALISATION OF ACID SECRETION BY THE HUMAN GALLBLADDER EPITHELIAL CELL

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ACRIDINE ORANGE.

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CHAPTER 9
EXPERIMENTS USING ISOLATED BOVINE AND HUMAN GALLBLADDER EPITHELIAL CELLS.

9.1 PRIMARY CULTURE OF BOVINE GALLBLADDER EPITHELIAL CELLS

9.1.1 BACKGROUND AND AIM:

Until recently, all studies on gallbladder epithelial cells functions have been restricted to using part or whole of the gallbladder. Several physiologists have used gallbladders from animal species to study ion transport and the electrical properties of the epithelium by applying either the Ussing chamber technique or whole organ preparation techniques. Both these techniques have been used in the present thesis and it has been realised that although these techniques are invaluable for ion transport or pharmacological studies, they provide little information on the intracellular events which are linked with the various biological properties of the gallbladder epithelium. On the other hand , the few existing techniques of isolating and culturing gallbladder epithelial cells are complicated and difficult to apply.

Another limiting factor in studying the properties of human gallbladder epithelium has been the lack of normal tissue. This has now become more readily available with the development of liver transplantation programmes. Alternatively, many animal models have been used to overcome the lack of availability of normal human tissue. The development of a simple and reliable method of obtaining and culturing gallbladder epithelial cells from a readily available source would permit a detailed study of the biological functions of these cells and would contribute to our understanding of the pathophysiology of diseases of the biliary tree.

The aim of this set of experiments was to develop a simple and reproducible method of culturing gallbladder epithelial cells from a readily available source, the bovine gallbladder and apply the same technique to human tissue.

9.1.2. MATERIAL AND METHODS

CELL ISOLATION

Bovine gallbladder was obtained from the local abattoir and transferred to the Laboratory on ice within 20 min of slaughter. The gallbladder was opened and the lumen rinsed with modified Hank's balanced salt solution *(HBSS, Sigma Chemical Co,Ltd St Louis, USA)* to remove bile and debris. The mucosa was stripped from the underlying muscular layer and fat by blunt dissection. Two circular pieces (2-3cm diameter) of mucosa were used from the fundus of the gallbladder for harvesting the cells. The mucosa was left for 15 min in preoxygenated HBSS and then rinsed in a series of Petri dishes containing 20 ml HBSS with 5ml N-Acetylcysteine (1mg/ml) *(Sigma Chemical Co, Ltd)* to dissolve the mucus. Each piece was then incubated for 20 min at 37°C in 20 ml Trypsin-EDTA *[0.5 g trypsin 1/250 and 0.2 g EDTA.4 Na /l, in Hank's balanced solution, Ca^{++} amd Mg^{++} free, (Sigma Chemical Co, Ltd)]* sufficient to cover the tissue completely. The tissue was subsequently transferred to a second beaker with 4ml bovine calf serum *(Sigma Chemical Co, Ltd)* added to inactivate the trypsin. The solution was then made up to 20 ml by adding HBSS. After incubation for 5 min at room temperature to allow trypsin inactivation, the gallbladder epithelial cells were detached by gently scrapping the mucosa with the end of a glass microscope slide held at an angle of 45°. The separated cells were resuspended in HBSS solution and filtered sequentially through a stainless steel sieve of 0.38mm (size 40) and 0.28mm (size 50) pore size respectively. The above procedures were performed in a laminar flow hood using conventional sterile techniques.

The filtered solution was then centrifuged at 3g for 5 min, the supernatant removed and the cells resuspended in 10 ml of HBSS solution. The cells were counted using a modified Neubauer haemocytometer. The percentage cell viability was checked by dye exclusion with 0.4% trypan blue under normal light microscopy.

TRYPAN BLUE EXCLUSION METHOD

This method is based on the principle that live cells do not take up certain dyes , whereas dead cells do. The following steps were followed:

a) 0.5 ml of trypan blue 0.4% solution were transfered to a test tube ; 0.3 ml of HBSS and 0.2 ml of cell suspension were added, mixed thoroughly and allowed to stand for 5-10 minutes.

b) With the use of a Pasteur pipette, a small amount of Trypan blue-cell suspension was added to both chambers of a haemocytometer with a cover-slip in place.

c) The number of viable (unstained) and non-viable (stained blue) cells were counted in the 1mm center square and four 1mm corner squares in each chamber .An average count per square for the viable and non-viable cells was calculated.

d) % Cell viability was determined by the following calculations:

% Cell viability = [Average number of unstained cells ÷ by average total number of cells (stained and unstained)] x 100%

CELL CULTURE

The final suspension was centrifuged and the cell pellet resuspended in Dulbeco's modification of Eagles Medium containing 10% fetal calf serum and added antibiotics (10,000 u/ml penicillin, 0.5mg/ml streptomycin and 25ug/ml amphotericin B) to a concentration of 1 x 10^6 cells/ ml. The cells were subsequently plated under aseptic conditions in plastic multiwell plates (15mm diameter well adding 1ml of the above suspension to each well). Both uncoated and coated (with collagen type IV or Cytodex 3 microbeads) plates were used and the plates incubated in a standard 95% air/ 5% CO2 incubator at 37°C. Round glass cover slips (15 mm diameter) were also used in some wells. The medium was routinely changed after 24 hr and thereafter every 48 hr. Reverse phase microscopy was performed regularly to monitor the progress of the cultures .

MONITORING OF CELL GROWTH AND CHARACTERISATION OF CELLS.

The cell cultures were inspected and photographed at daily intervals. DNA flow cytometry was performed on cells disrupted from the culture monolayer by EDTA treatment using standard techniques (Vindelar LL et al 1983), to define the percentage of cells in G_2/M phase (division), S phase (DNA synthesis) and G_0 phase (resting).The cell nuclei were stained with propidium iodide and analysed by an *EPICS V Flow Cytometer*

(Coulter, UK). The analysis of cell cycle activity was undertaken by EASY 2 computer programme.

Transmission Electron Microscopy (TEM) was used to analyse the morphological features of the growing cells.The cells were fixed in 4% glutaraldehyde for 24 hr, post fixed in osmium tetroxide and stained with uranyl acetate on nickel grids using standard techniques. Grids were viewed on a *Jeol 2 Electron Microscope.*

9.1.3. RESULTS

9.1.3.1. Harvesting of cells and monitoring of culture

A yield of around 5 x10^6 cells/ g tissue was obtained from the 5-10 g of gallbladder mucosa used. The viability of the harvested cells was in excess of 99% as assessed by the trypan blue exclusion method. Using a range of culture media (*MCDB -153, Eagles medium and Dulbeco modified Eagles medium)* satisfactory plating and growth occured with most media. This was assessed by two independent investigators by observing a) the cell appearance and their ability to attach the plate in the first 24h and b) the length of time the culture required to reach confluency from the same starting number of cells per well. *Dulbeco's modified Eagles medium* containing 10% fetal calf serum had an advantage in both plating efficency and growth. Cell attachment was clearly improved in the plates coated with collagen type IV; nevertheless, satisfactory results were obtained by using uncoated plastic plates and glass cover slips avoiding the cost of using type IV collagen.

Visible clusters of epithelial cells were present by day 2 ; the attached epithelial cells appeared flatened with prominent nuclei *(Figure 47a)*. Significant growth was present by day 6 *(Figure 47b)*. In the coated plates, (type IV collagen) confluency was reached at 8-10 days *(Figure 47c);* in the uncoated plates at 12-14 days. No problems with contamination of the cultures were encountered and the regimen of penicillin / streptomycin / amphotericin B was considered satisfactory. Experiments with Cytodex 3 microbeads as coating agent were unsuccessful.

9.1.3.2. Flow cytometry

Flow cytometry was performed at day 6 and day 10 *(Figure 48a)*. When the cells were in the log phase of development (day 6) there were 21% of the cells in S phase with 9% in G_2 phase as compared with 10% in S phase with 1.55% in G_2 phase when the culture was reaching confluency by day 10 *(Figure 48b)*. These results indicated that the cell culture maintained satisfactory growth until confluency had been reached.

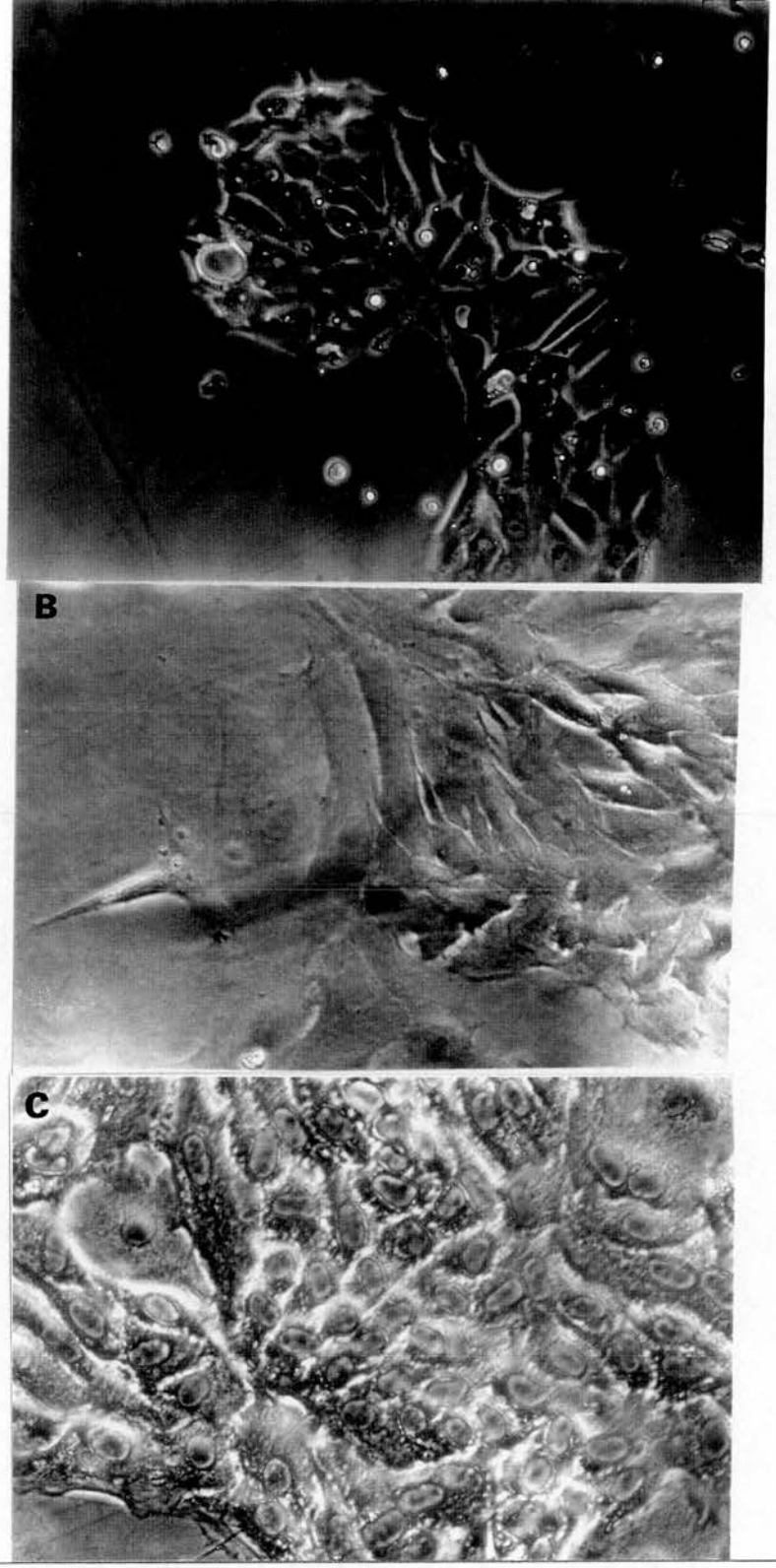
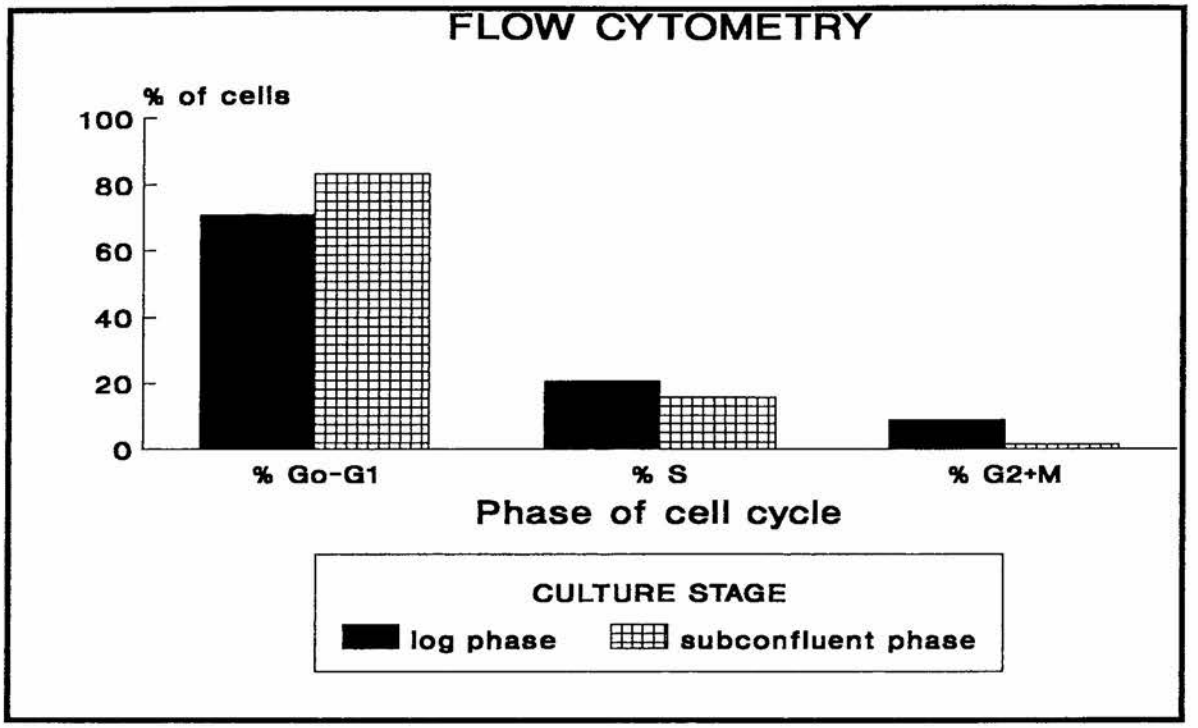


Figure 47: Phase contrast microscopy of bovine epithelial cells in culture.
(a) Clusters of epithelial cells at day 2 after plating.
(b) Culture appearance at day 6.
(c) Monolayer culture at day 10 when the culture is reaching confluency.



