Nucleation of cholesterol crystals from native bile and the effect of protein hydrolysis

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Abstract Nucleation time represents the terminal step in in vitro studies examining bile lithogenicity. Because of the concern that residual microcrystals, left after ultracentrifugation, may be responsible for the rapid nucleation time of gallbladder bile from patients with cholesterol gallstones, we have included a final filtration step. However, we found this procedure to considerably lengthen the nucleation time of abnormal biles. In view of the central importance of the nucleation assay we compared the effect of three commonly used gallbladder bile pre-treatment regimes (designed to remove endogenous crystals) on nucleation time. They were: *a*) immediate filtration of bile (0.22 μm filter); *b*) ultracentrifugation; and *c*) ultracentrifugation followed by filtration. The respective nucleation times were: *a*) 9.3 ± 3.7 days, n = 6; *b*) 2.9 ± 0.4 days, n = 10; *c*) 12.8 ± 2.3 days, n = 11. To determine whether the dramatic change in nucleation time was due to the removal of components other than seed crystals, we examined the mucus content, the total lipid composition of bile, and that of its cholesterol transport components following the different pre-treatments. No significant difference in total lipid, percentage cholesterol carried by the transport components, or their cholesterol/phospholipid ratio were found. Ultracentrifugation alone was sufficient to removal all detectable large molecular weight mucus glycoprotein. Although nucleation time of the abnormal gallbladder samples was extended in the ultracentrifuged/filtered biles, it was still significantly different ($P < 0.01$) from that of normal gallbladder biles, confirming an intrinsic difference between abnormal and normal biles, in cholesterol metastability. We also examined the effect of protein digestion on the nucleation time of native biles. Proteolysis of biliary proteins had little effect on the nucleation time of either normal gallbladder or abnormal gallbladder and common duct biles. The putative factors, if they are proteins, appear resistant to proteolytic cleavage or are of minor importance. —**Pattinson, N. R., and K. E. Willis.** Nucleation of cholesterol crystals from native bile and the effect of protein hydrolysis. *J. Lipid Res.* 1991. **32:** 215–221.

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Nucleation time (i.e., the appearance of cholesterol monohydrate crystals) represents the terminal step in in vitro studies examining bile lithogenicity (1, 2). Based on such measurements it has been determined that the formation of cholesterol crystals is not only dependent on the cholesterol saturation of bile and total lipid concentration (1–3) but also on the presence or absence of certain proteins with either pronucleating or antinucleating activity (4–12). However, a recent communication (13) has raised some doubts over when nucleation time of native biles can be considered as unequivocal. Moreover, recent work in our laboratory has pointed to yet another potential problem with this in vitro assay, in that the type of pretreatment of the native bile (designed to remove endogenous cholesterol crystals) prior to setting up the nucleation assay can also greatly influence the assays' outcome.

Because of the concern that residual microcrystals, left after ultracentrifugation, may be responsible for the rapid nucleation time of gallbladder bile from patients with cholesterol gallstones, we felt it prudent to include a final filtration step after ultracentrifugation (14). This had the added advantage of not only ensuring removal of any residual crystals, but also of maintaining sterility of the bile. In addition, previous studies (4, 7) have indicated that filtration after ultracentrifugation did not affect nucleation time. However, we have found this pretreatment regime to considerably prolong the nucleation time of abnormal gallbladder biles compared with the values reported by other groups.

In this study, therefore, we have compared the effect of three gallbladder bile pre-treatment regimes (commonly used by different laboratories) on nucleation time, and have examined whether the differences found could be explained by either the removal of mucus or changes in the lipid composition of the biliary transport components responsible for cholesterol solubilization. In addition, the effect of pronase digestion of biliary proteins on nucleation time was examined in gallbladder and hepatic biles from gallstone patients and in gallbladder biles from patients without cholesterol gallstones.

Abbreviations: CSI, cholesterol saturation index.

METHODS

Bile collection

Gallbladder bile was obtained from patients with gallstones at the time of elective cholecystectomy. Common duct bile was also obtained from a number of these patients by introduction of a catheter via the cystic duct prior to performing an operative cholangiogram.

