PART III: MATERIAL AND METHODS
Though the published data are not available, the incidence of biliary tract diseases among the Western Indian population, recorded during the last 8 years at New Civil Hospital, Ahmedabad (the largest hospital in the region), is relatively low (Table No. 5) in comparison with the Northern part of the country (Chhuttani, 1967). The kind of biliary tract diseases, the presence or absence of calculus and its nature, the association of various kinds and number of microorganisms with their significance under such conditions of biliary tract, is not investigated ever before.

The bacterial colonization of human biliary tracts and the significance of bacteria in the biliary tracts has been the subject of many investigations for almost a century (Finegold, 1977). Aerobic bacteria has been recovered from biliary tract of 25 to 50% of patients cultured (Mason, 1968; Edlund et al, 1959; Chaitin, 1973; Fukunaga, 1973; Gunn, 1975; Keighley et al, 1975). The organisms most commonly recovered, includes Escherichia coli, Klebsiella, Group D streptococci, Enterobacter, and Staphylococci. The high incidence of bactibilia is found in elderly patients (Fukunaga, 1973; Keighley & Graham, 1973; Ram & Charvi, 1974; Seik et al, 1975; Shimada, 1977) and in various disease conditions of the biliary tract (Fukunaga 1973; Keighley & Graham 1973; Keighley et al
<table>
<thead>
<tr>
<th>YEAR</th>
<th>AGE</th>
<th>Under 1 year</th>
<th>1-4</th>
<th>5-14</th>
<th>15-24</th>
<th>25-34</th>
<th>35-44</th>
<th>45-54</th>
<th>55-64</th>
<th>65-74</th>
<th>75 &amp; above</th>
<th>Total</th>
<th>Grand Total</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>SEX</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>M</td>
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<td>F</td>
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<td>F</td>
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<td>1973</td>
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<td>-</td>
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<td>1974</td>
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<td>4</td>
<td>-</td>
<td>2</td>
<td>6</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td>3</td>
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<td>1976</td>
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<td>-</td>
<td>-</td>
<td>1</td>
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<td>1</td>
<td>2</td>
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<td>6</td>
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<td>1977</td>
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<td>2</td>
<td>-</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Upto 31st July 1973</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>-</td>
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<td>2</td>
<td>2</td>
<td>5</td>
<td>2</td>
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<tr>
<td>1st August 1973 to Dec. 1978</td>
<td></td>
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<tr>
<td>1979</td>
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<td>1</td>
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<td>1</td>
<td>2</td>
<td>9</td>
<td>6</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Upto 31st July 1980</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

72 112 184
1975; Mautzer et al. 1974; Ram & Charvi 1974; Edlund et al. 1959; Anderson & Priestley 1951; Maddocks et al. 1973; Schoenfield 1971; Flehma et al. 1967; Scott & Khan 1969). Many a times (17 to 50 %) polymicrobial etiology has been observed in aerobic infections.

The attempts made in search of anaerobes by several investigators in the past, failed to isolate anaerobes from the biliary tract. On the other hand, the incidence of positive cultures from the biliary tract studied at surgery varied from 15 % to 80 % (England & Rosenblatt 1977; Lykkegaard Nielsen & Justesen 1976).

The anaerobic bacterial colonisation of human biliary tract and the role of these microbes in this region, has not been studied and it is yet to be understood. The great diversity in previous studies are probably due to the wide variability in kinds of specimen selection, specimen transport-tation procedures and inadequate methods for anaerobiosis.

In view of the renewed interest in the anaerobic bacteriology and non-availability of such documented study carried out in India, we attempted to investigate the presence or absence of anaerobic bacteria in human extrahepatic biliary tract and, in blood, using a more sophisticated, sensitive techniques of anaerobic culture and proper method of selection and transportation of the specimens.
(1) **Place of work:**

The work was carried out during the period of two years (August 1978 to July 1980) at D.J. Medical College and New Civil Hospital, Ahmedabad, the largest, 2000 bed teaching hospital situated in the Gujarat State, India.

(2) **Selection of human subjects:**

Selection of cases was random. Human cases were categorised into two groups and four subgroups as under:

- **Group I**: Surgical cases:
  - The patients operated upon for various diseases were subcategorised as under:
    - Group I a: 51 patients operated for the diseases of biliary tract.
    - Group I b: 103 patients operated for some diseases but they had normal extrahepatic biliary tract.

