*paper on methodology*

# A rapid, simple high capacity cholesterol crystal growth assay

**P. Robert C. Harvey,[1,*,†] and G. Aravinda Upadhya[*]**

Departments of Surgery* and Biochemistry and Molecular Biophysics,† Washington, University, St. Louis, MO 63110

**Abstract** Cholesterol crystal "nucleation time", more recently referred to as cholesterol crystal observation time, is defined as the first appearance of cholesterol crystals from isotropic crystal-free biles on light microscopy. This test is used to assess the potency of nucleating agents. Crystal appearance has conventionally been determined by polarizing light microscopy and crystal growth by counting the number of crystals. In this study we adapted a spectrophotometric method to a microtiter plate reader to generate cholesterol crystal growth curves. Model biles were prepared with a cholesterol saturation of 1.2 to 1.3 and total lipid concentration of 10.7 g/dl (taurocholate, 125 mM; cholesterol, 16.8–18.4 mM; phospholipid, 43 mM). Pronucleating IgM samples were used to establish and validate the assay. Cholesterol crystal growth curves were generated by reading absorption at 630 nm daily on a Dynatech microplate reader. Results were correlated to cholesterol crystal counts as determined by polarizing light microscopy. Standard curves generated from absorbencies of known masses of cholesterol crystals were used to quantify the mass of cholesterol crystals formed over the observation period. The assay was applied to known pronucleating biliary immunoglobulins. Results obtained were similar to our previous report that biliary IgM is more potent than biliary IgG. We conclude that using microplates and a microtiter plate reader provides a rapid high capacity method for detecting cholesterol crystal growth to assess potential nucleating agents in nucleation assays.—**Harvey, P. R. C., and G. A. Upadhya.** A rapid, simple high capacity cholesterol crystal growth assay. *J. Lipid Res.* 1995. **36:** 2054–2058.

**Supplementary key words** model bile • nucleation time • pronucleating proteins • biliary IgM • biliary IgG

Bile supersaturated with cholesterol is a prerequisite for cholesterol gallstone formation but does not discriminate between patients with and without gallstones. Forty to 50% of individuals without cholesterol gallstones have supersaturated bile (1–4). Cholesterol crystal "nucleation time" or the "crystal observation time" has been shown to discriminate between stone-forming bile and non-stone-forming bile (2, 4). Bile obtained from cholesterol gallstone patients has a crystal observation time of 1–2 days. In contrast, equally cholesterol-saturated bile from patients without stones is crystal-free well beyond this 1- to 2-day period (in some cases no nucleation is observed). Nucleating and antinucleating proteins have been shown to exist in bile and are now recognized to play a significant role in the formation of cholesterol crystals (5–13).

One of the difficulties in this area has been the lack of an easy, simple, and rapid method to assess cholesterol crystal formation and growth. The nucleation time or crystal appearance time test was introduced by Holan and Holzbach (2), and until recently was the only method available to detect pronucleating protein activity. This method relies on light microscopy to detect the formation of cholesterol crystals after a putative nucleating agent has been added to a model bile system. To assess crystal growth, investigators had the tedious task of examining an aliquot of bile samples by microscopy daily, each sample requiring up to 5 min to examine. Recently, a new procedure was introduced by Busch, Tokumo, and Holzbach (14) based on the detection of cholesterol crystals by spectroscopy. This method is less sensitive than the microscopic method of detecting cholesterol crystals because turbidity of a single crystal is negligible, and many crystals must be present before turbidity is detected. However, it has the advantage of ease and ability to follow crystal growth. We have modified this procedure to use microtiter plates and a microtiter plate reader. The modified procedure has the advantage in that samples can be read directly, without the need of taking aliquots for dilution.

## MATERIALS AND METHODS

### Model biles

Model biles were prepared as described by Kibe et al. (15) to obtain a cholesterol saturation index of 1.2–1.3

Abbreviation: CSI, cholesterol saturation index.