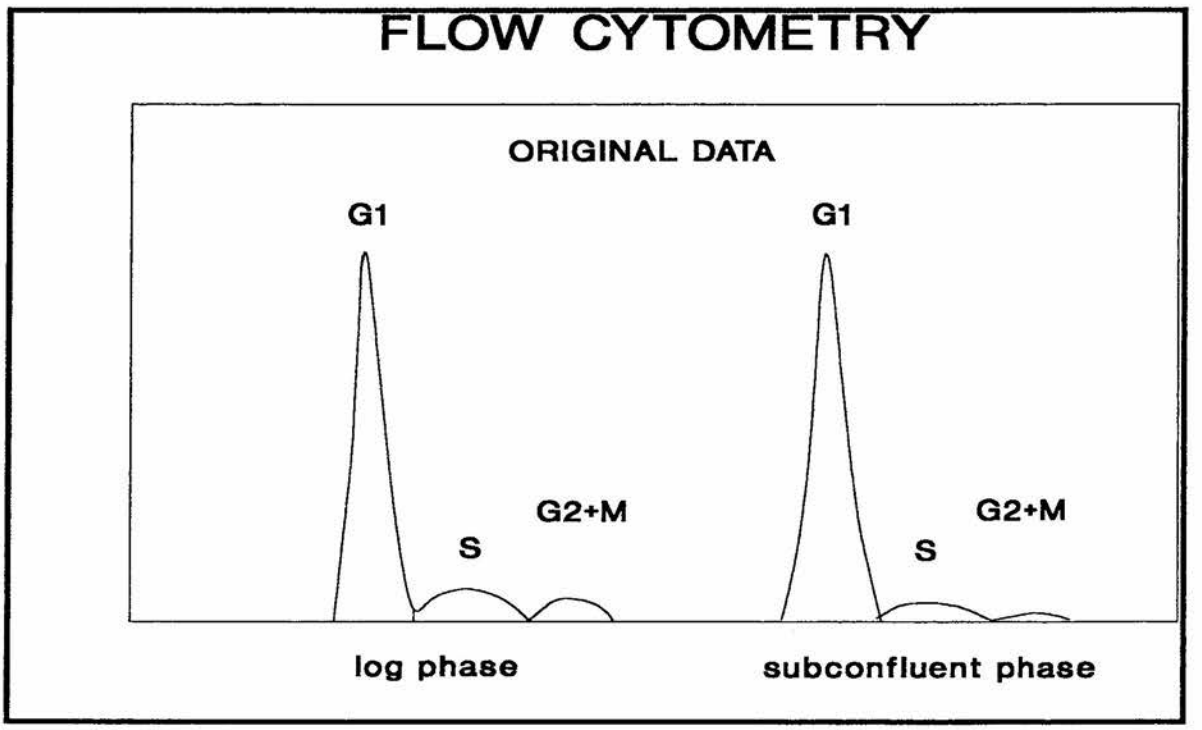
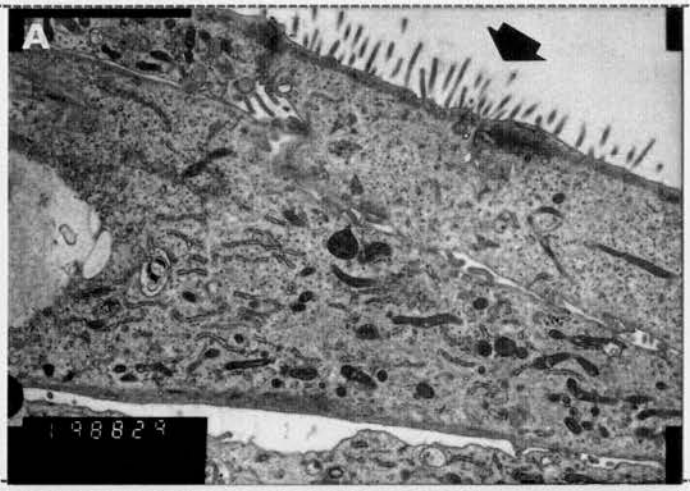


Figure 48: Flow cytometric study of gallbladder epithelial cells in culture. Cells were analysed at the logarithmic phase of development (day 6) and before reaching confluency (subconfluent stage, day 10); a) percentage of cells at each stage of cell cycle at the logarithmic and subconfluent phase of the cell culture; b) original data.

9.1.3.3 Characterisation of epithelial cells by Transmission Electron Microscopy

Transmission Electron microscopy of the cells taken from the monolayer on the subconfluent phase of the culture, showed typical epithelial cells with all the characteristics of the gallbladder epithelium. Cytoplasmic organelles and microvilli on the epithelial cells were prominent *(figure 49a)*. Using higher magnification, dilated endoplasmic reticulum and mucin-filled vacuoles were seen *(figure 49b)*. Focal tight junctions and interdigitating processes between adjacent cells, characteristic of epithelial cells were also present *(figure 49c)*.

A



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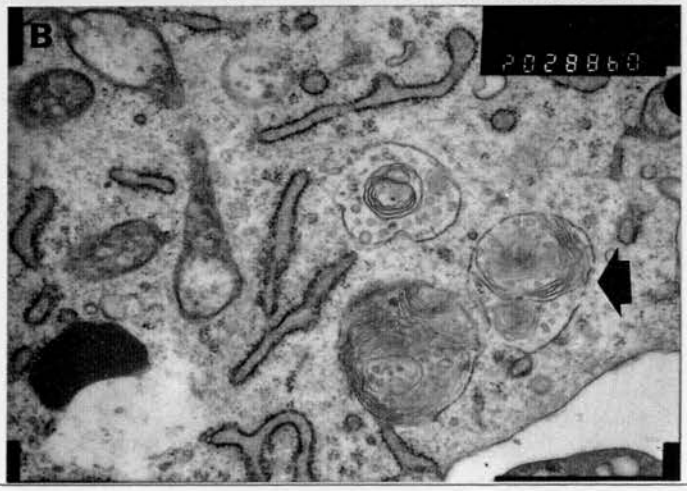


Figure 49a). Electron micrograph of cells taken from monolayer culture . Cytoplasmic organelles and microvilli (arrow) on the epithelial cells are prominent. (Mg x 6,000).

b). Micrograph showing dilated rough endoplasmic reticulum (left arrow) and mucin-filled vacuoles (right arrow). (Mg x 20,000).

C

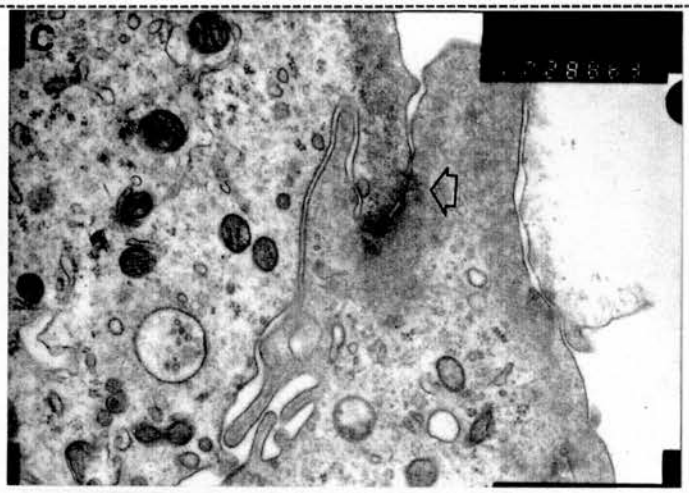


Figure 49c. Interdigitating processes between adjacent epithelial cells and focal tight junctions (arrow). (Mg x 20,000).

9.1.4. CONCLUSIONS

In this study we have developed a simple, reliable and reproducible method of isolation and culturing gallbladder epithelial cells, which remained viable for at least two weeks.

This method should be useful for the study of the biological properties of gallbladder mucosal cells both in experimental animals and in humans.

9.2 VISUALISATION OF ACID SECRETION BY THE HUMAN GALLBLADDER EPITHELIAL CELL

9.2.1 AIM

The aim of this study was to visualise the site of acid production from the viable human gallbladder epithelial cell in suspension, by colour changes of a pH - sensitive intracellular dye (acridine orange) under fluorescent microscopy and to study the effect of pharmacological agents on hydrogen ion production.

9.2.2 MATERIAL AND METHODS

ACRIDINE ORANGE.

Acridine orange is a positively charged molecule which has high affinity for negative charged structures such as *D.N.A.* In addition, it is a weak base and it is distributed across natural membranes as a function of *pH* gradient. At low concentrations it produces a green fluorescence, which is maximum at an emission wavelength of 510 nm, whilst at higher concentrations it produces a red fluorescence which is maximum at 660 nm. Acid production would therefore increase the accumulation of dye in the site of production resulting to a colour shift towards the red under the fluorescent microscope. This dye was used to provide chromatic evidence for the site of acid secretion by the human gallbladder epithelial cell.

EXPERIMENTAL PROTOCOL.

Fresh human gallbladder from open elective cholecystectomies was used. Cell suspensions were prepared immediately after the transfer of the gallbladder to the laboratory according to the method described in 9.1.2.

Suspensions were prepared to a concentration of $1x10^6$ cells/ml in *Dulbecco's modified Eagles medium* with 10% fetal calf serum.100uM of Acridine orange, which is a fluorescent dye, was added to the suspension and incubated at 37°.

The viability of the cell suspension was checked by the trypan blue exclusion method as previously described. The suspension was subsequently divided to three samples (groups) as follows:

Group 1 contained only human gallbladder epithelial cells in suspension.

Group 2 contained human gallbladder epithelial cells incubated with amiloride 2 mM.

Group 3 contained human gallbladder epithelial cells incubated with buffered histamine hydrochloride 10mM.

Group 4 contained non-viable human gallbladder epithelial cells (killed with formaldehyde).

Samples from each suspension group were visualised and photographed at 3 min, 15 min and 30 min following the addition of acridine orange under the fluorescent microscope.

9.2.3. RESULTS

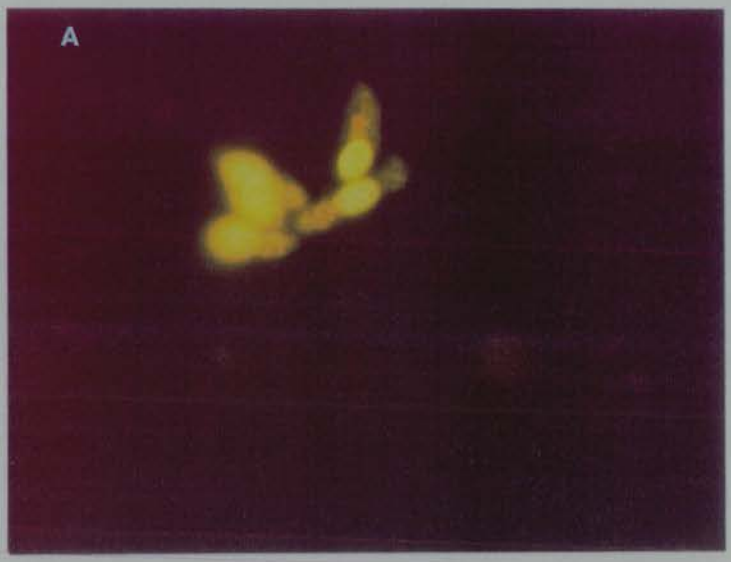
Human gallbladder epithelial cells had a columnar appearance with oval-shaped nuclei near the basal side of the cell. The nucleus of the viable cell (group 1) had an apple-green colour with yellow-green cytoplasm *(Figure 50a).* In contrast, in the non-viable cell (group 2) the nucleus was red , as a result of acridine orange accumulation to the uncoiled DNA. Similarly the cytoplasm of the non-viable cell had a diffuse red appearance presumably due to increased permeability of the cell membrane and diffusion of the dye within the cell *(Figure 50b).*

The viable gallbladder epithelial cells underwent signficant morphological changes compaired to baseline, at 15 and 30 min after addition of acridine orange. Initially yellow-red granules appeared towards the apical side of the cytoplasm which became confluent by 30 min *(Figure 51a,b,c).* Those areas corresponded to increased concentration of acridine orange presumably due to acid production (Berglindh T et al, 1980).

Group 2 (amiloride-incubated), however, did not show similar morphological changes and the cells retained the apple-greeen colour throughout the 30 min period *(Figure 52 a,b).*

. In contrast, group 3, which was treated with histamine, demonstrated much more florid morphological changes at 15 and 30 min of experiment compared with control group 1, and these were consistent with increased rate of hydrogen ion production *(Figure 53a,b).*

A



B

Figure 50 : a.) Viable , b.) Non-viable human gallbladder epithelial cell under the fluorescent microscope.

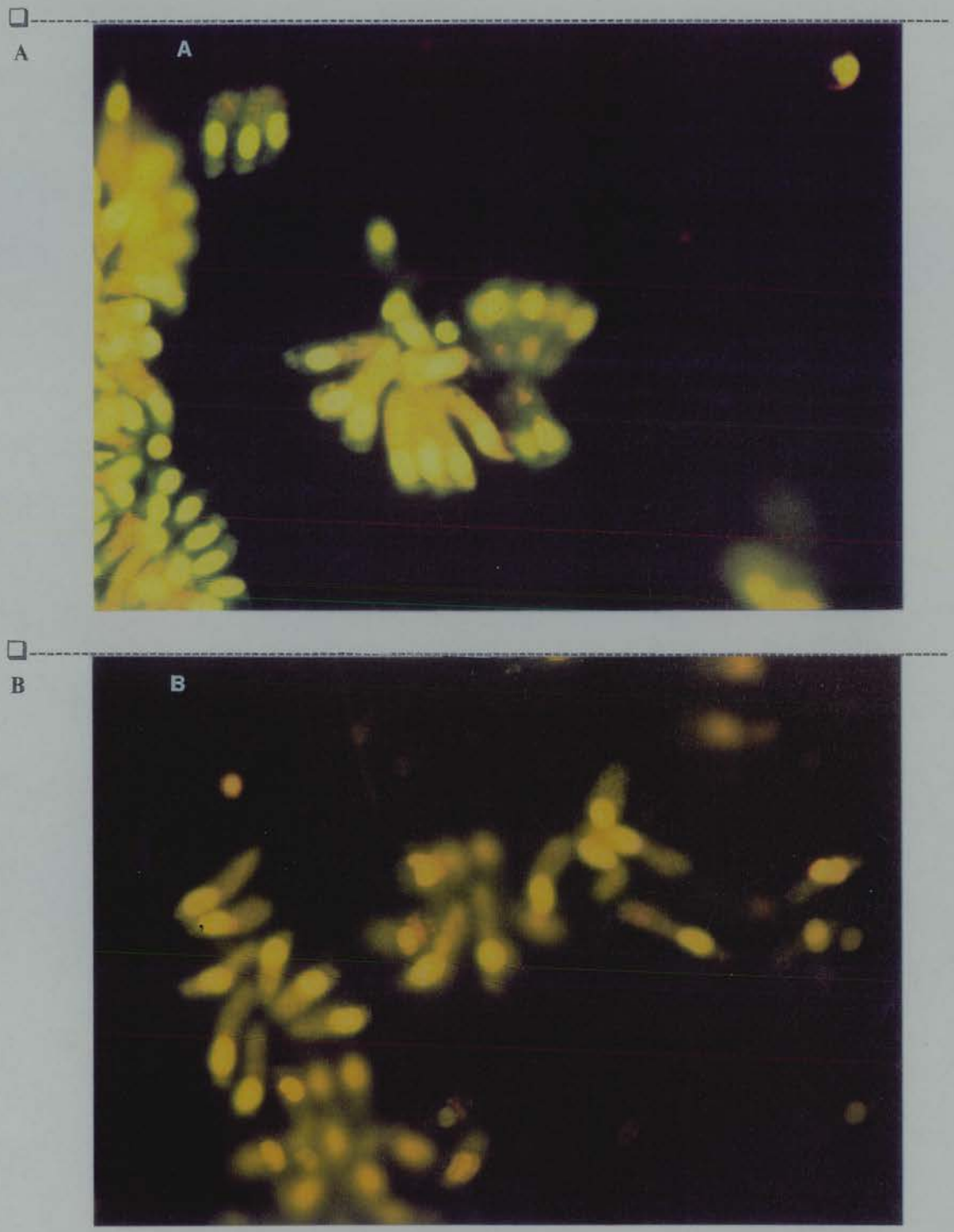


Figure 51: Human gallbladder epithelial cells in suspension with acridine orange at a) beginning b) 15 min. The changes into red colour of the cytoplasm indicate accumulation of acridine orange secondary to accumulation of acid.

