Liver and biliary

Nucleation of cholesterol monohydrate crystals from hepatic and gall-bladder bile of patients with cholesterol gall stones

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Summary Nucleation time and cholesterol saturation index of hepatic and gall-bladder bile were measured in 16 patients with cholesterol gall stones to determine whether a gall bladder or liver defect was responsible for the rapid nucleation time of gall-bladder bile in such patients. Although hepatic bile was consistently more saturated than gall-bladder bile, the in vitro nucleation time of gall-bladder bile was more rapid. Dilution of gall-bladder bile to hepatic bile concentrations did not affect nucleation time. The results indicate that the gall bladder plays an important role in the production of the rapidly nucleating bile which is found in patients with cholesterol gall stones, and that this role is not simply concentration of bile by the gall bladder. Normal and abnormal gall-bladder biles were also compared in a larger group of patients. The view that there is a nucleation defect in cholesterol cholelithiasis which is independent of cholesterol saturation was confirmed. Subgroups of normal and gall-stone population were defined by the nucleation time and saturation index. Results suggested that solitary stones may be produced under different conditions than multiple stones. Some putative nucleating factors were examined but none was found to distinguish between normal and gall-stone bile.

Supersaturation of gall-bladder bile is a prerequisite for cholesterol gall stone formation, however, it is not the sole requirement as many normal subjects have supersaturated bile. Holan and Holzbach showed that the in vitro rate of formation of cholesterol crystals (nucleation time) in gall-bladder bile from subjects with cholesterol gall stones was more rapid than that from normal individuals. Nucleation time provided a sharper discrimination between stone formers and normal persons than the cholesterol saturation index. Sedaghat and Grundy found that gall-bladder bile from patients with stones had, or developed, cholesterol crystals within 48 hours; bile from persons without gall stones did not develop crystals, although most had supersaturated bile. Thus, nucleation of cholesterol monohydrate from bile would appear to involve nucleating conditions in addition to supersaturation.

The major purpose of this study was to discover whether the characteristic rapid nucleation of gall-bladder bile from stone patients was because of a liver defect or whether the gall bladder contributed substantially to the production of the abnormal bile. Hepatic and gall-bladder bile were gathered in patients with gall stones and nucleation time and cholesterol saturation measured. The results suggest that a gall-bladder defect contributes to the production of rapid nucleation.

Gall-bladder bile was also obtained from normal individuals and compared with bile of stone formers in order to confirm and extend previous observations. Our results were in agreement with those of Holan and Holzbach. In addition, subgroups of normal and abnormal biles were defined, certain technical aspects of the method were investigated, and the relationship between some putative nucleating agents and in vitro nucleation time was examined.

Methods

Patients (Table 1)
Gall-bladder bile was obtained from 34 patients with gall stones at the time of elective cholecystectomy (Table 1). The bile ducts were normal by operative
Nucleation time of bile

Table 1  General details

<table>
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<th>Age (yrs)</th>
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<td>8</td>
<td>14</td>
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<tr>
<td>Gall stone patients</td>
<td>12-6 (SD)</td>
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<tr>
<td>Gall stone patients</td>
<td>50-3</td>
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<td>13</td>
</tr>
</tbody>
</table>

cholangiography and the patients were otherwise healthy. Hepatic bile was also obtained in 16 of these patients. The gall bladder was functioning on oral cholecystography in those patients who had this test preoperatively (24 of 34 in the total group, and 13 of 16 in those contributing hepatic bile). In the remaining patients diagnosed by sonography, gall-bladder bile obtained at surgery was dark green in appearance and had a total lipid concentration of at least 5 gm/dl. These criteria were found to be sufficient to exclude bile from non-functioning gall bladders, in agreement with previous observations.

Gall-bladder bile was also collected at laparotomy from 22 patients whose gall bladder and bile ducts were normal by sonography. Their diagnoses were duodenal ulcer (10 patients), carcinoma of colon (five patients), lymphoma (three patients) and gastric leiomyoma, pseudocyst of pancreas, recent appendiceal abscess, and undiagnosed abdominal pain (one patient each).