[1]To whom correspondence should be addressed.

and total lipid concentration of 10.7 g/dl. Final concentrations of the lipids were taurocholate, 125 mM; cholesterol, 16.8–18.4 mM; and phospholipid, 43 mM. Taurocholate was purchased from Calbiochem (La Jolla, CA), cholesterol from Sigma (St. Louis, MO), and phosphatidylcholine from Lipid Products (Surrey, UK). Briefly, bile salt was dissolved in methanol and mixed with cholesterol in chloroform and phospholipid in chloroform–methanol 2:1 to obtain the desired final concentrations. After a period of incubation (37°C for 4 h) to disperse the lipids, the organic solvents were evaporated under nitrogen and the residue solvent was removed by lyophilization over night. The dry lipid mixture was then reconstituted in 10 mM Tris-HCl, pH 7.4, containing 140 mM NaCl and 0.04% azide. To obtain isotropic solutions, model biles were incubated at 65–70°C for at least 5 h, cooled to 37°C, and filtered (0.2 μm filter, Corning, NY).

### Protein additions and crystal observation

Pronucleating biliary IgG and IgM were purified by affinity chromatography as previously described (7). Serum IgM was purified from a patient with Waldenstrom's macroglobulinemia and was kindly supplied by Dr. M. Schiff of the Department of Immunology, University of Toronto. Human serum albumin was obtained from Sigma Chemical Co. Biliary immunoglobulins dissolved in 10 mM Tris-HCl, 140 mM NaCl, pH 8.0, were added to sterile polystyrene microtiter plates (Corning, NY; capacity 300 μl) and the volume was brought to 20 μl before the addition of 200 μl of model bile to give a final concentration of 100–500 μg protein/ml. To prevent evaporation, peripheral wells were filled with water containing 0.02% azide (200 μl), plates were covered with parafilm, and then covered with a polystyrene lid (Corning, NY). Turbidity formed in wells was measured daily at 630 nm after a 5-sec agitation on a Dynatech microplate reader (MR5000, Dynatech Laboratories, Chantilly, VA). This reader is equipped with the ability to shake the microplate for a specific time to ensure that contents are completely mixed before the reading. Plates were maintained at 37°C throughout the observation period. Cholesterol crystal counts were performed in parallel wells by polarizing microscopy on 10-μl aliquots of sample. Once the number of crystals in a specimen was greater than 100, accurate counts could no longer be obtained due to massive overlayering and clumping of crystals.

### Statistical methods

Comparison among groups was made by one-way ANOVA. When ANOVA showed significance, multiple comparisons testing was performed by the Fisher's procedure. Significance was declared at $P < 0.05$.

## RESULTS

**Figure 1** shows microplate wells on day 9 of incubation with serum IgM at concentrations of 250 and 500 μg/ml with model bile of CSI = 1.25 and total lipid concentration of 10.7 g/dl. Turbidity is marked at both concentrations compared to control wells with no added protein. Turbidity was not due to precipitation of protein as control studies incubating IgG and IgM up to a concentration of 1000 μg/ml in model biles devoid of cholesterol resulted in absorbencies no greater than background readings throughout the incubation period (results not shown).

To correlate the microtiter plate assay with the standard crystal observation assay as determined by microscopy, two model bile solutions were prepared with CSI = 1.2 and 1.3. Serum IgM was added to these two model systems to obtain final concentrations of 250 and 500 μg/ml. Aliquots (10 μl) of parallel wells were taken for cholesterol crystal counts by polarizing light microscopy to confirm the formation of cholesterol crystals over the