- **Group II**: Autopsy cases:
  - The interval between death and necropsy was less than 10 hours in all those cases. The exact hours of necropsy after death is presented in Table No. 6. The selection of autopsy cases was at random. The cases were divided into two groups: Group II-a comprised of cases of immediate death and without known infection or any other kind of diseases. Group II-b consists of cases with an infections process and at the terminal stage of life.
# TABLE NO. 6: SPECIMEN SELECTION TIME FOR VARIOUS AUTOPSY CASES

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (Range)</th>
<th>1 hr.</th>
<th>1 to 2</th>
<th>2 to 3</th>
<th>4 to 5</th>
<th>6 to 7</th>
<th>8 to 9</th>
<th>9 to 10</th>
<th>Total</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GROUP II A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>18 - 52</td>
<td>0</td>
<td>5</td>
<td>3</td>
<td>12</td>
<td>21</td>
<td>16</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>M</td>
<td>14 - 57</td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>17</td>
<td>14</td>
<td>11</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td><strong>GROUP II B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>16 - 55</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>6</td>
<td>13</td>
<td>28</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>M</td>
<td>21 - 49</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>9</td>
<td>8</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>
bacteremia of septicemia was observed. The details of the causes of death of these cases are given in Table No. 7.

**TABLE NO. 7: CAUSES OF DEATH OF THE CASES STUDIED**

<table>
<thead>
<tr>
<th>Causes of death</th>
<th>No. of cases studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head injury</td>
<td>59</td>
</tr>
<tr>
<td>Bullet injury</td>
<td>6</td>
</tr>
<tr>
<td>Group II-A</td>
<td></td>
</tr>
<tr>
<td>Stab injury</td>
<td>21</td>
</tr>
<tr>
<td>Accidental blockage of Airway</td>
<td>4</td>
</tr>
<tr>
<td>Strangulation (Suicidal)</td>
<td>33</td>
</tr>
<tr>
<td>Electric shock</td>
<td>9</td>
</tr>
<tr>
<td>Poisoning</td>
<td>2</td>
</tr>
<tr>
<td>Burns (Septicemia)</td>
<td>63</td>
</tr>
<tr>
<td>Group II-B</td>
<td></td>
</tr>
<tr>
<td>Wound Infections (Bacteremia or septicemia)</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>235</td>
</tr>
</tbody>
</table>

(3) **Clinical Data:**

The clinical data of patients who underwent surgery were obtained from clinical record and charts. Similarly, relevant autopsy findings were also collected from the autopsy record (MLC & Pathology).

(4) **Kinds of Specimens Collected:**

The specimens of bile, blood, gallbladder wall and gallstone (whenever detected) were collected from the various groups understudy.
(5) **Collection of Specimens and Transportation**:

(A) **Bile**:

Five to 10 ml. of bile was obtained aseptically by sterile 21 gauze needle and syringe aspiration from gallbladder at the time of operation and from the autopsy cases, at the time of necropsy, prior to any procedural manipulation, to avoid contamination. After collection of bile, air was removed from the syringe and the needle was immediately plugged with a sterile rubber cork.

(B) **Blood**:

The specimens of blood (5 to 10 c.c.) was collected aseptically by venapuncture, preoperatively and post-operatively (3rd day), from the study group I (Ia and Ib).

A direct heart puncture (with sterile 16 gauze needle) procedure was adopted, with all precaution for asepsis, for the collection of blood sample (5 to 10 c.c.) from the selected autopsy cases.

Whenever found possible, the specimen of portal blood (5 to 10 c.c.) was collected from the portal vein or its tributaries of randomly selected cases of group I (a & b). The specimen of blood collected, was immediately inoculated aseptically (10% Vol/vol) into two, 50 ml. bottles containing blood culture broth medium (Brain Heart Infusion Broth with supplements) for aerobes and anaerobes.
(C) **Gall-bladder wall**

The piece of gall-bladder wall was collected in preweighed screw-capped sterile glass bottles (coming) containing 10 ml. of prereduced T₂ buffer solution (Meynell & Meynell, 1970). The specimens were obtained from study group Ia and study group II (a & b).

(D) **Stone**

Whenever stone was obtained from the biliary tract during surgery, it was collected in a sterile preweighed screw-capped glass bottle containing 10 ml. of prereduced T₂ buffer solution. The numbers and apparent characters of stones were noted at the time of collection.