There was no significant difference in sex, age, or weight, between the control and gall-stone groups (Table 1). Because there were more women in the gall-stone group and more men in the control group, however, a bias due to sex was sought in the key measurements, nucleation time and cholesterol saturation index. These were compared by sex within each group by Wilcoxon's rank test (nucleation time) or analysis of variance and F test (cholesterol saturation index). There were no significant differences in these measurements between sexes within groups. There was a non-significant tendency toward a higher cholesterol saturation index in the women in both groups. As there were more women in the stone group, this would tend to enhance rather than decrease the ability of the saturation index to discriminate between stone and control groups in our data.

Gall-bladder bile was also obtained at surgery from five patients with biliary colic and cholesterolosis of the gall bladder without gall stones.

Bile Collection

Upon opening the abdomen, bile was aspirated from the gall bladder using a sterile needle and syringe. Particular care was taken to obtain a complete aspiration of the gall bladder, in order to avoid the effects of stratification. In cholesterol gall stone patients in whom hepatic bile was also to be collected, the cystic duct was ligated as the first step after aspiration. A catheter normally used for cystic duct cholangiography was inserted into the common bile duct via the cystic duct. Hepatic bile was collected by gravity drainage until bile filled the catheter and 0-5 ml bile had been collected; this was discarded. As each subsequent 0-5-1-0 ml was collected it was immediately aspirated into a sterile syringe, until a total of 6 ml hepatic bile was obtained. The collections were performed or supervised by one of the authors. The cystic duct cholangiogram was performed after the collection.

Nucleation time analysis

After collection, bile was stored in sterile plastic syringes in the dark at 37°C with all air expressed from the syringe. Nucleation time was performed by the method of Holan and Holzbach with minor modification. Bile was maintained at 37°C throughout all manipulation and all instruments were sterile and pre-warmed to 37°C before manipulation of bile. Within one hour of collection, 6 ml bile was ultracentrifuged at 37°C in sterile polyallomer ultracentrifuge tubes for two hours at 100 000 g in a Beckman L5-50 preparative ultracentrifuge. The middle crystal-free layer was then isolated and maintained in sterile glass tubes with Teflon lined screw caps, at 37°C, in the dark, under nitrogen. An aliquot was examined immediately to confirm that no cholesterol monohydrate crystals or other particulate matter was present; the ultracentrifuged sediment was also examined. Aliquots were examined daily for the appearance of cholesterol monohydrate, calcium bilirubinate, and lecithin-cholesterol liquid crystals under plain and polarised light microscopy.

The appearance of cholesterol monohydrate crystals was the endpoint of the nucleation time determination, but in almost all cases the bile was examined for several days after nucleation. Observation was terminated at 21 days even if no crystals appeared. Bile was maintained at 37°C throughout the observation period.

Sterility of bile was confirmed in all samples by culture after ultracentrifugation and at the time of nucleation or at 21 days if nucleation had not occurred. Two samples from patients with stones grew E coli at initial culture. Five other samples became contaminated during the incubation period. Results from these samples are excluded.

To ensure that the nucleation time was not affected by the type of tube used during the incubation, a pilot study was done to compare the
nucleation time of human bile in Teflon tubes, siliconised glass tubes, and glass tubes. Nucleation times were identical in all tubes for the same bile samples. The glass test tubes were then used throughout the study.

On two occasions, enough normal gall-bladder bile was available to ultracentrifuge the bile again at the end of the first nucleation time analysis. In one case in which nucleation did not occur within 21 days, it was induced by the addition of a small number of cholesterol crystals. The original nucleation times were five and 21 days, while the second times were four and 21 days. On five occasions a bile sample was separated into four aliquots and nucleation time determined in each. In every case aliquots of bile from a single patient nucleated on the same day or on one of two consecutive days.

CHEMICAL ANALYSIS
Gall stones were obtained in every stone patient. Stones were dried, weighed, crushed, extracted in hexane and cholesterol was measured by GLC. All stones were greater than 70% cholesterol by weight.