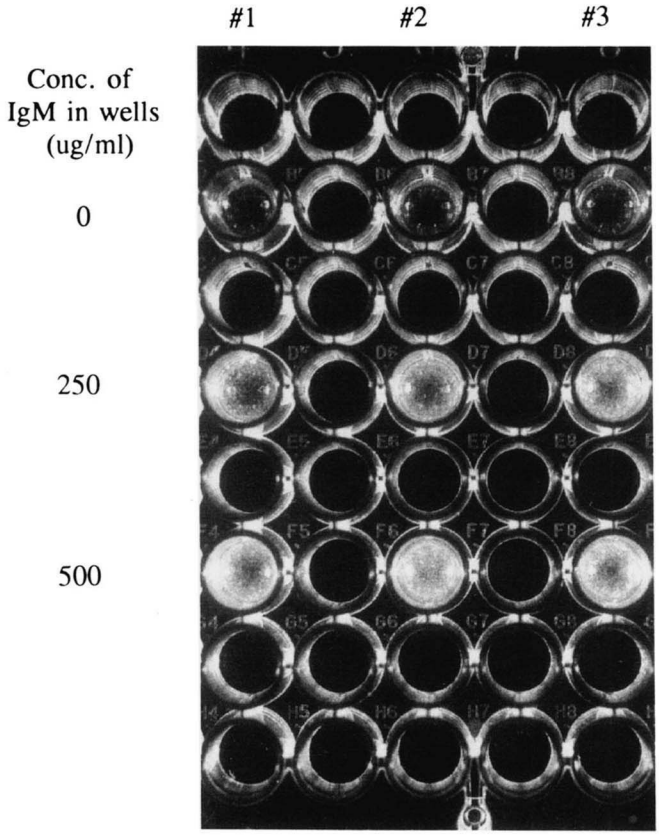


**Fig. 1.** Section of microtiter plate showing turbidity formed after 9 days of incubation of model bile with serum IgM at final concentrations of 250 and 500 μg/ml (n = 3). Turbidity is marked at both concentrations and absorbance corresponded to 0.252 ± 0.068 and 0.333 ± 0.026, respectively, for 250 μg/ml and 500 μg/ml (mean ± SD; n = 3). Note the lack of turbidity in control wells containing no protein additions. Controls with no protein added had an absorption similar to background reading on day 9 (0.046 ± 0.004). Model bile prepared with total lipid concentration equal to 10.7 g/dl and CSI = 1.25.