The specimens of bile, blood, gallstones and gall bladder wall were transported to the department of Pathology and Microbiology, B.J. Medical College, Ahmedabad within ten minutes to half an hour of the time of collection and processed within half an hour of the receipt of these specimens, in the following manner:

(6) **Bacteriological Methods**

(A) **Direct Gram's stain**

A direct Gram's stain of each of the bile specimen obtained from human cases, was performed.

(B) **Culture Methods**

(i) **Quantitative bacteriology**: This was performed by tenfold serial dilutions of the specimens.
(ii) Qualitative bacteriology:

(a) Aerobic bacteriology: The method of isolation and identification of aerobic bacteria was adopted from Cowan and Steel (1965) and Lennette et al. (1974).

(b) Anaerobic bacteriology:

(I) Method of anaerobiosis: Hungate's roll tube method was used (Hungate 1969).

(II) Pre-reduced anaerobically sterilised media were used.

(III) The methods of anaerobic bacterial isolation were adopted from Holdeman and Moore (1977); Dowel and Hawkins (1974) and Willis (1977). The scheme for the identification of anaerobes was as per Ellner et al. (1973); Dowel and Hawkins (1974) and Holand et al. (1977).

(7) Specimen processing:

(A) Bile:

The specimen of bile was subjected to six serial 1:10 dilution in molten (45 to 50°C), pre-reduced anaerobically sterilized Brain Heart Infusion agar (BHIA) roll-tubes, supplemented with hemin (5 ug/ml.), Vitamin K (0.5 ug/ml.), cysteine HCl (0.5 ml.), from original bile specimen was placed in molten (45 to 50°C) PRAS BHIA medium (4.5 ml.). After proper mixing, the subsequent dilutions were made.
by transferring 0.5 ml. content of the tube to another molten BHIA (4.5 ml.) medium.

Each medium containing tube was gassed with oxygen-free carbon dioxide and was rotated along its long axis on ice-plate until the agar cooled in a thin film along the walls. The sealed tubes were then incubated at 37°C for 24 to 46 hrs. The number of colonies in the tube containing between 30 and 3000 colonies was counted. Assuming that each colony is the product of a single organism, the result was reported as the number of bacteria per millilitre of original bile specimen. All tubes were observed for an additional 2 weeks to determine whether any further growth developed.

A portion (0.1 ml.) of original bile specimen and subsequent 1:10 serial dilution in sterile physiological saline, were placed and streaked on sheep blood agar (10%), chocolate agar and MacConkey's medium (oxoid) plates for the growth of aerobic and facultative anaerobic microorganisms. For the number and kind of microorganism, plates were observed after incubation aerobically at 37°C for 24 to 48 hrs. The plate of chocolate agar was incubated at 37°C in candle Jar (with 5 to 10 per cent CO₂), for 24 to 48 hrs.

(B) Gall-bladder wall and Gall-stone:

The specimen of gall-bladder wall and gall-stones
were subjected to three subsequent wash with 10 ml. of sterile pre-reduced \( T_2 \) solution, to eliminate maximum amount of bile present on the surfaces of these specimens. The exact weight of the specimen was calculated by a formula: 
\[
W = T_1 - T_2 (C + S)
\]
where \( W \) = specimen's exact weight, \( T_1 \) = total weight (I + C + S + W); C = glass containers weight with cap, \( W \) = weight of specimen, \( S \) = weight of pre-reduced salt solution & \( T_2 \) = weight of container & \( T_2 \) solution. The specimen was ground in a sterile tissue homogenizer chamber containing a 10 ml. volume of pre-reduced salt solution. The specimen was homogenised for one and half minutes, during which time, the chamber was immersed in ice water.

The homogenate was processed for qualitative and quantitative aerobic and anaerobic bacteriology as per the method described earlier. The quantitative bacteriological result was reported as the number of bacteria per gram of tissue/stone.

(C) Blood:

For the growth of anaerobic microorganisms, 50 ml. bottle containing pre-reduced anaerobically sterilized Brain Heart Infusion broth (oxoid), supplemented with hemin 0.5 \( \mu g/ml \), Vit. K\(_1\) 0.5 \( \mu g/ml \), and cysteine HCl 5 \( mg/ml \), was used. The bottle was flushed with oxygen free \( CO_2 \) and closed with sterile rubber cork before being incubated.
Plain Brain Heart Infusion broth (oxoid) was used for the growth of aerobic microorganisms.