Aliquots of bile and the isotropic phase of ultracentrifuged bile were stored at -20°C. Bile salts were measured by the 3-hydroxysteroidal dehydrogenase technique, phospholipids by the method of Bartlett and cholesterol by GLC. Cholesterol saturation was expressed as cholesterol saturation index. For measurement of non-dialysable hexoses in gall-bladder bile, 100 ul of gall-bladder bile was diluted with 250 ul distilled water. A 100 ul aliquot was dialysed for 48 hours in 8-32 dialysis tubing in a large volume of distilled water, under slow stirring. The dialysis bag was emptied and washed with distilled water and the collected volume dried down under nitrogen and then made up to 2 ml with distilled water. Four 300 ul aliquots were taken and 100 ul of a standard galactose solution was then added to two aliquots and 100 ul of distilled water added to the others. After precipitation with acid, hexoses were measured using the anthrone reaction with galactose standards. Recovery of added galactose was 92.3%±1.35 SEM. Addition of pure hexoses to bile before dialysis did not alter results, proving that dialysis was effective in removing free hexoses. No chloride was detectable in dialysed bile. Ultracentrifugation of bile did not alter the results of these measurements.

Total and unconjugated bilirubin were determined using the TLC method of Boonyapisit et al. To determine ionised and total calcium, an uncentrifuged sample of bile was filtered (pore size 0.45 μM) and analysed for ionised calcium using an automated analyser (Nova II Calcium Analyzer, Fisher Scientific), and for total calcium by atomic absorption spectrophotometry (Varian, Model 275). The ionised calcium value was corrected for the sodium concentration and activity coefficient of sodium in bile. Bilirubin and non-dialysable hexose measurements were performed on nine control and nine gall-stone bile samples. Calcium measurements were performed on four control and seven gall-stone bile samples.

STATISTICAL ANALYSES
Means between groups were compared using Student's t tests for parametric data and Wilcoxon's rank tests for non-parametric data. Non-parametric correlation was performed by Spearman rank correlation with correction for ties. All tests were two-tailed. Discriminant analysis was performed using the Statistical Analysis System (SAS) program on an IBM 3033 computer. The data was first transformed logarithmically to correct for unequal variances. Error rates were derived from the number of misclassified observations as determined by the discriminant analysis routine. These were compared by t test.

Results

COMPARISON OF HEPATIC AND GALL-BLADDER BILE IN PATIENTS WITH CHOLESTEROL GALL STONES (Table 2)
Gall-bladder bile nucleated significantly more rapidly than hepatic bile (p<0.02). Gall-bladder bile nucleated first in 13 of 16 patients and in 11 of these patients the difference in nucleation times between gall-bladder and hepatic bile was two days or more. Hepatic bile nucleated more rapidly in one patient and in two others both samples nucleated on day 1.

Gall-bladder bile was less saturated with cholesterol than hepatic bile in every patient (Table 2). Total lipid concentrations in hepatic and gall-bladder bile in the 16 patients were 2.77 gm/dl and 11.07 gm/dl respectively. The hepatic bile lipid concentrations were similar to previous studies in which they ranged from approximately 2.8 gm/dl to 4.0 gm/dl. When special precautions are taken to prevent contamination with gall-bladder bile, the total solute concentration of hepatic bile (lipids and other solutes) is between 4-6 gm/dl.

EFFECT OF DILUTION OF GALL-BLADDER BILE ON NUCLEATION TIME
In five patients, three with cholesterol gall stones and two control patients, an aliquot of ultracentrifuged gall-bladder bile was diluted with a solution containing 100 mM sodium chloride and 50...
Table 2  Comparison of gall-bladder and hepatic bile in 16 patients with cholesterol gall stones

<table>
<thead>
<tr>
<th>Patient</th>
<th>Nucleation time (d)</th>
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<th>Lipid concentration (gm/dl)</th>
<th>Nucleation time (d)</th>
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<th>Lipid concentration (gm/dl)</th>
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</table>

* Significantly different from values in gall-bladder bile (p<0.02).

mM sodium bicarbonate. The final concentration of biliary lipids was 33% of the original sample. The nucleation time of the diluted sample was determined at the same time as the original sample and was found to be identical in every case at two, one and one days in the gall-stone patients and 21 and 21 days in the controls. This dilution was chosen as 3:1 was the median gall-bladder-to-hepatic bile concentration ratio after collection of 10 of the 16 samples.