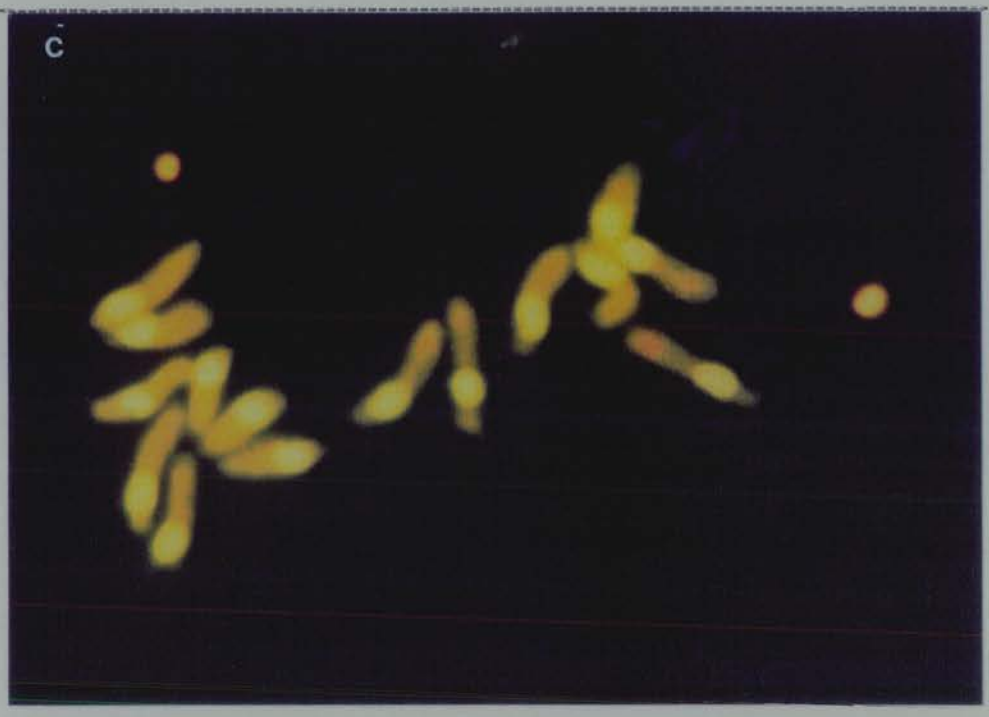


Figure 51c: Human gallbladder epithelial cells in suspension with acridine orange at 30 min of experiment (c). The changes into red colour of the cytoplasm indicate accumulation of acridine orange secondary to accumulation of acid.

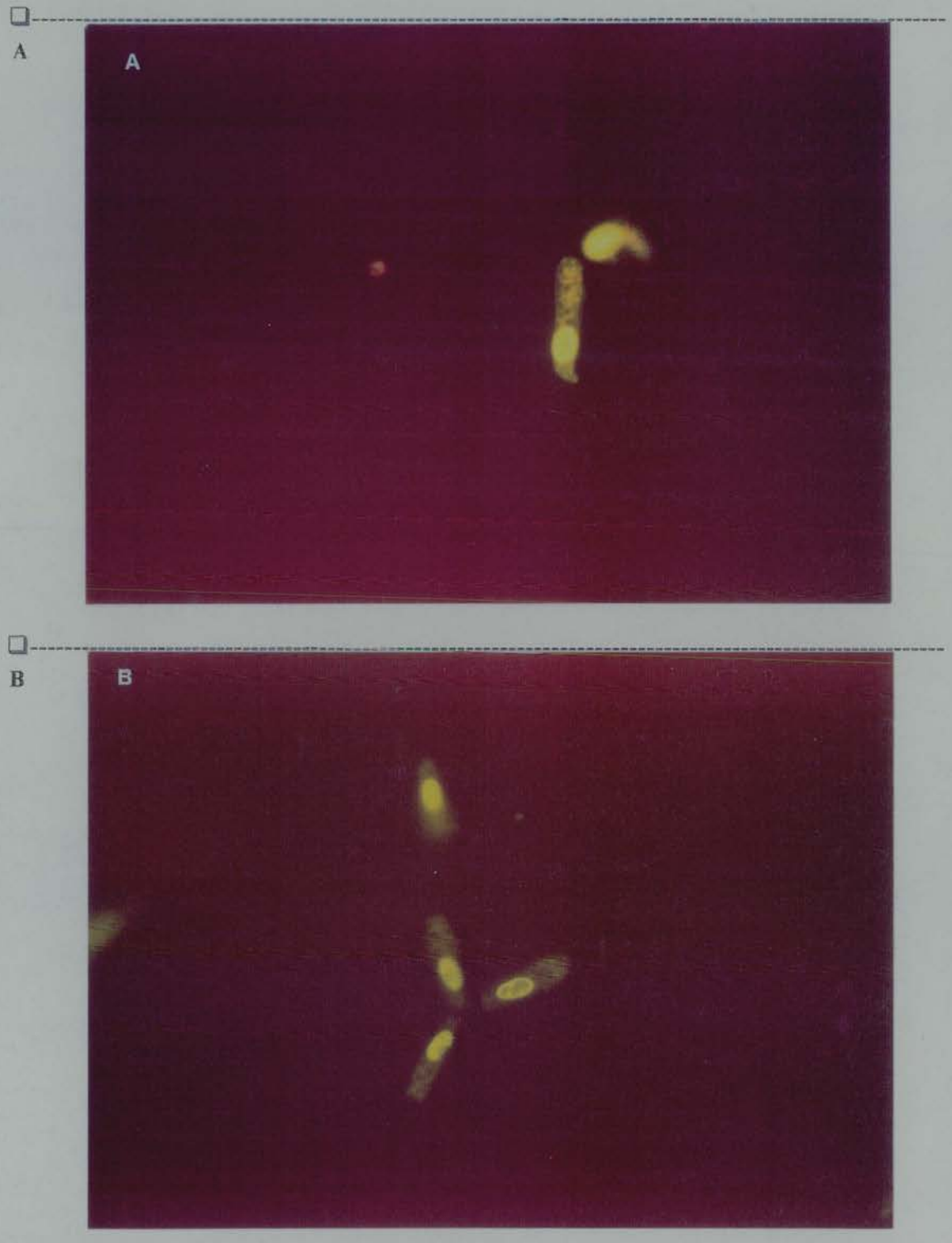


Figure 52: Human gallbladder epithelial cells in suspension after addition of amiloride a) at 15 min b) at 30 min; the cytoplasm retained the apple-green colour throughout the 30 min period .

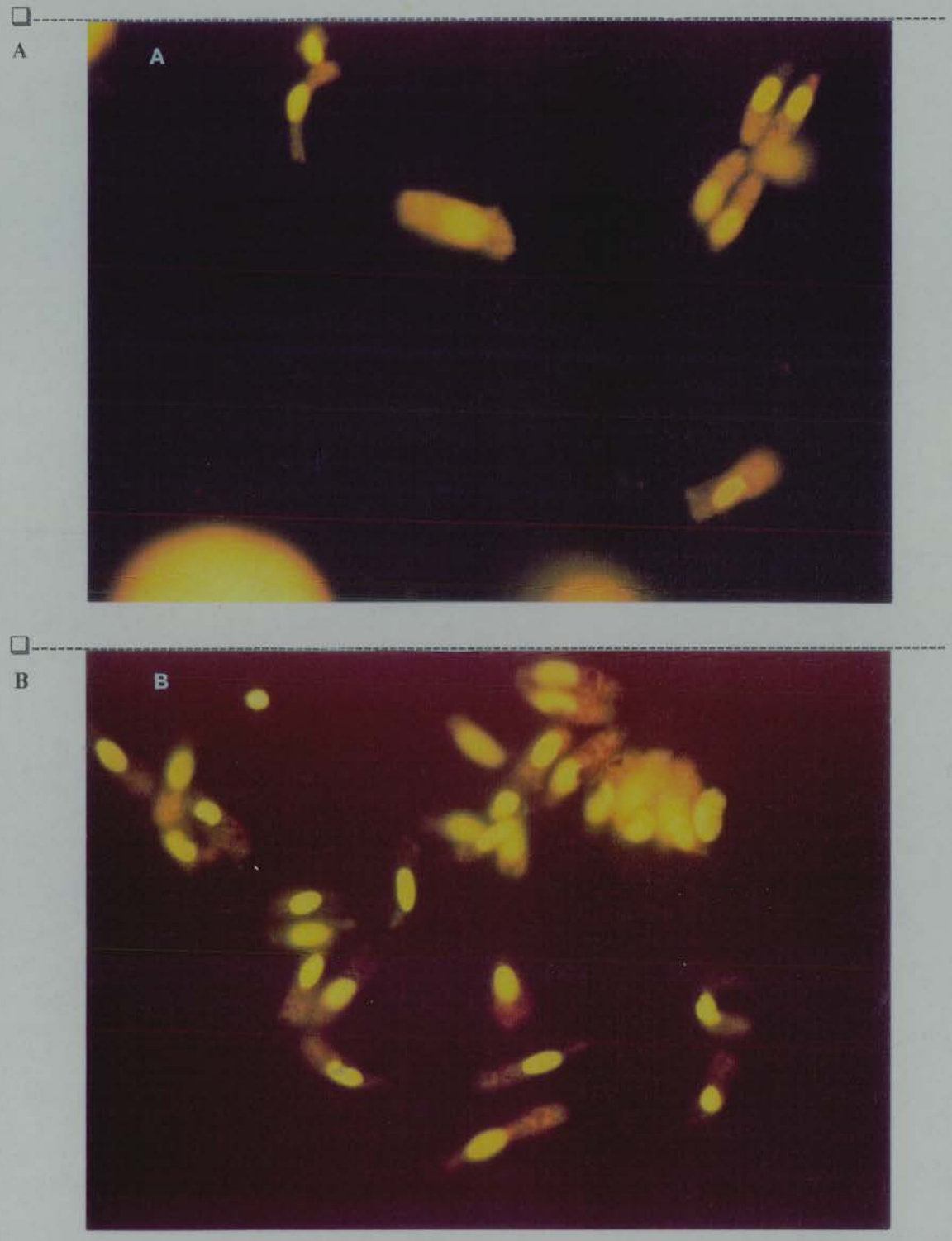


Figure 53: Human gallbladder epithelial cells treated with histamine demonstrated much more florid morphological changes at 15 (a) and 30 min (b) of experiment compa red with control group.

9.2.4 CONCLUSIONS

In this study we visualised under *fluorescent microscopy the process of acidification by the isolated human gallbladder epithelial cell.*

This process was *inhibited by amiloride and stimulated by histamine.*

The exact mechanism by which the gallbladder epithelial cell produces hydrogen ions is unclear.

From the present study, it can be inferred that *histamine through H-receptors stimulates the production of antiport proteins (Na+/H+), which in turn increase the amount of hydrogen ions produced by the epithelial cell.* The changes in the morphology of the cells in the present study are consistent with increased production of transport which initialy appear within the cytoplasm and gradually move towards the apical side of the cell where they are finally sited.

Further studies are necessary, using molecular biology techniques, to dissect the intracellular events which lead to apical hydrogen ion production by the gallbladder epithelial cell.

CHAPTER 10
DISCUSSION

CHAPTER 10
DISCUSSION

Gallstone disease is a common cause of morbidity and cholecystectomy represents the most common elective abdominal operation in Western Society. In the United Kingdom alone about 20% of the population may expect to develop cholelithiasis (Barker DJP et al 1979). Initially most research had been focused on the biochemical changes which occur in bile during gallstone formation. Recent years have seen significant advances in our knoweledge and it is now recognised that the gallbladder is not a passive reservoir but has several absorptive, secretory and motor functions which are intergrated to produce concentrated bile. The interactions between gallbladder bile and mucosa are of paramount importance to maintain the fine balance between concentration and precipitation of its constituents.

The gallbladder mucosa has one of the highest rates of water absorption in the body and an 80 -90% decrease in the initial volume of bile occurs within the gallbladder. This is achieved by the coupling of active sodium transport and passive water absorption resulting in isotonic fluid absorption (Diamond JM et al 1968). Fluid transport is subject to a variety of influences being higher in the daytime (Diamond JM, 1964) and be reversed to net secretion with feeding; it is abnormal in chronic inflammation or with the use of pharmacological agents (prostaglandins, prostacyclin and various gastrointestinal peptides) (Wood JR et al 1983). Electrolyte transport has been extensively investigated; chloride is actively absorbed in exchange for bicarbonate (Heintze K et al 1979), and potassium moves from the mucosa to the serosa according to electrochemical gradients (Rose RC, 1981).

Calcium is also absorbed and its distribution across the gallbladder epithelium is of importance in the formation of gallstones (Rege RV et al 1987). Studies have now shown that gallbladder bile from patients with either cholesterol or pigment stones is frequently supersaturated with calcium and thus liable to calcium precipitation (Shiffman ML et al 1992). Most gallstones contain a central core of calcium salts around which layers of either cholesterol or calcium bilirubinate are deposited as the stone enlarges (Bean JM et al 1979). This suggests that calcium precipitation may be critical initiating factor for gallstone formation and development. In addition , biliary calcium ions also reduce the solubility of biliary cholesterol , making cholesterol crystal formation more likely (Neithercut WD, 1989) and stimulate mucus glycoprotein (Malet PF et al 1986).

It has been postulated that one of the defence mechanisms of the gallbladder against calcium precipitation and gallstone formation, is the ability of the gallbladder to secrete hydrogen ions because acidifying gallbladder bile increases the solubility of calcium salts (Rege RV et al 1987). However direct evidence that the human gallbladder epithelium is capable of secreting hydrogen ions was lacking until the present work was undertaken.

In this thesis the ability of the galbladder epithelium to produce hydrogen ions and to acidify bile, the regulation of this process and its contribution in the formation of gallstones by affecting calcium salts solubility, were studied.

HYDROGEN ION SECRETION

Hydrogen ion secretion by fresh human and bovine gallbladder were studied by the Ussing chamber method (former) and a whole organ perfusion system (latter).

The Ussing chamber method has been adapted and used by many investigators since its introduction by Ussing H.H. and Zerhan K. in 1951. It is a valid method of keeping a biological preparation viable during the period of investigation and has been used to study electrophysiological, secretory and absorptive properties of tissues *in vitro*.Tissues previously used include frog skin, intestine, bladder and both animal and human gallbladder. For years it has been known that gallbladder bile is more acidic compared to hepatic and the difference in the pH has been attributed to bicarbonate absorption (Diamond JM and Cook CF 1968, Rose RC 1981).

In our study using human tissue mounted on the Ussing chamber *(chapter 5)*, it was demonstrated that fresh viable human gallbladder mucosa is capable of acidifying physiological solutions *in vitro*.This acidification is a function of viable tissue ; it was reduced in more inflamed gallbladders or when the epithelial cells became "sick" during the study and was lost when the mucosa was non-viable. Acidification was abolished when the gallbladder epithelium was exposed to sodium free solution or in the presence of high concentration of amiloride in the mucosal compartment. In our study where human gallbladders were used to investigate acid secretion *in vitro*, the gallbladder epithelium appeared capable of increasing the hydrogen concentration in mucosal side with simultaneous decrease of hydrogen concentration in the serosal side which suggests that hydrogen ions were transferred from the serosal to mucosal side of the tissue. The concomitant decrease of bicarbonate concentration with increased pCO_2 in the mucosal side indicates that this acidification is not simply due to bicarbonate re absorption. It appears that hydrogen ions produced by the mucosal cells react with bicarbonate to form

CO_2 and water with a decrease in bicarbonate ions. The observed differences in pCO_2 between the viable and non-viable gallbladders could be explained on the basis that a non-viable gallbladder mucosa loses its ability to secrete acid therefore less hydrogen ions are available to react with bicarbonate and also that the production of CO_2 from the mucosal cell metabolism is depressed. Studies on gallbladders of other animal species such as rabbit (Whitlock RT 1969), guinea pig (Heintz K 1981), necturus (Altenberg G 1990), and dog (Rege RV 1987) have shown that these tissues are capable of acidifying the mucosal solutions *in vitro* and there is evidence that a sodium /hydrogen antiport is present at the apical site of the epithelial cell. In the present experiments the use of sodium-free solutions and amiloride ,which is a specific sodium/hydrogen inhibitor, abolished acidification which suggests that hydrogen secretion in the human gallbladder depends upon a sodium /hydrogen antiport.