Normal gallbladder bile was obtained from patients by needle puncture aspiration and removal of the entire content during abdominal surgery for conditions other than those affecting the liver/pancreas or biliary tree [gastrectomy (4), sigmoid colectomy (3), pyloroplasty, and vagotomy (1)]. Approval of the protocol used in this investigation was obtained from the Ethical Committee of our Institution. All patients gave written consent prior to entering the study.

The sterility of all biles (prior to processing) was assured by aerobic culture on blood plates and Robinson's cooked meat medium, and anaerobic culture on supplemented agar plates (vitamin K 10 μg/ml; haemin 5 μg/ml) using standard methods.

Nucleation time: effect of pre-treatment

To determine the rate of de novo cholesterol monohydrate crystal formation, crystal-free fractions of abnormal gallbladder biles are required. To this end a series of 11 gallbladder bile samples were subjected to the following pretreatment regimes.

a) Filtration through a sterile 0.22-μm Flowfore D microdisc filter (Sartorius, West Germany) (11, 15). Prior to filtration the bile was labeled for 30 min at 37°C with a stock micellar solution (20 μl/ml bile) containing [^{14}C]cholesterol and [^{3}H]phosphatidylcholine. The 10-ml stock solution contained 700 μmol taurocholic acid, 25 μmol egg lecithin, 75 μCi dipalmitoyl phosphatidylcholine (choline-methyl-^{3}H) (New England Nuclear, Boston, MA), and 20 μCi [4-^{14}C]cholesterol (Amersham Lab., England). Radiolabel rapidly exchanged with endogenous lipid giving identical specific activities in each lipid transport form within 30 min. However, labeling of submicroscopic and microscopic crystals would be unlikely within this time period. Because of the high mucus content of all the abnormal biles, immediate filtration of bile was very difficult. Thus, we were only able to obtain sufficient bile for nucleation studies and lipid analysis in 4 of the 11 biles studied.

b) Ultracentrifugation at 100,000 g for 2–4 h (Beckman L7 Ultracentrifuge, rotor T1 70.1; 38K) (1, 2, 4, 5, 7, 16). The variation in ultracentrifugation time was necessitated by the high mucus content of a number of bile samples. The supernatant was then isolated and mixed thoroughly prior to chemical analysis and assay of nucleation time.

c) The same procedure as in (b) but the isolated supernatant was filtered immediately after aspiration, through a sterile 0.22-μm Flowfore D microdisc filter (4, 7, 14).

After microscopic examination of the processed bile for the absence of cholesterol monohydrate crystals, aliquots were removed for lipid analysis and gel filtration. Sampling of biles for assay prior to pretreatment was impossible because of the high mucus content in all abnormal gallbladder bile samples. Bile samples (0.5-ml volume) were then incubated at 37°C in sterile 1.5-ml screw-topped tubes and examined daily under a polarizing microscope for the first appearance of cholesterol crystals. Observation of one crystal in a single day was not considered as sufficient evidence for nucleation unless the result was repeated on at least 2 consecutive days. The nucleation time was then taken as the day on which the first crystal(s) appeared.

Nucleation time: effect of pronase digestion

Biles from patients with and without cholesterol gallstones were treated as per regime *c*). Aliquots of 0.5-ml were incubated at 37°C in sterile tubes in the presence and absence of added pronase (*Streptomyces griseus*, 2.5 mg/ml double distilled H_2O; 20 μl/0.5 ml bile; 3.5 U). Hydrolysis of pronase-sensitive biliary protein is essentially complete within 24 h as determined by silver staining, after SDS-polyacrylamide gel electrophoresis under reducing conditions (17) (**Fig. 1**). That the pronase was active in all bile samples was assured by placing an aliquot of sample on X-ray film covered with 1% agarose. After a 24-h incubation at room temperature, the film was thoroughly washed. In all samples containing pronase the film backing had been digested away.