**COMPARISON OF GALL-BLADDERS BILE FROM CONTROL PATIENTS AND PERSONS WITH STONES**

The data are shown in Figs. 1a, b. Gall-bladder bile from stone patients nucleated more rapidly than that from controls 2-5 days±3-7 SD, n=34 vs 14-0 days±8-0, n=22, p<0.02). The mean cholesterol saturation index was greater in the stone patients (1-27±0-31 vs 1-01±0-21, p<0-01). There was considerable overlap in cholesterol saturation between groups, shown as a horizontal bar in the Figure. Total lipid concentration was slightly less in gall-stone bile (12-09 gm/dl±4-79 than in control bile 14-74 gm/dl±4-10, p<0-05) as in other studies.2

The ability of the variables cholesterol saturation index and nucleation time to discriminate between control and stone former populations was tested by discriminant analysis. The error rate for cholesterol

![Fig. 1a Cholesterol saturation index vs nucleation time in gall-bladder bile of control patients. The horizontal bar indicates the range of overlap in values for cholesterol saturation index in control and stone former gall-bladder bile samples. Note that values for nucleation time have bimodal distribution.](http://gut.bmj.com/content/gut.24.9.836)

![Fig. 1b Cholesterol saturation index vs nucleation time in gall-bladder bile of patients with gall stones. Symbols indicate whether patients had multiple or single stones. Horizontal bar is the same as in Fig. 1a. □=multiple stones; ○=solitary stone.](http://gut.bmj.com/content/gut.24.9.836)
The cholesterol saturation index was 28·5% (16 of 56 observations) and for nucleation time 10·7% (six of 56 observations). Combination of both variables did not reduce the error rate obtained by nucleation time alone – that is, nucleation time analysis data improve the discrimination obtained by cholesterol saturation index, but the reverse does not occur. Discrimination provided by either variable was highly significant, but that given by nucleation time was better (p<0·0001 vs p<0·0006).

The nucleation time of normal bile appeared to be bimodal (Fig. 1a). Nucleation occurred between days 2–8 (5·6 days±2·1 days) in 10 patients; in the other 12 nucleation did not occur within 21 days. The cholesterol saturation index was greater in the bile samples which nucleated (1·17±0·18) than in those which did not (0·89±0·13, p<0·01). This is a large difference in means for control gall-bladder bile. Total lipid concentration was not different in the two groups of normals.

Patients with multiple cholesterol gall stones (n=26) appeared to be different from patients who had cholesterol solitaires (n=8), Fig. 1b. No patient with multiple stones had a nucleation time of greater than three days, whereas four of eight patients with solitaires had nucleation times from four to 21 days. In three other patients with solitaires the gall-bladder bile cholesterol saturation index was high, compared with patients with multiple gall stones.

A comparison was made of results in normal individuals whose gall-bladder bile nucleated within 21 days and patients with multiple cholesterol gall stones. The cholesterol saturation index in these two subgroups was almost identical, 1·17±0·18 in normals and 1·24±0·21 in stone patients. All results in the normal patients fell within the range of results for cholesterol saturation index of patients with multiple cholesterol gall stones. The nucleation time, however, was more rapid in patients with multiple cholesterol gall stones than in normals that nucleated (1·5 days±0·6 vs 5·6±2·1, p<0·02), and only one of the normal patients had a nucleation time within the range of nucleation times (one to three days) of those with multiple cholesterol gall stones.