All bottles were incubated at 37°C and examined macroscopically later on the day of receipt, once daily for 7 days, and finally on day 14 of incubation.

Routine subculture (approx. 0.1 ml.) to PRAS BHIA roll tube were performed from macroscopically positive bottles and macroscopically negative bottles after 14 days of incubation. Similarly, aerobic subculture (approx. 0.1 ml.) was done from BHIA Broth to chocolate agar, sheep blood agar and MacConkey's agar plates. The plate of chocolate agar was incubated at 37°C under 5 to 10% CO₂ concentration. The colony characteristics of anaerobes in roll tube and of aerobes and facultative anaerobes on various plating media were noted after 48 to 96 hours and 18 to 24 hours of incubation at 37°C, respectively.

(a) Procedure for pure culture and subculture of anaerobes:

Each anaerobic isolate was picked up with the help of sterile platinum were from a primary PRAS BHIA medium and was transferred to a 3 ml. volume of peptone yeast extract glucose broth (Wills 1977). The medium was incubated for 18 to 48 hours to obtain sufficient growth. After checking the purity of the broth culture, it was used as a source for the inoculation of subsequent test media and for other identification test procedures.

The preliminary group differentiation of anaerobes was made by morphological characteristics and cultural
characteristics of the isolates (Dowell & Hawkins 1974). Further identification of the anaerobes was made by biochemical characteristics, tolerance to various physical and chemical agents, and sensitivity to various antibacterial agents. The scheme for the identification of anaerobes was adopted from Holand et al (1977), Dowell & Hawkins 1974; Finegold et al, 1977; & Ellner et al, 1973.

The batteries of identification media were prepared as follows: (Willis 1977):

Fermentation media were prepared by adding filter-stereilized glucose, Mannitol, threhlose, xylose, salicin, rhamnose, arabinose, lactose or sucrose in recommended concentrations to thioglycolate medium without glucose and indicator. Media were steamed just prior to use and flushed with O₂ free CO₂ at the time of inoculation and then plugged with sterile rubber corks. Esculin hydrolysis was determined using the esculin broth recommended by the Centre for Disease Control (Dowell & Hawkins, 1974). Lecithinase and lipase activities were determined on modified egg yolk agar (Willis 1977) and incubated at 37°C in Mac Intosh anaerobic Jar with NO₂ and CO₂. Milk medium (3 ml.) for detection of acid, Gas, clot and digestion reactions was prepared by adding 0.5 g. of L-cysteine HCl to 1000 ml. of skim milk. Hydrogen peroxide degradation testing was done on BHIA with load acetate paper strip in 13 by 100 mm. tubes. Growth was exposed to air for at least
30 minutes prior to the addition of 3% $\mathrm{H}_2\mathrm{O}_2$. The indole test was performed on approx. 2 ml. sample of 48 hours incubated and Robertson cooked meat medium culture (Willis 1977). Vitamin $K_1$ and Hemin were added to all these media. In addition to these media, fermentation base was inoculated to serve as a fermentation medium control and as a pH check on test medium. The fermentation reactions were determined by the addition of 1% bromothymol blue to the carbohydrate broths after adequate growth of the isolate had been obtained (Dowell & Hawkins 1974).

All biochemical reactions were read after 18 to 48 hours of incubation at 37°C.

Sensitivity to Colistin 10 ug/ml.; Erythromycin 60 ug/ml.; Penicillin (2 ug/ml.), Kanamycin (1000 ug/ml.), Rifampin (15 ug/ml.), Vancomycin (5 ug/ml.), and Metronidazole (5 ug/ml.). Supplemented Muller-Hinton agar containing various concentration of these antibiotics, was used for the sensitivity testing (Willis, 1977).

PRAS-BHI broth containing 2% (wt/vol.) powdered oxgall (Difco) was used for bile tolerance test of the isolates.

A conventional method for the identification of the aerobic isolates was adopted from Cowan and Steel (1965) and Lennette et al (1974). The facultative anaerobes isolated were considered as aerobes in this study.
(9) Maintenance of stock cultures:

Stock cultures of anaerobes were maintained in tubes of supplemented Robertson cooked meat medium (Difco) and stored at room temperature in the dark. These cultures were transferred every 4 weeks and checked for contaminations and vitality of the culture, at the time of subculture.