A potential problem, with Ussing chamber studies is related to the viability of the tissue received at cholecystectomy. The ligation of cystic artery, which is part of the operative procedure, usually takes place between 5 to 20 min before the removal of the gallbladder. The effect of hypoxia during this period has been studied (Hopwood D. et al 1980) and about 70% of the gallbladders examined immediately after the operation showed evidence of mitochondrial change which was attributed to anoxia and mechanical damage. However, the human as well as animal gallbladder is a durable organ which can rapidly recover from hypoxia and can be preserved viable for *in vitro* experiments for up to 4 hours (Diamond J.M. 1965). The gallbladder has been used for years as a model to study ion transport across the epithelia . In the present study all the gallbladders demonstrated a transmural potential difference of more than 2.3 mV (range 2.3-11.9mV) serosal positive. This potential difference remained stable throughout the period of experiment and we, as well as other workers (Ross RC 1973, Nahrwold DL et al 1976., Jacyna MR 1986), could demonstrate that anoxia resulted in a drop at the transmural potential difference reflecting a reducing viability of the tissue which was reversible if the anoxic period was less than 10 min. Similar results were produced after immersion in formaldehyde with permanent drop of the potential difference indicating loss of viability.The continuous transpotential difference monitoring therefore has been used as a reliable method of monitoring the viability of the tissue and allow further study of the mucosal function.The transmural potential difference of the gallbladders under study was in accordance with the literature. Ross RC et al 1973 performed studies on the electrical properties of the human gallbladder and on 46 gallbladders the mean PD was 7.6 while other investigators (Nahrwold DL 1976) later showed that inflamed gallbladders have lower resting transpotential difference which is dependant upon the

degree of inflammation, the variation in time between the ligation of cystic artery and the transfer to the laboratory and the methodology used for studying the electrical properties of the tissue; for instance tissues which are clamped at the edges as in the Ussing Chamber technique suffer from edge damage which reduces the transpotential difference (Jacyna MR 1986).

Another potential problem with physiological studies using tissues from routine cholecystectomy is that most of those are not histologically normal. Although it is not appropriate to extrapolate to normal gallbladders in respect of hydrogen ion secretion, the fact that we could demonstrate changes in the acidification capacity with histology, the more inflamed gallbladders being less capable to secrete acid, implies that hydrogen ion secretion is a function of the normal human gallbladder mucosa.The acidification capacity was impaired when the cell morphology was abnormal and was abolished in the non viable gallbladder; these suggest that hydrogen ion secretion is a function of a viable tissue. There is recent supportive evidence from human studies that the gallbladder secretes acid;. Rege RV et al. have shown that the diseased human gallbladder is associated with decreased acid output (1988), but these observations were not based on studies in human gallbladder mucosa directly, but were inferred from biochemical analysis of gallbladder bile from laparotomy.

Our data would support the hypothesis by Moore EW and colleagues 1988, that reduced gallbladder hydrogen ion secretion is associated with gallstone formation. It is postulated that the observed pH changes in these experiments with diseased gallbladders represent only a fraction of the capacity which the normal human gallbladder epithelium might possess to secrete acid. In our study, it was also shown that acidification was sodium dependant, therefore it was closely linked with the concentrating ability of the gallbladder because it is known that water is passively absorbed consequent upon the absorption of sodium from the epithelial cell.

It however proved difficult to study hydrogen ion production and the factors which may influence acid secretion on normal human gallbladders, because of the lack of adequate numbers of normal human tissue. In addition, the Ussing chamber has the following limitations: ***a)*** only a very small piece of tissue was used, ***b)*** the fluid volume used was small and ***c)*** as a result of the above, the amount of hydrogen ions secreted per hour was small. These limitations made this technique unsuitable for pharmacological manipulations which were necessary to study the regulation of hydrogen ion secretion by the gallbladder epithelium. In order to overcome this, we used a whole gallbladder perfusion system. Whitlock RT and Wheeler HO in 1969 demonstrated that the rabbit gallbladder was able of acidifying the mucosal bathing fluid with simultaneous

alkalisation of the serosal fluid by using a whole gallbladder perfusion system . Our system was a modification of the method of Whitlock RT and Wheeler HO 1969, to enable the study of the electrophysiology and hydrogen ion production of the whole gallbladder. The advantages of such a system are: ***a)*** there was very little manipulation of the tissue and the structure of the organ was preserved for other studies, for example for histopathology studies and ***b)*** the magnitude of changes on hydrogen ion concentration was high and this facilitated pharmacological studies on the regulation of acid secretion. Our modified whole gallbladder perfusion system, has successfully permitted the measurement of electrical potential difference , tissue resistance and changes in the electrolyte concentrations of the mucosal and serosal fluids. Using this method, large amounts of fluid up to the physiological capacity of the gallbladder can be used. In addition there is uniform flow of gases during experiment which helps to limit the dangers of anoxia to the organ. In all gallbladders studied, the electrical behaviour observed was consistent with the experiments carried out by Whitlock RT and Wheeler HO on the rabbit gallbladder.

On histopathology in both human and bovine gallbladder a rich capillary blood supply is seen to lie immediately below the epithelial basement membrane and *in vivo* normal vascular perfusion approximates to direct perfusion of the gallbladder epithelial layer. When, however, the gallbladder is excised and studied *in vitro,* the vascular supply is interrupted and it assumed that the supporting structures of the gallbladder wall are responsible for the movement of water and solute observed during the experiments. Although it is postulated that the serosal side plays the functional role of the interrupted vascular supply the exact mechanism of solute movement remains obscure.

In this thesis an intergrated study of the bovine model was undertaken; in *chapter 6,* the bovine gallbladder was used to study hydrogen ion production by the normal gallbladder epithelium and its regulation; in *chapter 7* it was shown that there are significant morphological similarities between bovine and human gallbladder epithelium; in *chapter 8,* it was shown that bovine bile is a good model to study bile physiology particularly in relation to pigment stone formation, because the concentrations of most of its components are strikingly similar to human , with the exception of the lower cholesterol concentration. It is of interest that the bovine species does develop gallstones (4% in the present study) and this supports the concept that an imbalance of various components of bile other than cholesterol can also lead to gallstone formation, mainly of the pigment variety.

In our studies the bovine gallbladder demonstrated an initial mean tissue resistance of approximately 120 Ω-cm^2 which was subsequently reduced to approximately 80 Ω-cm^2.

Bovine gallbladder tissue resistance was higher than that of human tissue which has been reported to be approximately 50 Ω-cm^2 (Rose R.C. 1975). This higher resistance, however, may be attributed to the fact that in our experimental setting the gallbladder wall was left intact, while in most other studies only the mucosa was used for the experiments (the serosal was separated from the mucosa by blunt dissection). The potential difference across the bovine gallbladder epithelium was approximately 5mV serosal positive which is within the range reported for most other animal species (Rose R.C. 1975). Both the potential difference and tissue resistance were reduced by hypoxia. The results of the studies on the bovine gallbladder confirm that the normal gallbladder epithelium is capable of secreting hydrogen ions *in vitro*. Hydrogen ion secretion is a function of viable tissue, because hypoxia reduced hydrogen ion secretion in proportion with the changes in the electrophysiological parameters, which were used as markers of viability.

Mucosal acidification was associated with simultaneous serosal alkalisation, but the rate of mucosal alkalisation was approximately 30% higher than that of serosal alkalisation, indicating that the observed mucosal acidification was not only due to hydrogen ion transfer from serosa to lumen, but also due to generation and secretion of hydrogen ions by the gallbladder epithelium.

It is of interest that, although no differences in the rate of acidification were observed between male and female animals, the starting pH of the luminal solutions was significantly more acidic in male than in female gallbladders. Similarly, gallbladder bile from males was significantly more acidic compared with that from females. Gender is one of the most powerful influences on gallstones which are twice as common in females during their fertile years (Bouchier IAD 1991). The gallbladder contains receptors for oestrogens and progesterone (Singletary BK 1986) and it is therefore not surprising that female hormones may have a significant effect on both composition of bile and gallbladder epithelial function. Women on oral contraceptives have cholesterol supersaturated bile (Bennion LJ et al 1980, Kern F Jr et al 1987). Furthermore in experimental animals cholesterol-supersaturated bile depresses gallbladder motility and promotes mucin hypersecretion with the resultant formation of a mucin gel adherent to the gallbladder mucosa (Donovan JM 1993). In our observations male gallbladder bile was significantly more acidic than female bile, but both male and female gallbladders demonstrated similar acidification rates, and this suggests that the amount of mucin gel adherent to the gallbladder mucosa may be higher in the female than the male gallbladder thereby reducing the diffusion of hydrogen ions through the mucus into the bile. This may result, on one hand, in significantly acidic environment between the apical

side of the epithelium and the mucin gel and, on the other, to the bile in the gallbladder lumen being alkaline.

Mucus itself has long been recognised as a factor of importance in gallstone development. In animals fed lithogenic diet mucus hypersecretion precedes cholesterol crystal and stone formation (Lee SP et al 1981). It is possible among the many effects of increased mucus production on bile physical-chemistry, there might be reduced hydrogen ion diffusion into bile. This hypothesis requires testing.

Our data suggested that acidification reaches a steady state around pH 6; when mucosal pH was set below pH 6, acidification was reversed to alkalisation until pH reached 6. In most tissues the phospholipid bilayer forms the structural matrix and principal permeability barrier which prevents dissipative back fluxes of hydrogen ions into the cell, whilst the tight junctions prevent the paracellular back-diffusion. In addition mucus would provide another barrier. The ability of hydrogen ions for back diffusion depends upon a number of factors, such as the type of tissue and the degree of leak through the tight junctions , the presence of carrier-H^+ transporters and the compounds that might bind H^+; for example HCl is a small non-polar molecule which is formed in secretions rich in both chloride and H^+ (Gutknecht J, 1990). The results of our studies indicate that the gallbladder epithelium functions in such a way as to prevent bile pH to dropping below 6. It appears becoming leaky to hydrogen ions (back-diffusion) when their concentration exceeds 1000 nmol/l. Such a process may represent a protective mechanism to ensure that bile pH remains within a particular range to permit the optimum function of the biliary and pancreatic enzymes when bile enters the duodenum. In addition it has been shown that excessively acidic pH can induce polymerisation of calcium bilirubinate to form an insoluble polymer which can initiate the process of pigment stone formation (Donovan JM 1993).

MECHANISM OF HYDROGEN ION SECRETION

In this study it was shown that hydrogen ion secretion in the bovine gallbladder is a sodium-dependant process because it was abolished when sodium-free solutions or amiloride , which is a sodium / hydrogen antiport inhibitor, were used. The most likely system involved is an apical sodium / hydrogen antiport system in both the human and the bovine gallbladder. These results are in agreement with other studies on necturus (Altenberg G 1990), rabbit (Whitlock RT 1969) and guinea pig gallbladder (Heintz K 1981). The sodium /hydrogen exchange system appears to be an antiport rather than a pump, because hydrogen ion secretion was abolished by serosal but not mucosal ouabain

(sodium / potassium / ATP-ase inhibitor) indicating that hydrogen ion secretion is dependant upon the energy produced by sodium / potassium / ATP-ase pump. This pump is present at the basal side of the epithelial cell and extrudes 3 Na^+ ions in exchange for 2 K^+ ions, thus maintaining a low intracellular Na^+ high intracellular K^+ , and negative intracellular potential. The sodium / hydrogen antiport system exists in the membrane of most cells in the body and in the majority of circumstances functions to regulate the intracellular pH. It is of particular interest that in the case of the gallbladder epithelial cell the location of the system in the apical side (luminal) side may serve a dual function ; to regulate the intracellular pH as well as to acidify bile. Recent studies on the human sodium antiport system have revealed that the carrier protein involved is a phosphoglycoprotein of 110 *kD* present as a dimer with two functional domains; a transmembrane portion of 500 residues that has all the features to catalyse amiloride-sensitive Na^+/H^+ exchange with a built-in " H^+" sensor and a cytoplasmic regulatory domain that determines the set point value of the exchanger. The antiporter gene codes for a protein of 815 amino acids with two distinct domains (Anwer SM 1992). It has also been postulated that the Na^+/H^+ exchanger is involved in regulation of cell proliferation and activation of this transporter by mitogens may give rise in intracellular pH which acts as a trigger for initiation of cell division (Gleeson D 1992).

Fluid absorption by the gallbladder epithelium is a sodium dependent process (Diamond JM 1968). As we have shown, hydrogen ion secretion is linked with sodium reabsorption and therefore bile concentration and acidification are simultaneous phenomena. Amiloride , which we have shown that inhibits acidification , also reduces the concentrating ability of the gallbladder (Strichartz SD et al 1989).

Having established that the gallbladder epithelium was able to acidify bile, we investigated whether the regulation of hydrogen ion secretion by the gallbladder epithelium was similar to that of the stomach, which is the principal acid-secreting organ in the body. Hydrogen ion secretion was inhibited by a selective H_2 (Famotidine) and a non-selective (Diphenhydramine) histamine receptor antagonist. In contrast to the parietal cell, acid secretion by the gallbladder epithelial cell was only stimulated by high concentration histamine; this indicates that hydrogen ion secretion by the gallbladder epithelium is a histamine-dependant process, but the effect of histamine is much weaker and less receptor-specific compared with the stomach. Histamine receptors , both H_1 and H_2, have been found in the wall of primate (Schoetz DJ, 1983) guinea pig (Impicciatorre M, 1978) and dog gallbladder (Clanachan AS, 1982). However the amount and distribution of different types of histamine receptors varies with the animal model. Histamine is believed to exert a contractile effect on gallbladder muscle mainly

via H_1 receptors (Lennon F et al 1984) and also to modify the response of the gallbladder to cholecystokinin (Waldman DB et al 1977). However the nature of histamine receptors and the full role of histamine in the human bilary tract are unclear.

The source of histamine in the gallbladder is probably the mast cells, in analogy with the stomach, which are present within the deeper layers of the gallbladder wall. The presence of mast cells was demonstrated by immunocytochemistry by Hudson I and Hopwood D in 1986, who found that the number of mast cells in minimally inflamed gallbladders was significantly higher compared to gallbladders with chronic cholecystitis. Mast cells are potentially important in the pathogenesis of gallbladder disease because their granules contain a variety of chemical mediators. Histamine may induce, via H_2 receptors, the release not only of hydrogen ions but also prostaglandins which may enhance mucus secretion.

Omeprazole in high doses had a relatively weak inhibitory effect on acidification which indicates that the hydrogen / potassium exchanger is not the principal mechanism of hydrogen production by the gallbladder epithelium.