Biochemical methods

Gel filtration chromatography. Bile (0.2 ml) was chromatographed at room temperature on a Sepharose CL-4B-200 (Sigma Chemical Co., St. Louis, MO) column, 0.8 cm × 12 cm, pre-equilibrated with and eluted with phosphate-buffered saline, pH 7.4, 0.04% sodium azide, containing 6 mM taurocholate. Eluted fractions were monitored for [^{14}C]cholesterol and [^{3}H]phosphatidylcholine using a Packard Tricarb Scintillation counter. The scintillant contained 5.5 g PPO, 0.2 g POPOP, 330 ml Triton X-100, and 660 ml toluene. Color quenching and crossover of ^{14}C counts into the ^{3}H channel were corrected for by the external standard method. The composition of the bile transport components was determined from the calculated specific activity of cholesterol and phospholipid (18). Chemical analysis of the biliary components was as previously described (14).

Mucus glycoprotein assay. Human gallbladder mucus glycoprotein (in three bile samples) was determined essentially by the method of Pearson et al. (19). The following samples were examined: an aliquot of whole bile diluted 1:5 with phosphate-buffered saline containing 30 mM taurocholate; an aliquot taken after ultracentrifugation; and an aliquot of ultracentrifuged bile that had been

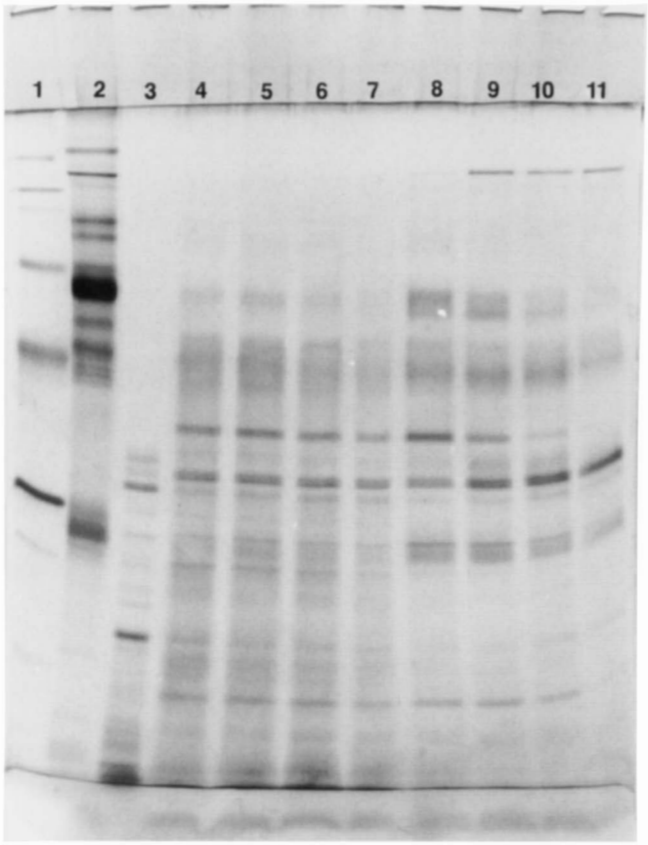

Fig. 1. Pronase digestion of bile: time dependence. Gallbladder bile was incubated with pronase (*Streptomyces griseus*) as described in Materials and Methods and subjected to SDS-polyacrylamide gel electrophoresis; lane 1, molecular weight markers (myosin, 205,000; β galactosidase, 116,000; phosphorylase B, 97,400; bovine serum albumin, 66,000; ovalbumin, 45,000; carbonic anhydrase, 29,000); lane 2, bile in the absence of pronase; lane 3, pronase (2.5 mg/ml); lane 4, digestion for 30 min; lane 5, digestion for 1 h; lane 6, digestion for 2 h; lane 7, digestion for 4 h; lane 8, digestion for 6 h; lane 9, digestion for 12 h; lane 10, digestion for 24 h; lane 11, digestion for 48 h. Each sample was diluted 1:3 in reducing buffer and 30 μl was applied per lane.

passed through a 0.22-μm microdisc filter. Samples were subjected to Sepharose CL-4B-200 gel chromatography (20 × 1.4 cm; flow rate 8.25 ml/h; fraction volume, 1.1 ml) in a column pre-equilibrated and eluted with phosphate-buffered saline containing 30 mM taurocholate. Void volume mucus glycoprotein, separated from free protein, bile acids, and pigment, was quantitated by the periodic acid-Schiff assay.