The Spearman rank correlation coefficient for nucleation time vs cholesterol saturation index in normals who nucleated was −0·063, (p>0·9) and −0·123 (p>0·3) in persons with multiple gall stones. Similarly the correlation coefficient between nucleation time and total lipid concentration was insignificant.

Total bilirubin concentration was significantly greater in normals than in gall-stone patients, as recently reported by Masuda. Table 3. There were no significant differences in unconjugated bilirubin concentration, per cent unconjugated bilirubin, ionised or total calcium concentrations or concentrations of non-dialysable hexoses (Table 3). Values for patients with multiple cholesterol gall stones and solitaires were similar.

**CHOLESTEROLOSIS**

The nucleation time and cholesterol saturation index of gall-bladder bile from a few patients with cholesterolosis, without gall stones are given in Table 4. Except for one patient the results were similar to normal subjects who do not nucleate. Hepatic bile was obtained in only two of these patients. Nucleation times were eight and 21 days with cholesterol saturation indices of 2·28 and 1·24 respectively. Gall-bladder nucleation time was 21 days in both patients.

**OTHER MICROSCOPIC FINDINGS**

Typical calcium bilirubinate precipitates were present at one day in every hepatic and gall-bladder

<table>
<thead>
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<th>Patient</th>
<th>Nucleation time (d)</th>
<th>Cholesterol saturation index</th>
<th>Total lipid concentration (gm/dl)</th>
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<td>AR</td>
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<td>1·22</td>
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</tr>
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<td>EH</td>
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<td>Mean</td>
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<tr>
<td>SD</td>
<td>8·9</td>
<td>0·22</td>
<td>4·25</td>
</tr>
</tbody>
</table>
Nucleation time of bile

Bile specimen except one. The precipitates were isolated in several instances and shown to be unconjugated bilirubin.

Typical lecithin-cholesterol liquid crystals (compressible Maltese cross figures) were frequently found in all groups, usually in small numbers (one to three per slide), but occasionally in larger numbers in normal gall-bladder bile, and in gall-bladder and hepatic bile of stone formers. There was no correlation between the nucleation time of cholesterol monohydrate and the time of appearance or number of liquid crystals. More than half of the gall-bladder bile samples which did not nucleate within 21 days had lecithin-cholesterol liquid crystals in bile after one day of incubation, and these persisted throughout the incubation.

Once nucleation of cholesterol monohydrate began it appeared to progress rapidly, and almost all samples showed a very large increase in crystal plates and clumping of crystal plates when viewed on the day after initial detection of cholesterol monohydrate.

The sediment obtained by ultracentrifugation on the day of collection was also examined (Table 5). All gall-stone patients had cholesterol monohydrate crystals in gall-bladder bile. One normal bile contained cholesterol crystals. Cholesterol monohydrate crystals were more common in gall-bladder bile than hepatic bile of stone formers. All sediments of normal and abnormal bile contained intact cells and cellular debris.

Discussion

Hepatic and gall-bladder bile nucleation time in patients with cholesterol gall stones

The cholesterol saturation of hepatic bile is greater than that of gall-bladder bile and consequently one would expect hepatic bile to nucleate more rapidly than gall-bladder bile. The results of this study which are opposite to this prediction indicate that an important alteration in bile occurs in the gall bladder in gall-stone patients.

Several lines of evidence make it extremely unlikely that the gall-bladder defect is simply concentration of the bile by the gall bladder. An increase in total lipid concentration would actually be expected to increase cholesterol solubility in bile, as it does in model bile solutions. Secondly, gall-bladder bile from normal individuals is more concentrated and in many instances as saturated as that from stone formers, yet it nucleates more slowly than bile from stone patients. Also, nucleation time and total lipid concentration correlate very poorly in either hepatic bile or gall-bladder bile samples.

Additional correlation analyses were also done in order to detect a relationship between nucleation time and concentration, including gall-bladder-to-hepatic bile concentration ratio vs gall-bladder nucleation time, and gall-bladder minus hepatic bile concentration vs nucleation time. None of the results suggested that the nucleation time was related to concentration of bile by the gall bladder. Finally, dilution of gall-bladder bile to one-third of initial concentration did not alter nucleation time.