CCK had a significant stimulatory effect on acidification. This has been previously documented in the stomach; CCK has structural similarities with gastrin and studies on isolated parietal cells have shown that CCK is a full agonist with high affinity for the gastrin receptor (Davison JS, 1989). Furthermore, there is a strong association between gastric motility and acid secretion; the explanation for such an association is complex and not fully understood but may involve other mechanisms such as neuronal, paracrine and humoral (Greenwood B, 1989). Whether similar mechanisms exist in the gallbladder is not known. Further studies are worthwhile to investigate whether the enhancement of acidification in the gallbladder is due to a specific CCK receptor effect or an indirect effect due to promotion of gallbladder contractility and clearing of mucus.

Carbonic anhydrase is the principal enzyme involved in the acid -base homeostasis within the cell by regulating the splitting of carbonic acid to produce free hydrogen ions necessary to balance the intracellular pH. In our studies acetazolamide , which is a carbonic anhydrase inhibitor, did not affect acidification in the pharmacological doses used; in addition no qualitative differences were noted in immunocytochemistry between normal and inflamed gallbladders in terms of the distribution of this enzyme within the gallbladder epithelium. This indicates that hydrogen ion secretion by the gallbladder is not dependant upon the function of this enzyme.

A potential limitation in common with all the *in vitro* studies is the degree of viability of the tissue used. In the present studies the vast majority of bovine gallbladders were transferred to the laboratory within 30 min and adequate viability was obtained by

measuring the electrophysiological parameters in the beginning and the end of each experiment. In addition, the pharmacological experiments were done on each gallbladder separately with a control period before and after each pharmacological manipulation.

ACID-BASE REGULATION IN BOVINE BILE

In the bovine bile there was a high proportion of taurine conjugated bile salts, which is unusual for a vegetarian animal (Haslewood GAD 1967). The main pigment is conjugated bilirubin which is rapidly oxidised to biliverdin. The latter is responsible for the greenish colour of the bovine bile (Sellers A.F. 1977).

Our observations on bovine bile are in agreement with those of other studies (Svanic J et al 1984, Shiffman ML et al 1990) which have demonstrated that gallbladder bile is a solution with a wide range of solute concentrations. The differences between individual samples may be accounted for by the variation in the length of time that the bile had been exposed to the concentrating capacity of the gallbladder. A recent study has shown that there is a continuous movement of bile in and out of the gallbladder and the extent rather than the rate of emptying is important in determining the degree of stasis of bile within the gallbladder (Howard PJ et al 1991). In this study we have demonstrated that the Henderson-Hasselbalch equation is applicable to bovine bile and that bicarbonate appears to be the main buffering system in bile because the calculated bicarbonate concentrations from the Henderson-Hasselbalch equation matches those measured enzymatically. Similar observations have been made in canine (Rege RV et al 1987) and human gallbladder bile (Marteau C et al 1990). pCO_2 was also linearly increased as bile became more acidic presumably due to increased CO_2 production as a result of the interaction between bicarbonate and hydrogen ion production from the gallbladder epithelium.

In *chapter 6* it was shown that the bovine gallbladder epithelium is capable of secreting hydrogen ions through a sodium /hydrogen exchange at the apical side of the epithelial cell and that this mechanism is amiloride-sensitive. In *chapter 8*, we found that the reduction in bile pH linearly correlated with sodium concentration in samples with sodium concentration above 200 mM. A similar correlation has reported by Marteau et al. 1990 in human but not in canine bile (Rege RV et al 1988). An explanation proposed for this pattern lies with the buffering capacity of bicarbonate; as the concentration of bicarbonate is gradually reduced after being either neutralised by the continuous production of hydrogen ions or by absorption by the gallbladder mucosa,

further secretion of hydrogen ions is unopposed. The pH therefore decreases more steeply in those samples with the highest sodium concentration.

Strong Gibbs-Donnan effects are exerted within bile mainly by total bile salts (Moore EW 1988) and these play an important role in the distribution of other ions such as potassium, chloride and calcium, which are passively transported across the epithelium. The concentration of these ions was found increased in the concentrated bile, apart from chloride which was reduced as bile became more concentrated presumably due to its active absorption and distribution according to electrochemical forces (Rose RC 1981). However the acid-base and electrolyte changes described above did not affect the osmolality of the concentrated bile which remained remarkably stable through a wide range of sodium and pH values. Similar observations have been made on human gallbladder bile samples (Shiffman ML 1990).

The mechanisms associated with the formation and growth of gallstones are complex and multifactorial. The role of the gallbladder epithelium in the formation of stones is recognised to be of importance as alterations in biliary composition, which may enhance its lithogenicity, can be partly induced by abnormalities in gallbladder epithelial function (Jacyna MR 1990). It is accepted that the sequence of events in the process of gallstone formation is supersaturation of bile, nucleation, precipitation and subsequent growth from microcrystals to microspheroliths to gallstones (Smith BF et al 1987). Supersaturation of bile with cholesterol is present in most patients with gallstone disease (Admirand WH et al 1968); but 40- 80% of individuals may have supersaturated bile in the absence of gallstones (Holzbach RT et al 1973, Hoffman AF et al 1982). This indicates the importance of nucleation which will be facilitated by either the presence of certain nucleating agents or the absence of the naturally occurring inhibitors of crystal formation or both. Calcium bilirubinate or mucous glucoproteins could serve as nucleating factors (Burnstein MJ et al 1983) while a bile protein which is a cholesterol crystal formation inhibitor has been proposed by Holzbach RT et al 1984, as a gallstone formation protective protein. Recently De Bruijn MAC et al 1992 and Abei M et al 1993, presented evidence of *42 KD* cholesterol crystalisation promoter protein (former) and of *130 KD* heterodimer inhibitor of cholesterol nucleation (latter) in human bile.

Over the last few years the importance of bile pH and electrolytes has become appreciated, in particular the role of calcium is considered of major relevance in the formation of both cholesterol and pigment stones (Rege RV et al 1985, Moore EW 1984 and 1990). Pigmented gallstones are predominantly composed of calcium salts of carbonate, bilirubinate, phosphate and long chain fatty acids and to a lesser extent carbonate (Sutor DJ et al 1977, Bean JM et al 1979, Moore EW et al 1982, Moore EW

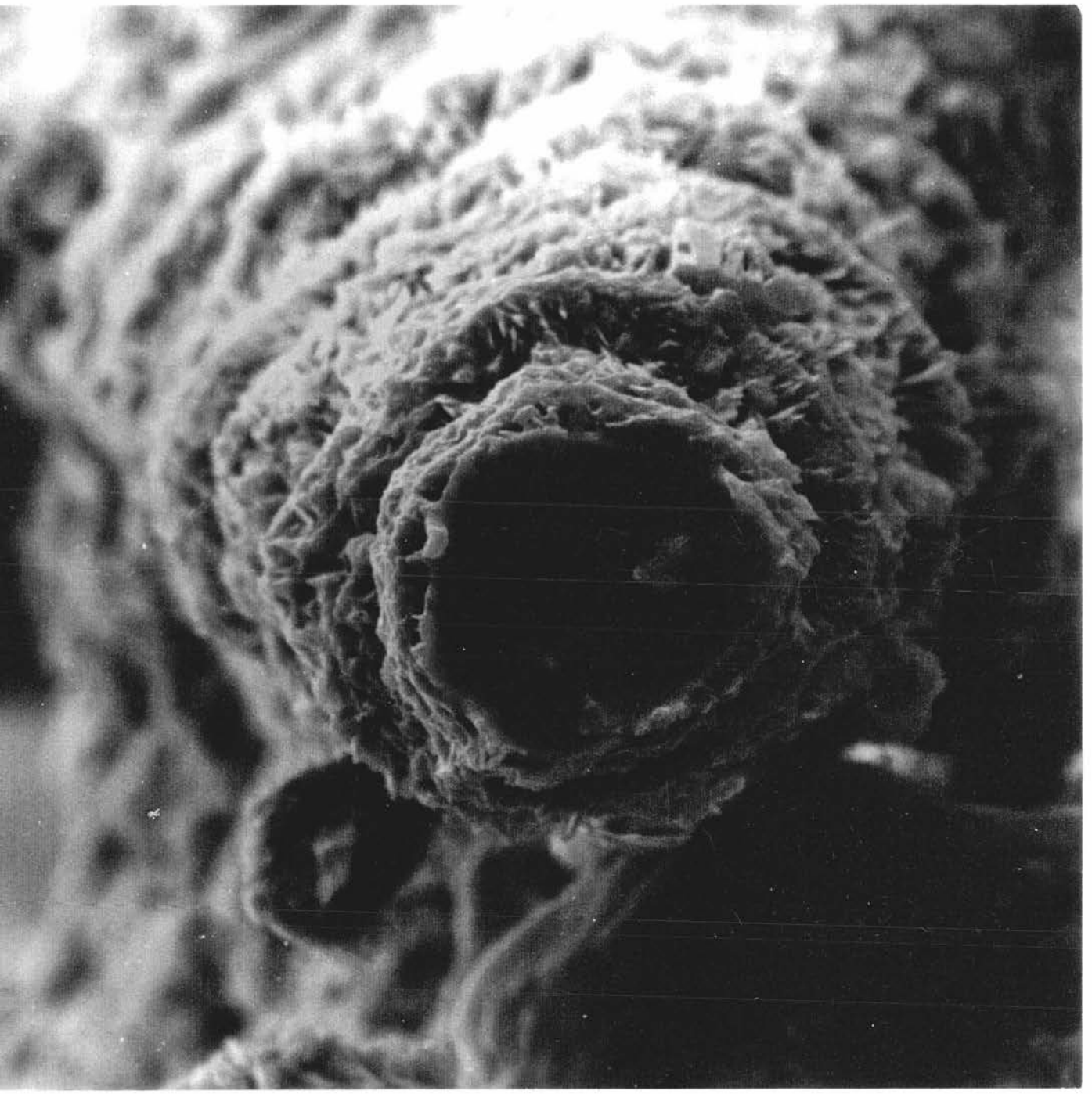


Figure 54: Periphery of a gallstone after removal of cholesterol showing a column of $CaCO_3$ under the scanning electron microscope (x 1,700). (Courtesy of Dr Lewis, Department of Educational Studies , University of Surrey, Guildford Surray)

1990, Heuman DM et al 1990). Cholesterol stones also contain calcium in their central nidus and often show peripheral calcification *(Figure 54)*. In particular, calcium carbonate precipitates on to the surface of the cholesterol gallstones and is present in most cholesterol gallstones (Wood JR 1983). Bile pH , the concentrating properties of the gallbladder, bicarbonate concentration, and the concentration of other ions present in bile such as sodium , chloride and phosphate can all influence calcium concentration particularly the free ionised component (Ca^{++}) (Heuman DM et al 1990). The regulation of calcium concentration in the gallbladder bile is therefore of critical importance. It is postulated that acid secretion may be biologically important because a reduction in the pH of the gallbladder bile effectively lowers the bicarbonate and reduces the risk of forming insoluble carbonate salts. As a result, the concentration of ionised calcium is increased in bile. The gallbladder epithelium , however , has the ability to absorb calcium and can reduce its concentration in bile by more than 50% (Conter RL et al 1986); bile acids also buffer the remaining ionised calcium. As a consequence of these various events less calcium is available to form insoluble salts (Moore EW et al 1982).

The relation between bile acidification and calcium carbonate solubility has been studied in dogs (Rege RV et al 1987). The findings that acidification of bile reduces the likelihood of precipitation of calcium carbonate would be explained by H^+ secretion and and could prevent gallstone formation. A recent study of human bile, however, failed to identify differences in acidification between gallstone-free individuals and patients with gallstones (Marteau C et al 1991). In that study, 80% of the patients studied had black (pigment) or mixed (containing <30% cholesterol) stones and only 20% pure cholesterol stones.

In this thesis *(chapter 8)*, the saturation indexes of two important calcium salts (calcium carbonate and calcium phosphate), whose precipitation in bile is considered important for the formation of gallstones, were calculated. The results suggest that relatively more alkaline gallbladder bile is associated with a higher saturation index for calcium carbonate; indeed when bile pH exceeded 7.10, 60% of the bile samples were supersaturated with calcium carbonate and the likelihood of precipitation of this salt was thermodynamically increased.

Studies on the solubility of calcium phosphate salts by Moore EW et al 1989, indicate that the only phosphate salt which is likely to precipitate within the pH range of bile is $CaHPO_4$. In contrast with calcium carbonate, $CaHPO_4$ solubility remained high within the pH values encountered in the bovine gallbladder in all but one of the bile samples ; however, the effect of acidification was still to enhance the solubility of this salt. The implications of the different magnitude of effect of pH on the solubility of the

two salts may be important; a reduction in bile pH would proportionally decrease the formation of $CaCO_3$ because the reaction :

$$[CO_3^{=}] \Leftrightarrow [HCO_3^{-}] + [H^{+}] \Leftrightarrow H_2O + CO_2$$

would be shifted to the right. This, in turn, would thermodynamically increase the amount of ionised calcium available for binding other anions in bile, in particular bilirubinate. This hypothesis is supported by the fact pCO_2 linearly increased with bile acidification in our study.

Although $CaHPO_4$ may be soluble within the physiological pH range, an excess of ionised calcium if combined with increased biliary phosphate production from hydrolysis of bile phospholipids (Moore EW 1989), would increase the formation of calcium phosphate and precipitation of this salt, if its concentration exceeds the saturation point.

It seems probable that differences in the gallbladder acidification capacity might be of importance in the process of gallstone formation; relatively more alkaline bile results in supersaturated with calcium carbonate bile, while if the bile is relatively more acidic, the formation of calcium carbonate would be significantly reduced. This would enable calcium ions to bind bile pigments. In addition the coexistence of other factors such as cholesterol supersaturation, excess of biliary phosphate or bilirubinate, the presence of nucleating factors or a deficiency in antinucleating factors (Sciarretta C et al 1984, Sutor DJ et al 1976) all play a role in determining whether crystals precipitate and which type of gallstone is formed.

Although the evidence from the studies on human gallbladders from cholecystectomy , normal bovine gallbladders and normal bovine bile suggests that the acid-base status of gallbladder bile is of major importance in the pathogenesis of gallstones and that defective hydrogen ion secretion by the epithelium significantly increases the risk of calcium salts precipitation, it is still not certain whether changes in acidification, all of which have been observed in calculus gallbladders in man, may be secondary to the accompanying inflammation and fibrosis, or whether some of the mucosal changes are secondary to impairment of acidification and that reduced hydrogen ion secretion actually precedes gall stone formation. It is also possible that supersaturated bile, or the hydrolysis of conjugated bilirubin, or other constituents of bile such as lysolecithin, may exert an injurious effect on the mucosa with resulting reduction of acidification.

PRIMARY GALLBLADDER EPITHELIAL CELL CULTURES

The gallbladder epithelium has been considered for many years to be an excellent model for the study of the physiological properties of epithelial cells and in particular ion transport and as it has already mentioned before, its role in the pathogenesis of gallstone formation is increasingly being recognised. Until recently, all studies on gallbladder epithelial cells have been restricted to using part or whole of the gallbladder. Several physiologists have used gallbladders from animal species to study ion transport and the electrical properties of the epithelium by applying either the Ussing chamber technique or whole organ preparation techniques. These techniques were used in this thesis to study several aspects of hydrogen ion secretion in the human and the bovine gallbladder. Although these techniques are valuable for transport studies, they provide little information on the biological properties of the gallbladder epithelial cell itself.

Another limiting factor in studying the properties of human gallbladder epithelium has been the lack of normal tissue. This has now become more readily available with the development of liver transplantation programmes. Alternatively, many animal models have been used to overcome the lack of availability of normal human tissue. The development of a simple and reliable method of obtaining and culturing gallbladder epithelial cells from a readily available source would permit a detailed study of the biological functions of these cells and would contribute to our understanding of the pathophysiology of gallstone formation by studying intracellular events.