Statistics

Statistical comparisons of nucleation times were made using Survival Analysis with the Montel-Cox statistic. Comparisons assessing the influence of pre-treatments on the lipid compositions were made using paired *t*-tests or ANOVA when the composition estimates for each pre-treatment sample involved replication. CSI and g/dl levels were compared between abnormal and the non-gallstone patients, and between the abnormal gallbladder and hepatic biles using *t*-tests. After these tests, stepwise discriminant analysis was used to see whether some linear combination of the variables would better separate the normal/abnormal gallbladder biles. All means are presented with standard errors (SE).

RESULTS

Effect of bile pre-treatment of nucleation time

Pre-treatment of the supersaturated gallbladder biles greatly affected their nucleation time. Mean nucleation times for the various pre-treatment regimes were as follows: *a*) immediate filtration, 9.3 ± 3.7 days, n = 6; *b*) ultracentrifugation, 2.9 ± 0.4 days, n = 10; *c*) ultracentrifugation/filtration, 12.8 ± 2.3 days, n = 11. Nucleation time of bile given pre-treatment *b*) was very rapid as previously documented (1, 2). Pre-treatment *c*) significantly increased ($P < 0.01$) the time for cholesterol monohydrate crystal formation compared with *b*). No significant difference on nucleation time was found between pre-treatments *a*) and *c*). The difference between *a*) and *b*) just failed to reach significance.

Effect of bile pre-treatment on biliary lipid composition and mucus content

To determine whether the filtration step was removing a labile lipid component of bile, we compared the total lipid composition of the biles after each pre-treatment. No significant difference between cholesterol, phospholipid, bile acid, total lipid concentration, or cholesterol saturation index between pre-treatments was found (**Table 1**). However, since the labile entity need not necessarily be a large percentage of the whole, we tried to increase the sensitivity of our measurements by separating the various biliary cholesterol transport components by gel filtration. Gel filtration of whole bile on Sepharose CL-4B-200 separated out three cholesterol-containing fractions: a high molecular weight component in the void volume corresponding to the nonmicelle/vesicular component (found predominantly in dilute hepatic biles), and a smaller vesicular component (18) seen as a shoulder leading up to the major transport component in gallbladder bile, the mixed micelle. The percentage distribution of cholesterol between these three components for five of the gallbladder bile samples is given in **Table 2**. No difference in the percentage distribution of cholesterol resulted from the various pre-treatments, irrespective of the nonmicellar/vesicular content. The cholesterol/phospholipid ratio of the transport forms is also given in Table 2. Again, no significant differences were seen between pre-treatment regimes. Direct measurements of cholesterol, retained on the filters (after hexane extraction), revealed no