Aerobic isolates were preserved by stabbing cultures in 0.7% semisolid nutrient agar. After incubating for 18 hours it was subsequently sealed with sterile paraffin and preserved at 4°C in the refrigerator. Subcultures were done every 4 weeks.
II : EXPERIMENTAL ANIMAL STUDY :

Introduction :

Anaerobic and aerobic bacteria recovered from the extra-hepatic biliary tree in most of the human biliary tract diseases, represent typical gut flora (Lykkegaard Nielsen & Justesen, 1976). The presence of intestinal polymicrobial biota in bile, favours the theory of an enterohepatic-biliary cycle of bacteria. However, this does not fit in some aspects of human biliary tract infections (Lykkegaard Nielsen & Justesen, 1976; Scott & Khan, 1967). Firstly, because, it is believed that a normal intestinal barrier does not allow the penetration of bacterium into the portal blood, and secondly, an obstructed (e.g. stricture or stone or carcinoma or pressure) bile duct does not favour the ascending travel of bacteria into the biliary tract. The previous experimental study in support of these claims furnish only indirect evidence of enterohepatic biliary cycle of bacteria. Moreover, these methods can be criticized from a methodological viewpoint.

Recently, it is strongly believed by several workers that like pyelonephritis, bacteria from blood can somehow be excreted through the liver cell into the bile capillaries or a passage of bacteria from blood through the gall-bladder wall, or through the common duct wall or through the wall of the small intrahepatic bile ducts surrounded by a plexus from the hepatic artery (Lykkegaard Nielsen & Justesen, 1976; Scott &

It is considered that if bacteria are excreted into a normal biliary tract, then they are simply passed into the intestine without giving rise to infection (Lykkegaard Nielson & Justesen, 1976). However, in biliary tract diseases, such bacteria multiply in the bile.

Many of these biliary tract invading microbes and especially anaerobes, are known to deconjugate and to dehydroxylate bile acids in human intestine. However, whether such bio-transformation of gall-bladder bile with anaerobes, in experimentally infected animals, occurs or not, is not well documented in the literature.

For these reasons, the present experimental work was undertaken to reevaluate the theory of haematogenous (systemic) route of anaerobic bacterial invasion of biliary tract and if so, whether the presence or absence of anaerobes in biliary tract have any role in the biotransformation of rabbit gallbladder bile acids.
MATERIAL AND METHODS

72 rabbits from a conventional colony (unspecified strains of white rabbits); weight 3.2 - 3.6 Kg.; (both sexes) were used. The animals were housed in individual cages with free access to water and food (hay).

(1) Inoculated bacterial strains:

One reference strain of B. fragilis-II (NCTC 8560), and one recent isolate (from biliary tract of Group-I human case) of B. fragilis, spp. fragilis-I, were used for this study.

Inocula were prepared from 18 hours culture in peptone-yeast extract glucose medium (Willis 1977) supplemented with heme (0.5 ug/ml), Vit.K. (5 ug/ml), cysteine-HCl (0.5 mg/ml) and ascitic fluid (25% final concentration). One ml. of broth culture was inoculated intravenously into an ear vein of rabbit. The viable bacterial count of the inoculum was detected by serial ten fold dilution of the broth culture and subsequently processed in PRAS EMIA roll tube. The average inoculated dose was, B. fragilis-II (NCTC-8560) 1.36x10^9 CFU/ml. and B. fragilis-I (Human strain) 2.12x10^9 CFU/ml.

(2) Grouping of Animals:

Rabbits were categorised into three experimental groups as under:

(A) Infected Groups:

Group I: Rabbits infected with B. fragilis strain-I. Total number of animals
under test were 24.
Group Ia : 12 animals were tested with their common duct ligated.
Group Ib : 12 animals were tested with unligated common bile duct.

Group II : Rabbits were infected with B. fragilis strain-II and were sub-grouped as under:
Group IIa : 12 animals with common duct ligated.
Group IIb : 12 animals with unligated common bile duct.

In each group (I & II) 24 animals were examined as under:

four ligated and four nonligated rabbits were sampled one day, four days, and seven days, respectively after inoculation, ligated animals being inoculated three days after common duct ligation.

(D) Non-infected group :

Group III : The control group of animals (n=24) were inoculated intravenously, with 1 ml. of sterile supplemented PYG medium. They were sub-grouped as under:
Group III a: 12 animals with ligated bile duct.
Group III b: 12 animals with unligated bile duct.

The sampling procedure was similar to that of infected groups of animals.