In *Chapter 9* we report a reliable and reproducible method for harvesting and culturing gallbladder epithelial cells from a readily available source, the bovine gallbladder. Over the past few years there has been increasing interest in the biological characteristics of biliary and gallbladder epithelial cells in an attempt to enhance our understanding of diseases affecting the biliary tree. Several investigators have attempted to culture biliary cells (Kumar V et al 1986, Grant AG et al 1977 and Ishii M et al 19891) but the methods described have tended to be complicated and difficult to apply.

A method of gallbladder tissue culture from the guinea-pig gallbladder has been developed (Elhamady MS et al 1983) in the hope of providing a direct tool to study the biological and immunological characteristics of these cells. The main disadvantage of tissue culture, however, is the simultaneous growth of other types of cells present in the gallbladder wall, particularly fibroblasts, which may suppress the growth of the epithelial cells ; for this reason the method has not been applied widely in the study of epithelial cell function. A few groups from the United States have reported some success in short term cultures of either normal human gallbladder cells (LaRusso NF et al 1989) or by using well differentiated gallbladder adenocarcinoma lines (Morgan RT et al 1981

,Purdum PP et al 1991). More recently, Oda et al. reported in detail a technique for the harvesting and long term culture of dog gallbladder epithelial cells (Oda D et al 1991). The dog gallbladder epithelial cells have similarities to bovine in a number of biological characteristics (time to reach confluency, division rates etc.) but the method we report in the present paper is simpler and quicker. Another significant advantage of the method presented in this thesis, is the use of bovine tissue which is easily obtainable and avoids the need for breeding costly experimental animals and subsequent operation required to retrieve the gallbladder. The availability of bovine tissue allows the use of frequent short term (up to two weeks) primary cultures rather than long term cultures avoiding problems of cell dedifferentiation which are likely to occur in long term cultures. In addition the method described for bovine tissue can also be applied to human gallbladder epithelial cells. The development of a simple method of gallbladder cell culture from a readily accessible source will facilitate the study of several aspects of gallbladder epithelial cell function and in particular the regulation of ion transport and hydrogen ion secretion, identification and cloning of the several antiport systems and channels operating on the cell membrane, regulation of mucin secretion and prostaglandin metabolism, and will enhance the understanding of the pathophysiological mechanisms of gallbladder disorders.

VISUALISATION OF ACID SECRETION BY THE GALLBLADDER EPITHELIAL CELL

In this thesis we have applied the method of isolation of gallbladder epithelial cells described in *chapter 9*, to visualise under fluorescent microscopy the process of hydrogen ion production by the isolated human gallbladder epithelial cell. The fluorescent dye acridine orange was used for this purpose. This dye is uptaken by the isolated cells with a resulting loss of fluorescence at emission of 510 nm. Acridine orange, which is a weak base, is distributed according to intracellulal pH gradient and accumulates in acidic environment; its accumulation is associated with an emission of red colour at 660 nm (Berglindh T et al 1980). Similarly, the dye is uptaken by uncoiled DNA (a sign of non-viabily of the cell) resulting to red discoloration of the nucleus.

In agreement with the Ussing chamber and whole organ perfusion experiments, the isolated epithelial cells appeared to produce hydrogen ions which resulted in a change of colour of the cytoplasm to red towards the apical side of the cell. This change was apparent after 15 min and it reached the maximum at 30 min. Addition of amiloride prevented any changes in the colour of the cytoplasm; in contrast, histamine enhanced

the red fluorescent emission. The exact mechanism by which the gallbladder epithelial cell produces hydrogen ions is unclear. From the present study using fluorescent dyes, it can be inferred that histamine stimulates the production of antiport proteins (Na^+/H^+), which increase the amount of hydrogen ions produced by the epithelial cell. The changes in cell morphology are consistent with increased production of antiport transporter proteins which initialy appear within the cytoplasm (where the rough endoplasmic reticulum is expected) and subsequently move towards the apical side of the cell where they are finally sited. Similar observations were made for the parietal cell by Berglindh T et al, 1980. However further studies to test this hypothesis are possible only by using molecular biology techniques to clone the antiport system and subsequently to look for changes to secondary messengers expression within the cell with the addition of histamine , amiloride or other pharmacological agents.

These results not only confirm that the gallbladder epithelial cell is capable of producing and secreting hydrogen ions, but also provide evidence that the method of isolation of epithelial cells is a valuable tool in the study of intracellular events of the gallbladder epithelial cell.

CONCLUSIONS

In conclusion in this thesis we have presented direct evidence for the first time in the literature that the viable human gallbladder is capable of secreting hydrogen ions. This acid secretion probably occurs through an apical Na^+/H^+ exchange at the mucosal site of the gallbladder epithelial cell and represents a protective mechanism against calcium precipitation. Gallbladders with gallstones and chronic cholecystitis had reduced ability to secrete hydrogen ions; whether reduced acidification was the primary defect which lead to gallstone formation or the result of acidification , remains unclear.

The experiments in the bovine gallbladder model have shown that acidification occurs through an ouabain and amiloride-sensitive, histamine dependent sodium / hydrogen exchange mechanism. In addition, it was demonstrated that bovine gallbladder bile is similar to human bile, in terms of acid-base and electrolyte concentration and can be used to study pH and electrolyte regulation in normal bile. Bile acidification significantly improved calcium carbonate but not calcium phosphate solubility. The latter's solubility was however high in the physiological bile pH range. This study also adds to the weight of evidence and enhances the hypothesis that pH changes in bile are important, in the pathogenesis of gallstone formation.

In this study we have attempted to advance our knowledge on the role of hydrogen ion secretion by the gallbladder epithelium in the pathogenesis of gallstones.The defective acid-base regulatory ability of the epithelium is undoubtfully another factor to be added in the long list of events involved in the formation of gallstones. Whether this understanding can be translated into therapeutic interventions remains speculative.

Finally in this thesis we have developed a simple method for culturing gallbladder epithelial cells and this method should be useful for the study of the biological properties of gallbladder mucosal cells both in experimental animals and in man.

FUTURE STUDIES

Several potential studies arise from the results of this thesis; the role of mucus and the role of oestrogens in the process of acidification would merit further investigation. Further studies towards identifying the characteristics and cloning the gallbladder epithelial cell sodium / hydrogen transporter would increase our understanding of the mechanisms of acid-base regulation in the gallbladder. Although the last few years the importance of calcium in the pathogenesis of gallstones has been appreciated, there are still several gaps in understanding the mechanisms by which the gallbladder handles calcium. The development of a simple method of isolation and culture of gallbladder epithelial cells in combination with other techniques will make possible the study of calcium handling and transport across the epithelial cell.

The distribution , type and role of histamine receptors in the gallbladder need further study. Similarly, very little is known about the interactions between histamine and cholecystokinin in modifying motility and possibly secretion; the effects of cholecystokinin on hydrogen ion secretion by the gallbladder are worthy of further investigation.

Epidemiological studies to identify whether the use of H_2 antagonists over the last 15 years has increased the incidence of gallstone disease, will be of considerable interest. Finally, pharmacological studies towards the development of agents, which amongst other properties, may promote acidification as a means to prevent gallstone formation will be of value.

CHAPTER 11
REFERENCES

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1. *Abei M, P Kawczac, H.Nuutinen, A.Langnas, J. Svanvik, R.T. Holzbach. Isolation and characterisation of a cholesterol crystallization promoter from human bile. Gastroenterology 1993; 104: 539-548.*
2. *Admirand WH, D.M.Small. The physicochemical basis of cholesterol gallstone formation in man. J. Cinical Investigation 1968;47: 1043-1052.*
3. *Afdhal NH and Smith BF. Current concepts on the pathogenesis of cholesterol gallstones. Gastroenterology International 1991; 4: 33-36.*
4. *Altenberg GA, Reuss L. Apical membrane Na^+/H^+ exchange in necturus gallbladder epithelium. J Gen Physiol 1990; 95: 369-392*
5. *Alvaro D, Angelico M, Gandin C, Corradini SG, Capocaccia L. Physico-chemical factors predisposing to pigment gallstone formation in liver cirrhosis. J Hepatol 1990; 10: 228-234.*
6. *Ananthanarayanan M, J.C. Bucuvalas, B.L. Schneider, C.J. Sippel, F.J. Suchy. An ontogenically regulated 48-kDa protein is a component of the Na^+- Bile acid co-transporter of rat liver. Am.J.Physiol. 1991;261: G810-G817.*
7. *Anwer MS, Berk PD, Suchy FJ and Wolkoff AW. Characterisation of membrane transport mechanisms :A summary of the 1991 AASLD single topic Conference. Hepatology 1992;16: 1179-1193.*
8. *Aronchick CA, F.P. Brooks. Anatomy and Physiology of the biliary tract. In Bockus Gastroenterology, Berk J.E. (Ed), WB Saunders Co, Philadelphia (4th Edition), 1985;6(117): 3449-3485.*
9. *Barker DJP, Gardner MJ, Power C, Hutt MSR. Prevalence of gallstones at neçropsy in nine British towns. Br Med J 1979;ii:1389-1392.*
10. *Barrett-Conor E.. Cholestasis in sickle cell anaemia. Am.J.Medicine 1968;45: 889-897.*
11. *Basmajian JV, C.E.Slonecker. Bile passages and gallbladder.In Grant's Method of Anatomy (11th edition), Williams & Wilkins, 1989: 168-170.*
12. *Bateson MC, I.A.D. Bouchier. Prevalence of gallstones in Dundee: a necropsy study. B.M.J. 1975;4: 427-429.*
13. *Bateson MC, McLean D, Ross PE, Bouchier IAD. Clofibrate therapy and gallstone induction. Digestive Diseas. 1978; 23: 623-628.*

14. *Beal S. Historical perspection of gallstone disease. Surg.Gyn &Obs. 1984;158:181-189.*
15. *Bean JM, Bills PM, Lewis D. Microstructure of gallstones. Gastroenterology 1979; 76: 548-555.*
16. *Benjamin IS. Histology, Gallbladder and biliary tree (chapter 12). In Gastroenterology:Clinical Science and Practice (2nd edition), I.A.D.Boucher ,R N.Allan, H.J.F.Hogson, M.R.B. Keighley (eds) , WB Saunders 1993:1672-1673.*
17. *Bennion LJ, Mott DM, Howard BV. Oral contraceptives raise the cholesterol saturation of bile by increasing biliary cholesterol secretion. Metabolism 1980; 29:18-22.*
18. *Berglindh T, Dibona DR, Ito S, Sachs G. Probes of parietal cell function. Am J Physiol 1980;238: G165-G176.*
19. *Biss K, K-J Ho, B. Mikkelson, L. Lewis, B.C. Taylor. Some unique biologic characteristics of the Masai of East Africa. New England J.Medicine 1971; 284: 694- 699.*
20. *Bouchier IAD, Neoptolemos J. Clinical features and non-surgical treatment of gallstones. In Gastroenterology: Clinical Science and Practice (2nd edition), I.A.D. Bouchier, R.N.Allan, H.J.F. Hodgson, M.R.B. Keighley (Eds), WB Saunders 1993: 1719-1731.*
21. *Bouchier IAD. Biochemistry of gallstone formation. Clinics in Gastroenterology.1983;12(1): 25-48.*
22. *Bouchier IAD. Gallstones and gender: sex differences matter. European Journal of Gastroenterology & Hepatology 1991;3:741-744.*
23. *Bouchier IAD. Gallstones: Formation and epidemiology. In Blumgart (Ed) Surgery of the Biliary Tract,1989 Chapter 38:503-516*
24. *Bouchier IAD. Postmortem study of the frequency of gallstones in patients with cirrhosis of the liver. Gut 1969;10: 705-710.*
25. *Braverman DZ, M.L. Jonhson, F. Jr Kern. Effect of pregnancy and contraceptive steroids on gallbladder function. New England J. Medicine 1980;302: 362-364.*
26. *Burnstein MJ, Ilson RG, Petrunka CN, Taylor RD, Strasberg SM. Evidence of a potent nucleating factor in the gallbladder bile of patients with cholesterol stones. Gastroenterology 1983; 85: 801-807.*
27. *Cahalane MJ, Neubrand MW, Carey MC. Physical-chemical pathogenesis of pigment gallstones. Seminars in Liver Disease1988; 8:317-328.*
28. *Cai W, Gabella G. Innervation of the gallbladder and biliary pathways in the guinea pigs. J. Anat 1983; 136: 97-109.*

29. *Campbell BA, Burton AC. Stratification of bile in the gallbladder and cholelithiasis. Surgery, Gynecology and Obstetrics 1949; 88: 731-738.*
30. *Cetta F. The role of bacteria in pigment gallstone disease. Ann Surg 1991;213: 315-326.*
31. *Clanachan AS, Courtney D, Scott GW. Stimulatory and inhibitory histamine receptors in canine cystic duct. Br J Pharmacol 1982;77: 717-723.*
32. *Cohen S, M. Caplan, L. Gottlieb, J. Patterson. Liver disease and gallstones in regional enteritis. Gastorenterology 1971; 60: 237-245.*
33. *Connerty HV, Briggs AR. Determination of serum calcium by means of orthocresolphthalein complexone. Am J Clin Pathol. 1966;45:290-296.*
34. *Conter RL, J.L. Roslyn, V. Porter-Fink, L.DenBesten. Gallbladder absorption increases during early cholesterol gallstone formation. Am.J.Surg. 1986;151:184-191.*
35. *Cremiaschi D, Henin S, Meyer G. Stimulation by HCO3 of Na+ transport in rabbit gallbladder. J Membr Biol 1979; 47: 145-70.*
36. *Curran P and McIntoch J. A model system for biological water transport. Nature 1962; 193: 347-348.*
37. *Cutter J. John S Bobbs and lithotomy of the gallbladder. Int Abs Surg1928; 47: 409-411.*
38. *Davison JS. Control of gastric secretion.. In Gastrointestinal Secretion, Davison J S (editor) , Butterworth & Co (Publishers), London 1989: 86-101.*
39. *De Bruijn MAC, Noordam C, Goldhoorn BG, Tytgat GNJ, Groen AK. The validity of the cholesterol nucleation assay. Biochim Biophys Acta 1992; 1138: 41-45.*
40. *Diamond JM. A rapid method for determining voltage concentration relations across membranes. J. Physiol. 1965;183(1): 83-100.*
41. *Diamond J M. Non-linear osmosis. J Physiol 1965; 183:58-82.*
42. *Diamond JM. Transport mechanisms in the gallbladder. Handbook of Physiology: Alimentary Canal, edited by C.F. Code , American Physiological Society ,Washington, D.C.,1968: 2451-2482.*
43. *Diemerbroek I. Anatome Corporis Humani. Utrecht 1672.*
44. *Dietschy JM. Water and solute movement across the wall of the everted rabbit gallbladder. Gastroenterology 1966;50:692-707.*
45. *Dietschy JM. Transport of water in rabbit and guinea pig gallbladder . J Gen Physiol 1964;48:1-14.*
46. *Donovan JM, M.C. Carey. Formation of cholesterol gallstones. In Gastroenterology: Clinical Science and Practice (2nd edition), I.A.D. Bouchier,*

R.N.Allan, H.J.F. Hodgson, M.R.B. Keighley (Eds), WB Saunders Co, Philadelphia, 1993: 1702-1708.