TABLE 1. Bile lipid composition

Patient	Pre-treatment	BA	PL	CH	Conc.	CSI	Nucleation Time
		mM	*mM*	*mM*	*g/dl*		*days*
AL	a	–	–	–	–	–	21
	b	–	–	–	–	–	–
	c	73.4	25.4	10.8	5.99	1.44	21
TO	a	–	–	–	–	–	–
	b	–	–	–	–	–	3
	c	–	–	–	–	–	7
RO	a	–	–	–	–		
	b	102.8	27.7	11.2	7.63	1.25	2
	c	99.7	26.8	11.5	7.42	1.29	21
CH	a	–	–	–	–	–	–
	b	96.3	33.9	14.4	7.90	1.37	6
	c	88.9	32.6	13.2	7.40	1.34	21
FN	a	88.0	29.1	16.7	7.22	1.78	2
	b	82.9	25.3	15.0	6.61	1.84	3
	c	80.9	23.4	12.4	6.26	1.67	7
BL	a	–	–	–	–	–	–
	b	94.5	61.1	24.2	10.31	1.62	3
	c	108.1	46.4	21.2	9.72	1.50	10
C1	a	95.8	26.4	12.1	7.22	1.42	21
	b	95.6	26.1	11.2	7.15	1.33	4
	c	97.7	24.3	11.3	7.12	1.40	21
RS	a	–	–	–	–	–	6
	b	151.4	37.0	13.5	10.80	1.04	2
	c	155.6	31.3	13.1	10.57	1.14	18
HO	a	65.6	36.4	15.7	6.65	1.72	3
	b	68.5	34.6	16.0	6.66	1.74	2
	c	69.0	36.0	15.3	6.77	1.64	7
CO	a	–	–	–	–	–	–
	b	119.0	27.0	10.2	8.33	1.11	2
	c	115.6	26.8	10.7	8.17	1.17	2
ED	a	86.6	20.7	11.4	6.30	1.65	3
	b	79.1	19.1	11.7	5.82	1.86	2
	c	81.2	18.7	11.4	5.88	1.85	6

The effect of bile pre-treatments (a, filtered immediately; b, ultracentrifuged-supernatant; c, ultracentrifuged-filtered supernatant) on biliary lipid composition and nucleation time; BA, bile acid; PL, phospholipid; CH, cholesterol; CSI, cholesterol saturation index.

measurable levels (phospholipid and mucin were not measured).

In addition, we examined the effects of ultracentrifugation and filtration on mucus glycoprotein concentration. Mucus glycoprotein was separated on a column of Sepahrose CL-4B-200 and measured by the periodic acid-Schiff assay with absorbance at 555 nm. High levels of absorbance were found in the void volume fraction of the column with whole bile samples (diluted 1:5) prior to pre-treatment (absorbance 0.2–0.4). No measurable void volume glycoprotein was found in ultracentrifuged samples (undiluted) or those that had been ultracentrifuged and filtered.

Nucleation time

Abnormal bile duct and gallbladder biles and gallbladder biles from non-gallstone patients were obtained and subjected to identical pre-treatments, i.e., ultracentrifugation/filtration, prior to setting up the nucleation assay.

Nucleation time, CSI, and concentration (g/dl) for the abnormal gallbladder and common duct and normal gallbladder biles are given in **Tables 3, 4, and 5**. Gallbladder bile from cholesterol patients nucleated significantly ($P < 0.01$) more rapidly than from non-gallstone patients (10.9 ± 11.4 days, n = 25 vs. 18.8 ± 1.5 days, n = 10), although there was considerable overlap. We attempted to separate the groups using a linear combination of the three variables: nucleation time, CSI, and concentration using stepwise discriminant analysis. Only one variable (nucleation time) met the entry criteria ($F > 4.0$). A cut-off point between 13–16 days on nucleation time gave a 68.8% successful classification overall; 80% nongallstone formers; 64% gallstone formers. Abnormal gallbladder bile nucleated

TABLE 2. Cholesterol solubilization

Patient		% CH			CH/PL		
		Ves	S-Ves	Mic	Ves	S-Ves	Mic
FN	b	14.0	7.9	78.2	1.43	0.79	0.52
	c	17.1	5.9	77.0	1.53	0.81	0.45
RS	b	0	6.5	93.5	–	0.61	0.36
	c	0	4.8	95.2	–	0.61	0.41
HO	a	2.4	9.7	88.0	0.74	0.72	0.40
	b	1.4	7.3	91.3	0.93	0.80	0.44
	c	1.0	9.1	89.8	0.35	0.56	0.42
CO	b	0.7	11.8	82.5	–	0.62	0.34
	c	4.5	10.8	84.7	0.86	0.57	0.38
ED	a	36.0	0	64.0	1.62	–	0.40
	b	37.1	0	62.9	1.60	–	0.45
	c	39.4	0	60.6	1.72	–	0.43

The effect of bile pre-treatment (a, filtered immediately; b, ultracentrifuged-supernatant; c, ultracentrifuged-filtered supernatant) on cholesterol solubilization by its respective transport forms separated by gel filtration chromatography (Sepharose 4B); Ves, vesicle; Mic, micelle; CH, cholesterol; PL, phospholipid; S-Ves, small vesicle.

significantly more rapidly than hepatic bile ($P < 0.01$), despite the latter having a significantly higher mean CSI (Tables 3 and 5).