It seems reasonable to conclude that bile is altered in the gall bladder by a change in composition, either the addition of a nucleating agent or deletion of an anti-nucleating agent normally present in hepatic bile. As model bile systems seem to nucleate more rapidly than native hepatic bile the latter possibility is attractive.

Sedaghat and Grundy also studied nucleation in bile samples by a different technique but all patients had common duct stones and therefore the results cannot be compared.

Nucleation of gall-bladder bile in patients with gall stones and control patients

Holan and Holzbach stated that nucleation time improved distinction of normal from stone former gall-bladder bile, a conclusion confirmed in this study, by discriminant analysis. Subgroups of normal individuals and stone patients appear to exist.

The bimodal distribution of normal gall-bladder bile into nucleating and non-nucleating groups was associated with a highly significant difference in cholesterol saturation between groups suggesting that cholesterol saturation is an important determinant of whether normal bile will nucleate in vitro under these conditions.

Holan and Holzbach did not observe the same bimodal pattern. Gall-bladder bile from some control patients did nucleate between 10-21 days, but bile incubation temperature was decreased to

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control</th>
<th>Gall-stone</th>
<th>Gall-stone</th>
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<tr>
<td>Cholesterol crystals</td>
<td>1/22</td>
<td>34/34</td>
<td>7/16</td>
</tr>
<tr>
<td>Calcium bilirubinate crystals</td>
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<td>34/34</td>
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<td>Lecithin-cholesterol liquid crystals</td>
<td>11/22</td>
<td>11/22</td>
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Table 5 Microscopic findings in bile sediment after ultracentrifugation

Table refers only to sediment obtained after ultracentrifugation on day of collection.
4°C at 10 days in their study. This would have increased the chance of nucleation in their samples after 10 days and this probably explains why our observations differ. Even Holzbach’s data may be considered bimodal since, as in our study several patients did not nucleate at all.

Nucleation time and cholesterol saturation index also appeared to distinguish patients with single or multiple gall stones. For the purposes of the questions asked in this study, separation of stone formers into two groups is important because it permits comparison of nucleation times between a clinically defined stone group (multiple stones) and a normal group whose cholesterol saturation was almost identical (normals who nucleated). The convincing separation of these groups on the basis of nucleation time also strongly supports the suggestion that there is a nucleating factor involved in the pathogenesis of cholesterol gall stones, which is independent and additional to the effect of cholesterol supersaturation.1

A summary of the picture suggested by these findings is that some normal individuals are protected from stone formation by low cholesterol saturation of bile. Others whose saturation is in the range at which stones form appear to lack the gall-bladder defect responsible for rapid nucleation. Both saturation and nucleation factors exist in patients with multiple cholesterol gall stones. Patients with solitary stones whose saturation is similar to patients with multiple stones frequently have high nucleation times. Others with low nucleation times often have very supersaturated gall-bladder bile. Several reasons why either condition might result in single stones rather than multiple stones could be advanced, but would presently be only hypothetical.

Caution is required in extrapolating data presented in this study to the in vivo state. Certainly the actual values for nucleation time in vivo may differ considerably from that in vitro and be affected by such important factors as rate and frequency of gall-bladder filling and emptying.

NUCLEATING AGENTS IN BILE

Bilirubin,22–25 mucous glycoproteins26,27 and lecithin-cholesterol liquid crystals28,29 may be nucleating agents or precursor crystals for cholesterol monohydrate. The appearance of calcium bilirubinate crystals at one day in almost every sample means that bilirubin precipitation does not determine the nucleation time. Bilirubin precipitation, however, may still be necessary for nucleation to occur at all. No support for this putative nucleating agent can be found in the measurements of unconjugated bilirubin or calcium concentrations. The method used for bilirubin analysis10 was the best method available at the time that our samples were gathered. Recently HPLC methods have appeared30 which are more accurate, but the method used was probably quite adequate for present purposes, considering the large overlap between results in the groups and the fact that the mean concentration and mean per cent of unconjugated bilirubin were actually greater in the control group.