47. *Dowling RH, Bell GB, White J. Lithogenic bile in patients with ileal dysfunction. Gut 1972; 13: 415-420.*
48. *Down RHL, M.J. Whiting, J.McK Watts, W. Jones. Effect of synthetic oestrogens and progesterones in oral contraceptives on bile lipid composition. Gut 1972;24: 415-420.*
49. *Duane WC. Formation of gallstones (chapter 10). In Liver & Biliary diseases N.Kaplowitz (Ed), William & Wilkins, Baltimore 1992: 148-161.*
50. *Dumont M, Uchman S, Erlinger S. Hypercholeresis induced by ursodeoxycholic acid and 7-ketolithocholic acid in the rat. Possible role of bicarbonate transport. Gastroenterology 1980; 79: 82-89.*
51. *Elhamady MS, Hopwood D, Milne G, Ross P, Bouchier IAD. Tissue culture of guinea-pig gallbladder epithelium. J Pathol 1983;140:221-235.*
52. *Erlinger S. Physiology of bile secretion. In Gastroenterology: Clinical Science and Practice (2nd edition), I.A.D. Bouchier, R.N.Allan, H.J.F. Hodgson, M.R.B. Keighley (Eds), WB Saunders 1993: 1693-1701*
53. *Evett RD, J.A. Higgins, A.L. Brown. The fine ultrastructure of the normal mucosa in human gallbladder. Gastroenterology 1964: 47;49-60.*
54. *Farquhar MG, G.E. Palade. Junctional complexes in various epithelia.J.Cell. Biol. 1963;17:375-412.*
55. *Festi D, Frabboni R, Bazolli F, Sangermano A, Ronchi M, Parini P et al. Gallbladder motility in cholesterol gallstone disease. Effect of ursodeoxycholic acid administration and gallstone dissolution. Gastroenterology 1990: 99; 1779-1785.*
56. *Fisher RS, Stelzer F, Rock E, Malmud LS. Abnormal gallbladder emptying in patients with gallstones. Dig. Dis.& Sci. 1982; 27: 1019-1024.*
57. *Fletcher DM, C.G. Clark. Gallstones and gastric surgery. British J. of Surgery 1968;55: 895-899.*
58. *Forrester RL, L.J. Wataji, D.A. Silverman, K.J.Pierre. Enzymatic method for determination of carbon dioxide in serum. Clin.Chem. 1976;22:243-245.*
59. *Furlong TJ, Spring KR. Mechanisms underlying volume regulatory decrease by Necturus gallbladder epithelium. Am L Physiol 1990; 258: C-1016-C1024.*
60. *Gallinger S, Harvey PRC, Petrunka CN and Strasberg SM. Effect of binding of ionised calcium on the in vitro nucleation of cholesterol and calcium bilirubinate in human gallbladder bile. Gut 1986; 27: 1382-1386.*

61. *Gelarden RT, Rose RC. Electrical properties an diffusion potentials in the gall bladder of man , monkey, dog, goose and rabbit. J Membrane Biol 1974;19:37-54.*
62. *Gilloteaux J, Pomerants B, Kelly T R . Human gallbladder mucosa Ultrastructure: Evidence of Intraepithelial Nerve structures. Am. J. Anatomy 1989; 184: 321-333.*
63. *Gleeson D. Acid-base transport systems in gastrointestinal epithelia. Gut 1992;33:1134-1145.*
64. *Godfrey PJ, Bates T, Harrison M, King MB, Padley NR. Gallstones and morbidity. A study of all gallstone-related deaths in a single health district. Gut 1984; 25: 1029-1033.*
65. *Grace PA, G.J.Poston, R.C.N. Williamson. Biliary motility. Gut 1990; 31: 571-582.*
66. *Grant AG, Billing BH. The isolation and characterization of a bile ductule cell population from normal and bile duct ligated livers. Br J Exp Pathol 1977;58:301-310.*
67. *Greco AV, Mancinelli R, Mingrone G, Racanicchi S. A glucagon-secretin- like peptide stimulates the intrinsic nervous plexus of guinea pig gallbladder. Experimentia 1990; 40: 452-454.*
68. *Greenwood B. Relationship between gastrointestinal motor activity and secretion . In Gastrointestinal Secretion, Davison J S (editor) , Butterworth & Co (Publishers)1989: 230-244.*
69. *Grider JR, G.M. Makhlouf.Distinct receptor for cholecystokinin and gastrin on muscle cells of stomach and gallbladder. Am.J.Physiol (Gastrointest Liver Physiol 22) 1990;259: G184-G190.*
70. *Gurantz D, Schteingart CD, Hagey LR, Steinbach JH, Grotmol T, Hofmann AF. Hypercholeresis induced by unconjugated bile acid infusion correlates with recovery in bile of unconjugated bile salts. Hepatology 1991; 13: 540-550.*
71. *Gutknecht J. Proton permeation through lipid bilayers and biological membranes. European J Gastroenterol Hepatol 1990; 2(3): 172-174.*
72. *Hamada Y, A. Karjalainen, B.A. Setchell, J.E. Millard, F.L. Bygrave. Acute effects of cholestatic and choleretic bile salts on vasopressin and glucagon-induced hepatobiliary calcium fluxes in the perfused rat liver. Biochem J. 1992;283: 575-581.*
73. *Haslewood GAD. Bile salt evolution. J Lipid Research 1967;8:535-550.*

74. *Hayes PC, A.Patrick, J.E Roulston, J.T. Murchison, P.Allan, J.N.Plevris, B.E. Clarke, I.A.D. Bouchier. Gallstones in diabetes mellitus: Prevalence and risk factors. European J. Gastroenterology & Hepatology 1992;4: 55-59.*

75. *Heaton GB, K.W. Heaton. Lipid composition of bile in diabetics and obesity matched controls. Gut 1979;20: 518-522.*

76. *Heintz K, K.U. Petersen, J.R. Wood. Effects of bicarbonate on fluid and electrolyte transport by guinea pig and rabbit gallbladder:stimulation of absorption. J.Membr. Biol. 1981;62: 175-181.*

77. *Heuman DM, E.W. Moore, Z.R. Vlachevic. Pathogenesis and dissolution of gallstones. In Diseases of the Liver and the Biliary Tree. Edited by D.Zakim & T.D. Boyer, WS Saunders Co, Philadelphia 1990; IV:1480-1516.*

78. *Hofmann AF, Grundy SM, Lachin JM, Baum RA, Hanson RF et al. Pretreatment biliary composition in white patients with radiolucent gallstones in the National Cooperative Gallstone Study. Gastroenterology 1982; 83: 738-752.*

79. *Holan KR, R.T. Holzbach, R.E. Hermann, A.M. Cooperman, W.J. Claffey. Nucleation time: a key factor in the pathogenesis of cholesterol gallstone disease. Gastroenterology 1979;77: 611-617.*

80. *Holzbach RT, M.Marsh, M.Olszewski, K.Holan. Cholesterol solubility in bile. Evidence that supersaturated bile is frequent in healthy man. J. Clinical Investigation 1973;52: 1467- 1479.*

81. *Howard PJ, Murphy GM, Dowling RH. Gallbladder emptying patterns in response to a normal meal in healthy subjects and patients with gallstones: ultrasonographic study. Gut 1991;32(11):1406-1411.*

82. *Howard PJ and Murphy GM. Bile physiology. Current Opinion in Gastroenterology 1993; 9: 791-799.*

83. *Hudson I, Hopwood D. Macrophages and mast cells in chronic cholecystitis and 'normal' gallbladders. J Clin Pathol 1986 ; 39: 1082-1087.*

84. *Impicciatorre M. Occurrence of H_1 and H_2 histamine receptors in the guinea pig gallbladder in situ. Br J Pharmacol 1978; 64: 219-222.*

85. *Ishii M, Vroman B, LaRusso NF. Isolation and morphologic characterization of bile duct epithelial cells from normal rat livers. Gastroenterology 1989;97:1236-1247.*

86. *Jacyna MR, P.E.Ross, M.A. Bakar, D.Hopwood, I.A.D. Bouchier. Characteristics of cholesterol absorption by human gallbladder: relevance to cholesterolosis. J.Clin.Pathol. 1987;40:524-529.*

87. *Jacyna MR, Ross PE, Hopwood D, Bouchier IAD. The effect of secretin on sodium ion absorption by the isolated human gallbladder. Aliment Pharmacol Ther 1989; 3: 293-297.*

88. *Jacyna MR. Aspects of mucosal function in the human gallbladder 1986:108-110 (Thesis). University of Dundee,Scotland.*

89. *Jacyna MR. Interactions between gallbladder bile and mucosa; relevance to gallstone formation. Gut 1990; 31:568-570.*

90. *Janquera LC, J. Carneiro, A.Contopoulos. Histology of the gallbladder. In Basic Histology (2nd edition), Lange ,Los Altos, California USA 1977:325-328.*

91. *Jelinek DF, S. Anderson, D.A. Slauchter, D.W. Russel. Cloning and regulation of cholesterol 7a- hydroxylase: the rate-limiting enzyme of bile acid biosynthesis. J.Biol.Chem. 1990;265: 8190-8197.*

92. *Kaminski DL. Arachidonic acid metabolites in hepatobiliary physiology and disease. Gastroenterology 1989; 97: 781-792.*

93. *Kern F Jr , Everson GT. Contraceptive steroids increase cholesterol in bile:mechanisms of action. J LIpid Res 1987; 28:828-839.*

94. *Kouroumalis E, Hopwood D, Ross PE, Bouchier IAD. Human gallbladder epithelium: non specific esterases in cholecystitis. J Pathol 1984; 142: 151-159.*

95. *Kouroumalis E, Hopwood D, Ross PE, Milne G, Bouchier IAD. Gallbladder epithelial and hydrolases in human cholecystitis. J Pathol 1882; 139: 179-191.*

96. *Kumar V, Jordan TW. Isolation and culture of biliary epithelial cells from the biliary tract fraction of normal rats. Liver 1986;6:369-376.*

97. *Kyosola K, Penttila O. Adrenergic innervation of the human gallbladder. Histochemistry 1977; 54: 209-218.*

98. *LaMont JT, F.Bernard, J.R.L.Moore. Role of gallbladder mucin in pathophysiology of gallstones. Hepatology 1984;4(5): 51S-56S.*

99. *LaMorte WW. Biliary motility and abnormalities associated with cholesterol cholelithiasis. Current Opinion in Gastoenterology 1993; 9:810-816.*

100. *LaRusso NF, Hoerl BJ, Vroman BT, Scott RE: Biological characteristics of cultured human gallbladder epithelial cells. Hepatology 1989;10:636 (Abstr).*

101. *LaRusso NF. Proteins in bile: How they get there and what they do. Am J Physiol 1984; 247: G199-205.*

102. *Lee SP, Carey MC, LaMont JT. Aspirin prevention of cholesterol gallstone formation in prairie dogs. Science 1981; 211: 1429-1430.*

103. *Lee SP , LaMont JT, Carey MC. Role of gallbladder mucus hypersecretion in the evolution of cholesterol gallstones. J Clin Invest 1981; 67: 1712-1723.*

104.Lennon F, Feeley TM, Clanachan AS, Scott GS. Effects of histamine receptor stimulation on diseased gallbladder and cystic duct. Gastroenterology 1984; 87: 257-262.

105.Leyssac P, Bukhave K, Frederiksen O. Inhibitory effect of prostaglandins on isosmotic fluid transport by rabbit gallbladder in vitro, and its modification by blockade of endogenous PGE-biosynthesis with indomethacin. Acta Physiol Scand 1974; 92: 496-507.

106.Lillemoe K, Webb TH, Pitt HA. Neuropeptide Y; a candidate neurotransmitter for biliary motility. J Surg Res 1988; 45: 254-260.

107. Lundgren O, Svanvik J, Jivegard L. Enteric nervous system . II Physiology and pathophysiology of the human gallbladder. Digest. Diseas. Sci 1989; 34:284-288.

108.Machen TE, D.Erlij, F.B.P. Wooding. Permeable Junctional complexes: The movement of lanthanum across rabbit gallbladder and intestine.J.Cell.Biol.1972;54:302-312.

109.Malet PF, Locke CL, Trotman BW, Soloway RD. The calcium ionophore A23187 stimulates glucoprotein secretion by the guinea-pig gallbladder. Hepatology 1986;6:569-573.

110.Martau C, B.Sastre, N.Iconomidis, H.Portugal, A.M. Pauli, A. Gerolam. pH regulation in human bile : study in patients with and without gallstones. Hepatology 1990;12: 997-1002.

111.Masclee AAM, Jansen JBMJ, Driessen WMM, Geuskens LM, Lamers CBHW. Effect of truncal vagotomy on cholecystokinin release, gallbladder contraction and gallbladder sensitivity to cholocystokinin in humans. Gastroenterology 1990; 98: 1338-1344.

112.Messing B, Boris C, Kunstlingen F, Bernier JJ. Does total parenteral nutrition induce gallbladder sludge formation and lithiasis? Gastroenterology 1983; 84: 1012-1019.

113.Meyer G, G. Botta, C. Rossetti, D.Cremaschi. The nature of the neutral Na+/Cl- coupled entry at the apical membrane of rabbit gallbladder epithelium:III analysis of transport on membrane vesicles. J.Membrane Biol. 1990;118:107-120.

114.Milov DE, W-S. Jou, R.B. Shireman, P.W.Chun.The effect of bile salts on carbonic anhydrase.Hepatology 1992;15: 288-296.

115. Moore EW, Keith FB, Shiffman ML, Kelley EH, Krell H. Pathogenesis of calcium-phosphate containing stones: VI Gallbladder bile is unsaturated with CaHPO4 in patients with gallstones or morbid obesity. Gastroenterology 1989; 96:A633 (abstr.)

116. *Moore EW, Kelley EH, Keith FB, Krell H. Pathogenesis of calcium -phosphate containing gallstones.II CaHPO4 precipitation and solubility constant product. Gastroenterology 1989;96:A632 (Abstr).*

117. *Moore EW, Kelley EH, Keith FB, Krell H. Pathogenesis of calcium -phosphate containing gallstones. V Saturation limits for total inorganic phosphate in bile as a function [Ca^{++}] and pH. Gastroenterology 1989;96:A633 (Abstr).*

118. *Moore EW, Ross JW Jr. NaCl/CaCl2 activity coefficients in mixed aqueous solutions. J Appl Physiol 1965;20:1332-1336.*

119. *Moore EW. Biliary calcium and gallstone formation. Hepatology 1990;12:206S-218S.*

120. *Moore EW. The role of calcium in the pathogenesis of gallstones.Ca^{++} electrode studies of model bile salt solutions and other biological systems. Hepatology 1984;4:228S-243S.*

121. *Moore EW.The regulation of ionised calcium (Ca^{++}) in bile ; Taurocholate induces powerful Gibbs-Donnan effects in vitro.Gastroenterology 1988;94:A572 (abstr.)*

122. *Morgan RT, Woods IK, Moore EW, McGavron L, Quinn LA, Semple TV. Human gallbladder adenocarcinoma cell line. In Vitro 1981;503:17-24.*

123. *Moseley RH. Cholestasis (chapter 11). In Liver & Biliary diseases ed. by N.Kaplowitz, William & Wilkins, Baltimore 1992: 163-179.*

124. *Neithercut WD. Effect of calcium , magnesium and sodium ions on in vitro nucleation of human bile. Gut 1989;30:665-670.*

125. *Nicholas P, P.A. Rinaudo, H.O. Conn. Increased incidence of cholelithiasis in Laennec's cirrhosis, Gastroenterology 1972;63: 112-121.*

126. *O'Grady SM, Wolters PJ, Hiltebrand K, Brown DR. Regulation of ion transport in porcine gallbladder: effects of VIP and norepinephrine. Am J Physiol 1989; 257: C53-C57.*

127. *Oda D, Lee SP, Hayashi A. Long term culture and partial characterization of dog gallbladder epithelial cells. Laboratory Invest. 1991;64(5):682-692.*

128. *Ohya T, J. Schwarzendrube, N.Busch, S.Gresky, K.Chandler, A. Takabayashi, H.Igimi, K.Egami, R.T. Holzbach. Isolation of a human biliary glucoprotein inhibitor of cholesterol crystallization. Gastroenterology 1993; 104: 527-538.*

129. *Ostrow JD. The etiology of pigment stones. Hepatology 1984; 4: 215S-222S*

130. *Paroli JE, Becker JM. Gallbladder and sphincter of Oddi motility. Current Opinion in Gastroenterology 1990; 6: 668-676.*

131. Plevris J N, Harrison D J, Bell E B, Bouchier I A D. The immunological characteristics of human gallbladder innervation. Europ. J. Gastroenterol. & Hepatology 1994 ; 6:151-158.