Effect of pronase digestion on nucleation time

Because biliary proteins have been implicated as having important roles in both preventing and accelerating nucleation, we examined the effect of proteolysis on cholesterol monohydrate crystal formation. Although the great majority of biliary protein was hydrolyzed within 24 h, not all proteins were susceptible to pronase digestion (Fig. 1).

Proteolysis of biliary protein from nongallstone patients reduced nucleation time in only one of nine biles. Similarly, protein hydrolysis had no significant effect on the nucleation time of gallbladder or hepatic biles from gallstone patients (Tables 3, 4, and 5).

DISCUSSION

The results of this study demonstrate that the way gallbladder bile samples are handled prior to setting up nucleation studies dramatically alters the time for cholesterol monohydrate crystals to form. Although we found as did others (1, 2), that gallbladder biles of similar cholesterol saturation collected from patients with cholesterol gallstones nucleated more rapidly than those without, the magnitude of the difference was considerably reduced under our conditions such that it was impossible to completely discriminate between the two populations on this basis.

As nearly all gallbladder bile samples from patients with cholesterol gallstones already contain cholesterol crystals, it is critical to ensure their complete removal prior to incubation for new crystal growth. It is possible, however, that the filtration step rather than removing residual crystals could have removed some other critical structure, such as liquid crystals, vesicles (or their aggregates), or mucus. Vesicle aggregation has been shown to precede nucleation (16); however, we were unable to show any quantitative change in the gel filtration profile of the lipid components as a result of the various pre-treatment regimes. That the difference in in vitro nucleation time could be due to the incomplete removal of mucus glycoprotein also seems unlikely, as we found that ultracentrifugation alone was sufficient to remove all large molecular weight (void volume) periodic acid-Schiff-positive material.

Direct attempts to measure precipitated cholesterol crystals on the microdisc filters were made but we failed to find measurable levels. This may be due to our inability to measure with any accuracy small yet potentially critical quantities of lipid material. For the same reason, a role for a quantitatively small vesicular fraction cannot be totally excluded.

TABLE 3. Gallbladder bile from cholesterol gallstone patients

Patient	CSI	Conc.	Nucleation time	
			No Pronase	Added Pronase
		g/dl	*days*	
WI	0.77	11.31	10	6
HU	1.17	5.16	2	2
CR	1.85	3.30	2	2
GR	1.08	11.56	3	2
OS	1.10	7.58	7	7
SP	1.17	6.32	20	11
JO	1.12	5.44	5	5
DO	1.09	2.70	21	21
VH	1.13	11.08	8	10
CK	1.09	4.47	21	21
NI	1.57	3.38	10	3
AV	1.94	5.50	21	21
PR	1.97	5.39	5	5
BE	1.82	5.60	5	5
HD	1.50	9.71	7	7
FI	1.15	8.28	21	21
HP	1.31	5.29	17	17
MI	2.23	3.68	16	15
FN	1.84	6.61	7	5
BL	1.51	9.61	10	10
CL	1.40	7.11	21	21
RS	1.25	10.65	18	6
HO	1.64	6.77	7	7
CO	1.17	8.17	2	2
ED	1.82	5.88	6	3
Mean	1.42	6.85	10.88	9.40
± SE	0.07	0.52	1.42	1.40

Nucleation time of gallbladder bile from gallstone patients in the presence or absence of the proteolytic enzyme, pronase (*Streptomyces griseus*); CSI, cholesterol saturation index.