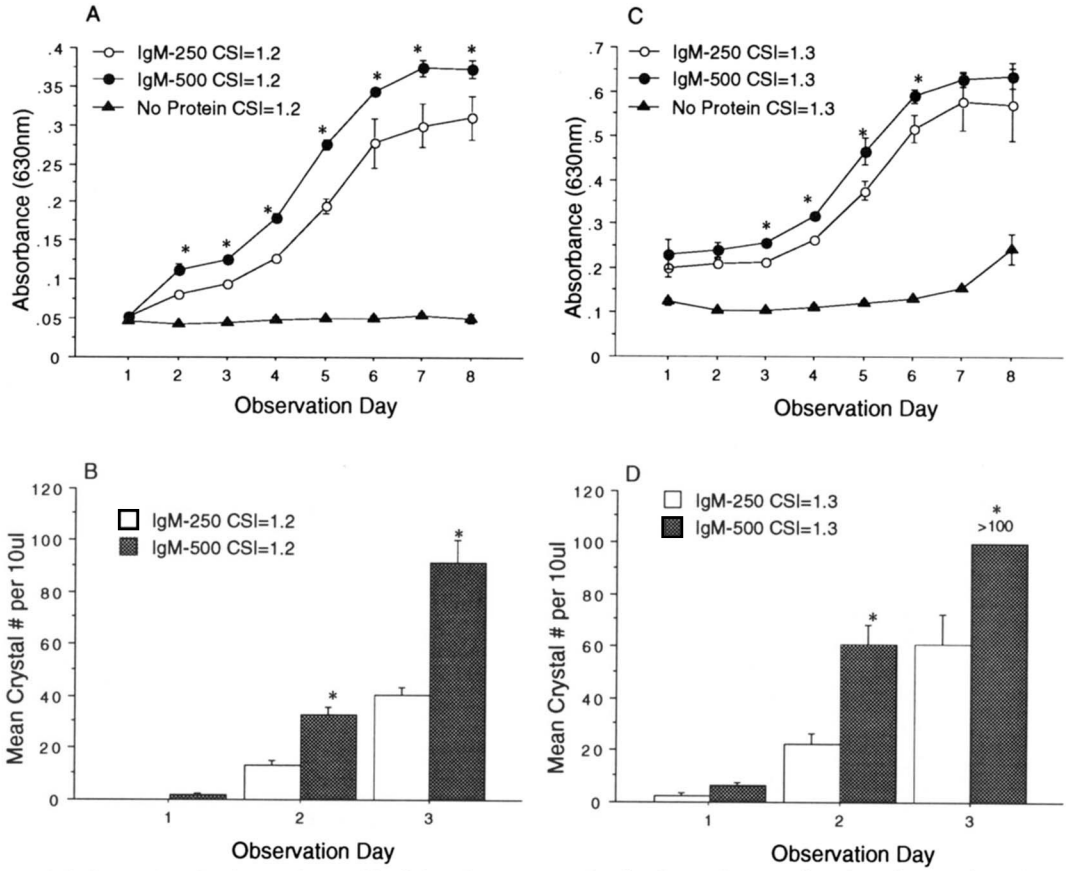


**Fig. 2.** Comparison of cholesterol nucleation as detected by light microscope to that by absorption on microtiter plate reader using serum IgM. A: Cholesterol nucleation by microtiter plate reader with model bile of CSI = 1.2. Turbidity was detected by absorbance at 630 nm of parallel wells to that used for crystal counting in Fig. 2B. Absorbance was significantly greater on days 2 to 8 for addition of IgM at a concentration of 500 μg/ml compared to the addition of 250 μg/ml (ANOVA, $P < 0.05$, indicated by asterisks). Results were similar to that found by counting cholesterol crystals. B: Cholesterol nucleation by light microscope with model bile of CSI = 1.2. Mean cholesterol crystal number per 10-μl aliquot formed over the observation period was detected under the polarizing light microscope for additions of serum IgM to model bile at final concentrations of 250 and 500 μg/ml. Number of crystals formed on days 2 and 3 was significantly greater for the 500 μg/ml addition compared to the 250 μg/ml addition (ANOVA, $P < 0.005$). For practical reasons, crystals were not counted beyond 4 days. On day 4 the number of crystals for both IgM additions was greater than 100 and therefore no statistics could be performed at this time point. Controls with no added protein formed no crystals over the observation period. C: Cholesterol nucleation by microtiter plate reader with model bile of CSI = 1.3. Model biles with higher CSI resulted in higher background readings due to interference by vesicles. Absorbance was significantly greater on days 3 to 6 for addition of IgM at a concentration of 500 μg/ml compared to the addition of 250 μg/ml (ANOVA, $P < 0.05$, indicated by asterisks). D: Cholesterol nucleation by light microscope with model bile of CSI = 1.3. Results were similar to model bile with CSI = 1.2 but significantly more crystals formed in model bile with CSI = 1.3 compared to model bile with CSI = 1.2. Crystals started to appear in control bile with no added protein on day 6. Vesicles were observed to be present from day 1.

observation period. **Figure 2A** shows the cholesterol crystal growth by microplate reader for CSI = 1.2. The mean crystal number per 10 μl of model bile with CSI = 1.2 from day 1 to day 3 for the additions of 250 and 500 μg/ml of serum IgM is shown in Fig. 2B. No crystals were observed over the observation period in controls with no protein additions. Significantly more crystals were present on days 2 and 3 with IgM at 500 μg/ml compared to IgM at a concentration of 250 μg/ml (ANOVA; $P < 0.005$). On day 4 the number of cholesterol crystals as determined by the microscope was greater than 100 for both 250 and 500 μg/ml additions and therefore no statistics could be performed at this time point. Absorbencies were significantly higher on days 2 to 8 for the 500 μg/ml addition compared to the 250 μg/ml addition (ANOVA, $P < 0.0007$ for days 2 to 5, $P < 0.05$ for days 6 to 8) (Fig. 2A). By comparing the results shown in Fig. 2A to those of Fig. 2B it can be seen that crystal number per 10-μl aliquot must reach 10–20 before an increase in absorbance is seen. Twenty crystals per 10 μl equals 400 crystals in the 200 μl model bile in the well.