132. Plevris JN, Hayes PC, Bouchier IAD. Mechanism of acid secretion in the bovine gallbladder epithelium. Evidence of Na^+/H^+ exchange. Gut 1990;31(10):A1215.

133. Polak JM, Bloom SR, Sullivan SN, Facer P and Pearse AGE. Enkephaline-like immunoreactivity in the human gastrointestinal tract. Lancet 1977;i:972-974.

134. Pozo MJ, Salido MD, Madrid JA, Salido GM. In vitro effect of pirenzepine on motility of canine gallbladder. J Pharm Pharmacol 1990; 42: 89-93.

135. Purdum PP, Hylemon PB, Moore EW. Luminal acidification by cultured human gallbladder epithelium: Validation of a new experimental model. Gastroenterology 1991;100(5):A335.

136. Rege RV , Moore EW. Evidence for H+ secretion by the in vivo canine gallbladder. Gastroenterology 1987;92:281-289.

137. Rege RV, E.W.Moore. Convective movement of Ca^{++} across guinea pig gallbladder epithelium. Am.J.Physiol. 1992;262: G990-G995.

138. Rege RV, Moore EW, Nahrwold DL. Pathogenesis of calcium containing gallstones:relationship of total calcium and free ionised Ca^{++} in canine gallbladder and duct bile. Surg Forum 1985;36:132-134.

139. Rege RV, Moore EW. Pathogenesis of calcium containing gallstones;canine ductular bile, but not gallbladder bile is supersaturated with calcium carbonate. J Clin Invest 1986; 77:21-26.

140. Rege RV, Nahrwold DL, Moore EW. Absorption of biliary calcium from the canine gallbladder; protection against the formation of calcium - containing gallstones. J Lab Clin Med 1987;110:381-6.

141. Reuben A. Biliary proteins. Hepatology 1984;4:465-505.

142. Reuss L. Ion transport across gallbladder epithelium. Physiol. Reviews 1989; 69: 503-545.

143. Reuston RH, Maloney D, Jones AL, Hradek GT, Wong KY, Goldfire ID. Biliary secretory apparatus: Evidence for vesicular transport mechanism for proteins in the rat using horseradish peroxidase and [I^{125}] insulin. Gastroenterology 1980; 78: 1373-1388.

144. Rhodes M, A.Allen, T.W.J. Lennard. Mucus glucoprotein biosynthesis in the human gallbladder inhibition by aspirin. Gut 1992; 33: 1109-1112.

145. Ritter JK, F. Chen, Y.Y. Sheen, H.M. Tran, S. Kimura, M.T. Kimura, M.T. Yeateman, I.S. Owens. A novel complex locus UGTI encodes human bilirubin ,

phenol and ohter UDP glucuronyl transferase isozymes with identical carboxyl termini. J.Biol.Chem. 1992;267: 3257-3261.

146.Rose RC, R.T.Gelarden, D.L.Nahrwold. Electrical properties of isolated human gallbladder. Am.J.Physiol.1973;224:1320-1326.

147.Rose RC. Absorptive functions of gallbladder. In Functions of the stomach and intestine by Friedman MHF (ed), HM+M Medical and Scientific publishers,1975;329-347.

148.Rose RC. Absorptive functions of the gallbladder.In Jonhson L.R.,ed. Physiology of the gastrointestinal tract.Vol 2. New York:Raven Press,1981; 1021-1033.

149. Roslyn JJ, Abedin MZ, Strichartz SD, Abdou MS, Palant CE. Regulation of gallbladder ion transport; role of biliary lipids. Surgery 1989; 105: 207-212.

150.Roslyn JJ, H.A. Pitt, L.L. Mann, M.E. Ament, L. Den Besten . Gallbladder disease in patients on long term total parenteral nutrition. Gastroenterology 1983;84: 148-154.

151.Ruetz S, Hugentobler G, Meier PJ. Functional reconstitution of the canalicular bile salt transport system of rat liver. Proceed. Nat. Acad. Sci. (USA.) 1988; 85: 6147-6151.

152.Sampliner RE, P.H. Bennett, L.J. Commess, F.A. Rose, T.A. Burch. Gallbladder disease in Pima Indians. Demonstration of high prevalence and early onset by cholecystography. New England J Medicine 1970;832: 1358-1364.

153.Scheeres DE, T.H.Magnuson, H.A.Pitt, J.A.Bastidas, C.A. May, K.D.Lillemoe. The effect of calcium on gallbladder absorption. J.Surg. Res. 1990;6:547-551.

154.Schjoldager B, Powers SP, Miller IJ. Affinity labelling the bovine gallbladder checystokinin receptor using a battery of probes. Am J Physiol (Gastroint Liver Physiol) 1988; 255; 579-586.

155.Schoetz DJ, Wise WE, LaMorte WW, Birkett DH, Williams LF. Histamine rečeptors in the primate gallbladder. Dig Dis Sci 1983; 28: 353.

156.Sciarretta G,Ligabue A, Garuti G, Pieromaldi S, Verri A, Giacobazzi G. Inhibitory activity of gallbladder bile on calcium carbonate crystallization in vitro. Scand J Gastroenterol 1984;19:626-630.

157. Scott RB, Diamant SC.. Biliary motility associated with gallbladder storage and duodenal delivery of canine hepatic biliary output. Gastroenterology 1988; 95: 1069-1080.

158.Scragg RKR, A.J. McMichael, R.F. Seamark. Diet, alcohol and relative weight in gallstone disease: a case control study . B.M.J. 1984b;288: 1113-1119.

159.Scragg RKR, G.D. Clavert, J.R. Oliver. Plasma lipids and insulin in gallstone disease. A case control study. B.M.J. 1984;289: 521-525.

160. Segal Y, Reuss L. Ba++, TEA+ and quinine effects on apical membrane K+ conductance and maxi K+ channels in gallbladder epithelium. Am J Physiol (Gastrointest Liver Physiol) 1990; 258: G745-G752.

161. Sellers AF.Neurohumoral regulation of Gastrointestinal function, secretion and motility.In Dukes Physiology for domestic animals. MJ Swenson (Edit.). Cornell University Press Ltd. 1977;20:285.

162.Severi C, Grider JR, Maklouf GM. Functional gradients in muscle cells isolated from gallbladder, cystic duct and common bile duct. Am J Physiol (Gastrointest Liver Physiol) 1988; 255: 647-652.

163.Sewell RB, S.J.T. Mao, T. Kawamoto, N.F. LaRusso. Apolipoproteins of high, low and very low density lipoproteins in human bile. J. Lipid Research 1983;24: 391-401.

164.Shaffer EA, Small DM. Biliary lipid secretion in cholesterol gallstone disease. The effect of cholecystectomy and obesity. Journal Clin Investigation 1977; 59: 828-840.

165.Shaffer EA. The effect of vagotomy on gallbladder function and bile composition in man. Annals of Surgery 1982; 195: 413-418.

166. Shiffmam ML, Sugerman HJ, Moore EW. Human Gallbladder Mucosal Function. Gastoenterology 1990;99:1453-1459.

167.Shiffman ML, E.W.Moore. Acidification of gallbladder bile is defective in patients with all types of gallstones. Gastroenterology 1984;94:A591.

168.Shiffman ML, H.J. Sucerman, J.M. Kellum, E.W. Moore. Calcium in human gallbladder bile. J.Lab.Clin.Med. 1992;120: 875-884.

169.Shiffman ML, H.J. Sucerman, J.M.Kellum, E.W.Moore. Change in gallbladder bile composition following gallstone formation and weight reduction. Gastroenterology 1992;103: 214-221.

170.Simion FA, Fleischer B, Fleischer S. Subcellular distribution of bile acids, bile salts and taurocholate binding sites in rat liver. Biochemistry 1984; 22: 6459-6466.

171.Singletary BK, Van Thiel DH, Eagon PK. Estrogen and progesterone receptors in human gallbladder. Hepatology 1986; 6: 574-578.

172.Snell RS, Ed. The abdomen (chapter 5): Part II. The abdominal cavity. In Clinical Anatomy (2nd edition), Little Brown and Co, 1981: 207-208 & 244-246.

173.Soloway RD, E.B.Fayasal, D.W.Trotman, N.E.Weston, J.F.Jr Ficca. Water content of gallstones:location and contribution to a hypothesis concerning stone structure. Hepatology 1982;2: 223-229.

174.Soloway RD, Trotman DW, Ostrow JD. Pigment stones. Gastroenterology 1977; 72: 167-182.

175.Spivak W, D. Divenuto, W. Yuey. Non-enzymatic hydrolysis of bilirubin mono- and diglucuronide to unconjugated bilirubin in model and native bile systems.potential role in the formation of gallstones. Biochem.J. 1987;242: 323-329.

176. Sternberger LA, Hardy PH, Cuculis JJ, Meyer HG. The unlabelled antibody enzyme method of soluble antigen-antibody complex (horseradish peroxidase anti-horseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 1970;18:315-333.

177. Steward M C and Case R M. Principles of water and ion transport across epithelia. In Gastrointestinal Secretion, Davison J S (editor) , Butterworth & Co (Publishers)1989: 1-27.

178. Stolz A, Takikawa H, Ookhtens M, Kaplowitz N. The role of cytoplasmic proteins in hepatic bile acid transport. Annual Review of Physiology 1989; 51: 161-176.

179.Stone BG, J.S. Gavaler, S.H. Belle, D.P. Shreiner, R.R. Peleman, R.P. Sarva. Impairement of gallbladder emptying in diabetes mellitus. Gastroenterology 1988;95: 170-176.

180.Strichartz SD, Abedin MZ, Abdou MS, Roslyn JJ. The effects of amiloride on biliary calcium and cholesterol gallstone formation. Ann Surg 1989;209: 152-156.

181.Sutor DJ, L.I. Wilkie. Calcium in bile and calcium salts in gallstones. Clin. Chem. Acta 1977;79: 119-127.

182. Sutor DJ, Percival JM. Presence or absence of inhibitors of crystal growth in bile.I Effect of bile on the formation of calcium phosphate , a constituent of gallstones. Gut 1976;17:506-510.

183.Sutor DJ and Wooley SE. X-ray diffraction studies of the composition of gallstones from English and Australian patients. Gut 1969; 10: 681-683.

184. Svanic J, Allen B, Pellegrini C, Bernhoff R, Way L. Variations in the concentrating function of the gallbladder in the conscious monkey. Gastroenterology 1984;86:919-925.

185. Svanvik J, PellegriniCA, Allen B, Bernhoft R, Way LW. Transport of fluid and biliary lipids in the canine gallbladder in experimental cholecystitis. J Surg Res 1986;41:425-431.

186. Symmers W S-C. In Systemic Pathology., 2nd Edition. Churchill Livingstone 1978; Vol 3: 1304-1330.

187. Thistle JC, Cleary PA, Lachin JM, Tyor MP, Hersh T. The natural history of untreated cholelithiasis during the National Cooperative Gallstone Study (NCGS) . Gastroenterology 1982; 82: 1197 (Abstr).

188. Thomas PJ, A.F. Hofmann. A simple calculation of the lithogenic index of bile: expressing biliary lipid composition on rectangular coordinates. Gastroenterology 1973; 65: 698-700.

189. Thornton J, C.Syme, K.Heaton. Moderate alcohol intake reduces bile cholesterol saturation and raises HDL cholesterol. Lancet 1983;ii: 819-821.

190. Thudichum. A treatise of gallstones. J. Churchill &Sons, London 1863.

191. Tormey J, Diamond J R. The ultrastructural route of fluid transport in gallbladder. J Gen Physiol 1967; 50: 2031-2060.

192. Toth JL, Harvey RC, Upadyha A, Strasberg SM. The gallbladder mucosa albumin absorption and protein secretion by the gallbladder in man and in the pig. Hepatology 1990; 12: 729-737.

193. Trotman BW, T.A. Morris III, H.M. Sanchez, R.D. Soloway, J.D. Ostrow. Pigment versus cholesterol cholelithiasis:identification and quantification by infrared spectroscopy. Gastroenterology 1977;72: 495-498.

194. Tucker LE, T.N. Tangedahl, S.R. Newmark. Prevalence of gallstones in obese Caucasian American women. Internatinal Journal of Obesity 1982;6: 247-251.

195. Ulissi A, P.P. Purdum III, E.W.Moore. Convective movement of Ca++ ions across cultured human gallbladder epithelia. Hepatology 1991;14(4): 267(A).

196. Ussing HH, K.Zehran. Active transport of sodium as the sourse of electric curremt in the short-circuited isolated frog skin. Acta Phys. Scand 1951;23: 110-127.

197. Van der Werk SDJ, Van Berge Henegouwen, Palsma DMH, Ruben AT. Motor function of the gallbladder and cholesterol saturation of duodenal bile. Neth J Med 1987:30; 160-171.

198. Vindelar LL, Christensen IJ, Nissen NI. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 1983;3:323-327.

199. Vlahcevic ZR, C.C.Jr Bell, L. Swell. Significance of the liver in the production of lithogenic bile in man. Gastroenterology 1970a;59: 62-69.

200. Wahlin T, Thornell E, Jivegard L, Svanvik J. Effects of intraluminal prostaglandin E2 in vivo on secretory behaviour and ultrastructural changes in mouse gallbladder epithelium. Gastroenterology 1988; 95:1632-5.

201. Waldman DB, Zfas AM, Maklouf GM. Stimulatory H_1 and inhibitory H_2 histamine receptors in gallbladder muscle. Gastroenterology 1977; 22: 932-936.

202. Walter WA & Snell RS. Diseases of the gallbladder and bile ducts . WB Saunders Co Philadelphia, USA 1940:1-10.

203. Weinman SA, Reuss L. Na+/H+ exchange at the apical membrane of the Necturus gallbladder. J Gen Physiol 1982; 80: 299 - 319.

204. Wenckert A and Robertson B. The natural course of gallstone disease.Eleven year review of 781 nonoperated cases.Gastroenterology 1966; 50: 376-381.

205. Wentz PW, Savory J, Cross RE. Improved method for the measurement of inorganic phosphate in serum with a centrifugal analyser. Clin Chem 1976;22:257-260.

206. Whitlock R T, Wheeler H O. Coupled transport of solute and water across rabitt gallbladder epithelium. J Clin Invest. 1964; 43: 2249-2260.

207. Whitlock R T, Wheeler H O. Hydrogen ion transport by isolated rabbit gallbladder. Am J Physiol 1969; 217:310-316.

208. Wildgrube HJ, Füssel U, Lauer H, Stockhausen . Measurement of conjugated bile acids by Ion-Pair High Performance Liquid Chromatography. J Chromatography 1983;282:603-608.

209. Wood JR, Svanvik J. Gallbladder water and electrolyte transport and its regulation. Gut 1983;24: 579-593.

210. Ziegler K, Elsner H. Functional molecular mass of the 14C - azidobenamidotaurocholic acid binding proteins in hepatocellular bile acid transport systems. Biochim Biophys Acta 1992; 1103: 229-232.

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