Mucous glycoproteins are important in the prairie dog model of cholesterol gall stones.26 Purified mucin gels made from human gall-bladder mucosa and added to prairie dog hepatic bile causes precipitation of cholesterol monohydrate crystals.27 This type of mucin gel which can be seen with the microscope was not observed in our samples. Mucous glycoproteins estimated by the anthrone reaction after dialysis and acid precipitation were not greater in gall-stone gall-bladder bile in this study; in fact, the mean concentration was higher in the control group. Furthermore, there was no difference in non-dialysable hexose concentration between groups of animals who did and did not form gall stones in a study using the standard hamster model of gall stones.31 It is a formality to state that failure to find a difference between concentrations of a diffuse group of compounds does not exclude the possibility that one particular mucous glycoprotein is a critical nucleating agent.

In addition to the method used in this study, bile mucous glycoproteins have been measured by hexosamine assay32 and the Alcian blue technique after separation on a Sepharose column.26 Using the former technique discrimination of normal from stone former gall-bladder bile was equivocal.32 Results in humans using the latter technique are not available. The rationale for use of the Sepharose column is that it excludes compounds whose molecular weight is less than 1 million from the assay. While this is sensible when measuring mucous glycoproteins of intestinal origin whose molecular weight range is defined, its value in measuring bile mucous glycoproteins is not clear. As the molecular weight of bile mucous glycoproteins had not been determined, and as bile mucous glycoproteins of very large molecular weight might be degraded into smaller potentially important nucleating compounds in the biliary system we felt that determination of non-dialysable hexoses was the appropriate technique for the purposes of this initial study.

An important role for lecithin-cholesterol liquid crystals in nucleation of cholesterol has been noted in model systems.28,29 Results of this study do not support this connection. It is incorrect to consider, however, lecithin-cholesterol liquid crystals as a
single class of structures with identical composition. For example, some crystals float and others sediment upon ultracentrifugation and they are somewhat variable in appearance. Liquid crystals important in nucleation of model systems might be present and active in human bile, but not be morphologically separable from other crystals which are also present in native bile and do not stimulate nucleation. In a recent study of gall-bladder bile from 81 patients obtained by duodenal intubation liquid crystals were frequently present without cholesterol monohydrate crystals in individuals without gall-bladder disease.

CHOLESTEROLIS
Both nucleation time and saturation index were similar to results in control patients, indicating a distinct pathogenesis for this disease. Tilvis et al have recently presented interesting studies of this disease with which our data agree. The results of the small number of hepatic bile analyses are in keeping with the view that a gall-bladder defect is present in patients with stones.

TECHNICAL CONSIDERATIONS
Holzbach concluded that the ultracentrifugation technique satisfactorily removed cholesterol crystals of all sizes which would be present in a sample already containing large cholesterol crystal faces—that is, the possibility of residual microcrystals explaining the results was remote. This conclusion was supported in this study by the finding that resusp normal bile behaved similarly to the original sample. Also in three patients (one control, one patient with cholesterolosis, and a patient with a cholesterol solitaire) cholesterol crystals were present in the ultracentrifuged sediment but the isotropic phase did not nucleate within 21 days.

Culture of bile at the time of nucleation appears to be a wise precaution against including results from contaminated specimens. The type of container (glass, Teflon, siliconised glass) in which bile is incubated does not affect results of nucleation time analysis.

Finally it is unlikely that the extensive precautions to exclude dust which are required in model systems are necessary in native bile nucleation time analysis. All samples were treated identically and it seems unlikely that the distinctions between groups could have been found if contamination with dust had been important. Perhaps the difference between native bile and model bile can be explained by again proposing the presence of antinucleating factors in normal bile. Cellular material was consistently found in normal bile in this study and if the native system was as sensitive as the model system to particulate material one might expect cholesterol crystal formation to be a regular feature of normal bile.

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