(3) Common duct ligation:

A: Anesthesia:

The rabbits were anesthetized with ether. The anesthesia was administered uniformly for each individual animal and only to the necessary extent to perform a laparotomy.

B: Operative Procedure:

The operation was performed in an closed room.
Sterile gowns, mask and gown were put on before operative procedures were done. Operation table was covered with a sterile towel.

After saving the entire abdomen, skin was disinfected thrice with 2.5 per cent iodine in 70 per cent alcohol.
Operations were performed with all antisepctic precautions and techniques. Laparotomy was performed through an upper midline incision, no later than five minutes after the initiation of anesthesia. The common bile duct was identified; one centimeter from the duodenum it was ligated twice with two sterile 3.0 silk ligatures. The abdominal wall was closed in two layers.
(4) **Sampling Procedures**:

Anaesthesia was accomplished as mentioned above and all antiseptic precautions were taken as already described. The laparotomy was performed through a right para-median incision parallel to and a good distance from the former midline incision. After clamping the cystic duct, 0.2 to 0.5 ml. of Gall-bladder bile was aspirated with a 21 G sterile needle and tuberculin syringe assembly. The air from the syringe was removed and the needle was plugged with a sterile rubber cork (00).

The empty gall-bladder was removed. A portion of G.B. wall was taken into screw capped (with rubber seal) container containing 10 ml. of prerduced I₂ buffer solution. Care was taken not to contaminate the portal vein.

The specimen of blood (0.2 – 0.5 c.c.) was taken from portal vein and also at the same time, from the ear vein, with the help of 24 gauze needle and tuberculin syringe. The blood was inoculated aseptically into prerduced anaerobically sterilized supplemented Brain heart infusion broth (25 ml.).

The specimens of bile and gall-bladder wall were processed within one hour of sample collection, as per the method described earlier.

The cultural technique and identification methods of anaerobes were similar to that described earlier.
The remainders of bile acid specimen was kept at -20°C and subsequently processed for thin-layer chromatography to detect the deconjugation of bile acids from the ligated and non-ligated, infected and non-infected experimental animals.

(5) Animals excluded from the series:

Five animals never entered the trial, since they died in the post-operative period after common duct ligation.

Two animals died of shock, after common duct ligation but before being inoculated with any microbes. None of these two animals showed the presence of any microbe in gall-bladder bile or venous blood or portal blood.

Other two animals died abdominal wound infections by Pseudomonas and Staph.aureas respectively.

One animal died on 4th operative day during the night time. The animal was discarded without any autopsy being performed.

(6) Contamination:

The presence of Staphylococcus epidermidis, Diptheroides and B.subtilis were considered to be contamination.

(7) Statistical Methods:

Chysquares two way classification tests have been employed.
III : METABOLISM OF BILE ACIDS : IN VIVO (HUMAN & RABBITS) IN VITRO STUDY

Introduction:

Anaerobic bacteria are known to play a major role in bile acid metabolism. The extent of such activities of anaerobes in man is not fully understood, partly because of inadequate anaerobic cultural methods used in previous studies and partly due to the difficulties in obtaining proper bile specimen from the unoperated human being.

In the gut, several anaerobes deconjugate bile acids, dehydroxylate them at the 7 alpha position, and modify them in various other ways (Hofmann, 1977). Such changes are necessary for the maintenance of the normal pattern of bile acids in the enterohepatic circulation to keep normal metabolic and other activities of the body.

In the normal human, biliary bile acids, are thought to be present exclusively in the conjugated form. Many anaerobes known to metabolise bile acids, may also occur in the bile of infected human biliary tract (Edlund et al 1959). There is then, possibility that the bile acids may be deconjugated by such anaerobic bacteria present in the biliary tract.

Unfortunately, the biological significance of most of the metabolites of bile acids are not known. At present, pathophysiological significance of only some of the few free bile acids are known. Dawson & Isselbacher (1960) and
others have shown that free bile acids are more toxic than conjugated. Free bile acids are strong detergents with membrane toxic properties, hence, they could have role in the pathogenesis of the development of liver parenchymal cell injury, and the development of cholecystitis in the patients with infected gall-bladder contents. Free bile acids also favour the gallstone formation (Bouchier et al 1968; Editorial 1971). It is also known that many normal biota of the intestine are inhibited by free bile acids, which is subsequently responsible for abnormal metabolism in the host and in the development of several disease states.