TABLE 4. Gallbladder bile from nongallstone patients

Patient	CSI	Conc.	Nucleation time	
			No Pronase	Added Pronase
		g/dl	*days*	
PA	1.00	11.99	21	21
DE	1.21	10.28	13	13
ME	1.09	7.30	21	21
WO	0.83	4.40	21	21
SM	1.30	5.37	21	21
LO	0.98	10.91	21	21
MO[a]	1.01	11.02	21	21
ES	1.91	5.72	7	7
MU	1.59	8.37	21	11
MC[a]	1.14	10.27	21	ND
Mean	1.21	8.56	18.80	17.44
± SE	0.10	0.86	1.53	1.85

Nucleation time of gallbladder bile from patients without cholesterol stones in the presence or absence of the proteolytic enzyme, pronase (*Streptomyces griseus*); CSI, cholesterol saturation index.
[a]Pigment stone.

Previous work by Holzbach and colleagues (6) suggested that the delayed onset of nucleation observed in normal gallbladder bile was produced by factors present in the biliary protein fraction. When bile proteins were digested with pronase and subsequently recombined with model bile, the observed extension of nucleation times seen with intact biliary protein was lost. We therefore anticipated, particularly in biles from patients without cholesterol gallstones, that pronase digestion would accelerate the nucleation time in these biles. In only one out of nine normal gallbladder biles did pronase digestion cause more rapid nucleation. Conversely, in the abnormal gallbladder samples we expected pronase digestion to extend nucleation time. A slight but nonsignificant decrease was observed. These results suggest either that protein is not an important determinant of nucleation time or that the putative factors are resistant to pronase digestion. Although the great majority of biliary protein was hydrolyzed by pronase, silver staining did reveal some intact protein bands (Fig. 1). Recent work would indicated that the pronucleating factor identified by Groen et al. (10) is indeed resistant to proteolytic digestion. On the other hand, the antinucleating factor(s) described by Holzbach et al. (6) were pronase-sensitive. A role for biliary protein as antinucleating agents would therefore appear negligible. The difference between normal and abnormal gallbladder biles with respect to their nucleating times is, therefore, likely to be due to the inactivity or absence of a pronucleating factor in the former, rather than the presence of an antinucleating factor. Although pronase digestion of biliary protein did not significantly change the nucleation time, we did observe an apparent increase in both the number and size of cholesterol crystals. Thus a role for antinucleating proteins in slowing down subsequent crystal growth cannot be excluded.

TABLE 5. Common bile duct bile from cholesterol gallstone patients

Patient	CSI	Conc.	Nucleation time	
			No Pronase	Added Pronase
		g/dl	*days*	
BL	1.42	2.74	21	18
CR	2.33	1.79	4	4
SP	1.67	3.77	21	21
JO	1.20	3.02	21	14
VH	0.92	6.35	21	21
NI	2.39	1.52	21	21
MO[a]	1.22	5.68	21	21
AV	2.36	3.15	21	21
PR	–	–	21	21
HE	1.99	2.62	4	2
BR	1.48	3.24	15	12
Mean	1.70	3.39	17.36	16.00
± SE	0.17	0.49	2.06	2.16

Nucleation time of common bile duct bile from cholesterol gallstone patients in the presence or absence of the proteolytic enzyme, pronase (*Streptomyces griseus*); CSI, cholesterol saturation index.
[a]Pigment stone.

In conclusion, our results would indicate that, depending on the pre-treatment regime chosen to remove endogenous cholesterol crystals, the outcome of the nucleation assay will vary markedly. No significant change in lipid composition or mucus content could be shown to account for this phenomenon. We suggest that only after ultracentrifugation and filtration can one absolutely exclude the possibility of residual cholesterol crystals being responsible for the very rapid nucleation seen in these abnormal biles. The results indicate a need to standardize the assay procedure. Although nucleation time of the abnormal gallbladder samples was extended in the ultracentrifuged/filtered biles, it was still significantly different from that of normal gallbladder biles, confirming an intrinsic difference between abnormal and normal biles in cholesterol metastability.

Proteolysis of biliary proteins had little effect on nucleation time of either normal or abnormal gallbladder biles. The putative pro-/anti-nucleating factors, if they are proteins, appear unaffected by proteolysis or are of minor importance.

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