Figures 2C and 2D compare the crystal growth assays as determined by crystal counts by the microscope to that by turbidity using a microtiter plate reader at a higher CSI of 1.3. Comparing the results by microtiter

plates of CSI = 1.2 to 1.3 shows that the background readings with the CSI = 1.3 are higher. High CSIs result in the formation of cloudy model bile preparations and therefore high background readings. This is due to excessive vesicles that are present with higher CSIs. Thus this assay may be inappropriate to use with model biles of high CSIs. Vesicles that are present at lower CSIs are of such a concentration as not to interfere with the assay. We found that the optimum cholesterol saturation of model biles for the cholesterol nucleation assay by the microtiter plate reader was between 1.2 and 1.25. This is an appropriate CSI to study promoters and inhibitors of cholesterol nucleation. Cholesterol saturations of 1.1 gave a poor response particularly for less potent pronucleating proteins (data not shown).

A standard curve of known mass of cholesterol crystals is shown in **Fig. 3.** Cholesterol microcrystals were prepared by sonication of recrystallized cholesterol from hot ethanol and aliquots were pipetted into microtiter wells. Results were linear up to 5 mg of cholesterol/ml. **Figure 4** shows the crystal growth curves generated by pronucleating biliary immunoglobulins IgG and IgM on undiluted original model bile samples (n=3; three different samples and model preparations with CSI = 1.2). Biliary IgG was added at a concentrations of 100 and 250 μg/ml to show the dose response of the assay. Absorbencies with additions of IgG at 250 μg/ml were significantly greater than those with addition of 100 μg/ml. Results are consistent with our previous observations that biliary IgM is a more potent pronucleating protein than biliary IgG. No crystal growth was observed for addition of albumin at a concentration of 500 μg/ml.

## DISCUSSION

"Nucleation time", or more recently referred to as "crystal observation time", is defined as the time of appearance of cholesterol crystals in a bile sample that has been initially cleared of crystals. Patients with cholesterol gallstones have rapid crystal observation times compared with equally saturated bile from persons without gallstones. An excess of pronucleating proteins in the bile of cholesterol stone patients has been proposed to explain this observation. Antinucleating proteins in non-stone-forming bile has also to be considered. Much recent research has been directed at trying to identify these pro- and anti-nucleating proteins. Typically, an isolated protein fraction from bile is added to a model bile system prepared in the laboratory by mixing together in the desired proportions bile salt, phospholipid, and cholesterol to simulate native gallbladder bile. Pronucleating activity is suggested when the protein accelerates the crystal observation time compared to model biles without protein additions. Some investigators terminate their assay after the appearance of one or two crystals as seen under the light microscope. The question remains whether this is physiologically significant as patients that form a large number of cholesterol crystals are more likely to form gallstones than those that form the occasional crystal. Therefore, cholesterol crystal growth assays have been introduced.

The original and widely used method of measuring "nucleation time" involves the use of light microscopy to detect and count crystals (2). Although sensitive, the method is very time-consuming and crystal counts cannot be accurately done once crystal number exceeds 100. Busch et al. (14) introduced a spectrophotometric method to ease the measurement of cholesterol crystal growth. The method is somewhat less sensitive than the microscopic method because many hundreds of crystals must be present before detection, but it is easier to perform on a large number of samples. The method requires the use of model biles of CSI in the 1.4 range which results in turbidity from vesicles. Each day an aliquot is taken, diluted in a taurocholate buffer 15–50 times to eliminate vesicles, and measurements are taken and corrected by considering the dilution. The present method has certain additional advantages. Because of the use of microtiter plates no handling of sample or corrections as a result of dilution are required. Readings are done quickly, efficiently, and directly on the whole sample using a microtiter plate reader. The use of model bile of lower CSI is possible thus eliminating interference from vesicular turbidity. The concentration of vesicles at the CSI used does not contribute significantly to the absorbance.