In view of the above facts, the present study was undertaken (1) to discover the possible presence of the free bile acids in the infected gall-bladder bile of human and experimentally infected rabbits. (2) to correlate the findings observed in human study with those of animal experimentations and (3) to specify the role of anaerobic isolates in bile acid metabolism. In vitro study was carried out to study the deconjugation of bile acids, under controlled conditions.

(1) **Analysis of human and rabbit bile to detect the presence of free bile acids:**

(A) **Bile specimens:**

After cultural inoculation, the remainder of the bile specimens obtained from human surgical cases (Group Ia & b) and from experimental animals (Group I,II
and Group III), were preserved at -20°C in deep freeze. All specimens were heated and agitated at 37°C for 5 minutes prior to application to thin-layer chromatography (TLC) plates.

(B) Bile acid standards:

The bile acids used were glucocholic, glycodeoxycholic, taurocholic, taurodeoxycholic, and deoxycholic acids (Maybridge Chemicals Co.). Their purity was checked by TLC as described below. n-butanol solution containing 1 μg/lul. of each of the above bile acids standard was used.

(C) Analytical Methods:

Bile and bile acid standards were analysed by TLC on silica gel G (E Merck, Germany) plates of 0.25 mm. thickness.

5 ml. samples of human bile (Group I, a & b) and Rabbit bile (infected and non-infected groups) were applied in 4 to 5 mm. diameter area to each of eight prescored channels on activated TLC plates.

5 ml. of standard of each of the above bile acids were also applied to each plate. The plates were heated to 60°C and were kept at that temperature for 15 minutes. After plates cooled to room temperature, they were placed in a developing tank which had been allowed to equilibrate for one hour with solvent system of iso-octane : isopropyl
ether : glacial acetic acid : isopropanol (2:1:1:1) (Gregg 1966). When the solvent front was reached to a distance of 10 cm. from the base line where the bile acids were applied, the plates were allowed to evaporate. The dried plates were sprayed with a 10 per cent ethanolic solution of phosphomolybdic acid and heated at 100°C for 20 minutes to visualize the bile acid standards and various bile acids present in the bile specimen.

(2) **In vitro study of bile acids deconjugation** :

(a) **Bacterial strains** :

The anaerobic bacterial strains tested include, recent isolates (from human biliary tract), of various anaerobic strains; viz, B. fragilis spp. species fragilis (5 strains), peptostreptococcus anaerobius (2 strains), Veillonella spp. (2 strains), C. perfringens (5 strains) and streptococcus faecalis (2 strains).

Following reference strains (obtained kindly from Prof. J.C. Collee, Department of Bacteriology, University Med. School, Edinburgh, Scotland) were also tested: Two strains of B. fragilis (NCTC 8560; NCTC 9343); one strain of B. fragilis asccharolyticus (NCTC 9337); two strains of Fusobacterium necrophorum (NCTC 10575; NCTC 10577) and one reference strain of Enter group V (NCTC 9816).

(b) **Assay Technique** :

Pure culture of anaerobic strain was obtained from roll
tube agar culture and was inoculated in 10 ml. of fluid thiol broth (Difco) supplemented with hemine (0.5 ug/ml.), Vit. K (0.5 ug/ml cysteine HCl (0.5 mg/ml.) and Ascitic fluid (20%), containing 0.2 per cent of standard bile acids (glycocholic, glycodeloxycholic, taurocholic and taurodeoxycholic acids) and 10 per cent (Vol/Vol) human gall-bladder bile (sterile). The organisms were allowed to grow at 37°C. At the end of 18 hours incubation; after shaking, 25 ml. of the broth content was taken aseptically and applied to an activated TLC plate and analysed. Simultaneously, 0.5 ml.of the alliquot of the broth culture was inoculated in 4.5 ml. of molten (45°C to 50°C) (PRAS BHA roll tube and seven serial tenfold dilutions were made in the roll tube medium, for the quantitative analysis of bacteria present in the content of the medium (after 18 hours of incubation). The number of colony forming units/ml. of the medium was counted after incubation at 37°C for 48 hours. The broth culture tubes were further incubated for a period of total of 48 hours. The analysis of bile acids and the quantitative bacteriology of the content of the broth medium, were done after 48 hours as mentioned above.

The broth culture medium and broth medium (without bacteria) with respective bile acids and human bile, were incubated similarly (i.e., 18 hrs., 48 hrs.). These were taken as controls for TLC examination.