The present study is a direct comparison of cholesterol crystal growth assays performed by the microscope

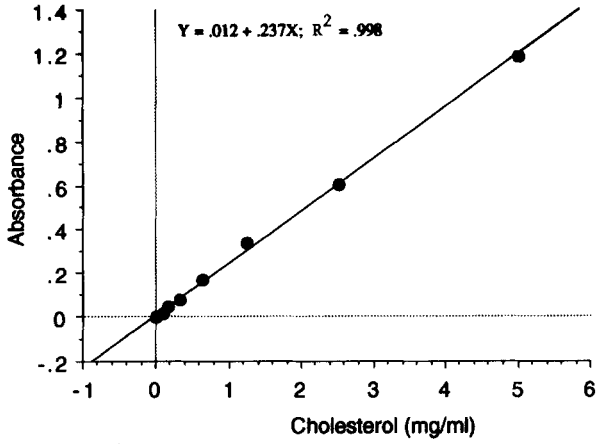


**Fig. 3.** Standard curve of cholesterol microcrystals read by the microtiter plate reader. Recrystallized cholesterol from hot ethanol was added to model bile buffer (Tris-HCl, pH = 7.4, containing 140 mM NaCl and 0.02% sodium azide) and sonicated to generate microcrystals. Aliquots (200 μl) of the cholesterol crystal suspension ranging from 0.081 to 5 mg/ml were placed into wells of the microtiter plates and read by the microtiter plate reader.

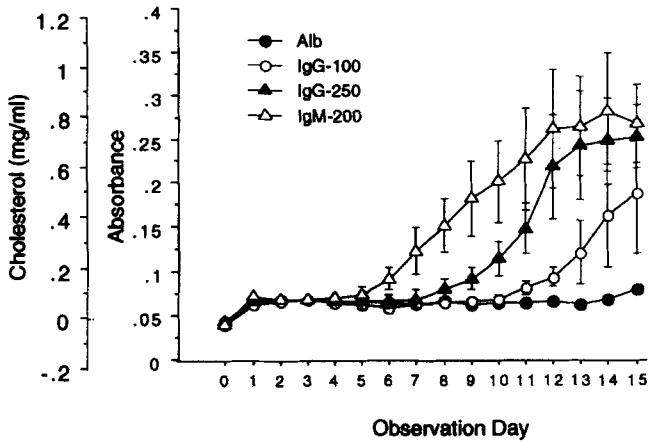


**Fig. 4.** Absorbencies of biliary immunoglobulins added to model biles with CSI = 1.2. Biliary IgG acts in a dose-response manner and biliary IgM is a more potent nucleator than biliary IgG. Serum albumin had an insignificant effect on cholesterol crystal growth.

to those determined by a spectrophotometric method. Cholesterol crystals appeared first detected by the light microscope without any change in the absorbance indicating the increased sensitivity of the microscopic method. However, both cholesterol growth assays measure late events in the overall process of cholesterol nucleation. Cholesterol nucleation is a complex process involving fusion, aggregation, or rupture of cholesterol-enriched vesicles. It is now believed that anhydrous cholesterol crystals in the form of needle, helical, and tubular microstructure can be the precursors to cholesterol monohydrate plates (16). Cholesterol crystal growth assays shed no light on these earlier events of nucleation but may represent the final common result of increased cholesterol crystal numbers.

We conclude that using microplates and a microtiter plate reader provides a rapid high capacity method for detecting cholesterol crystal growth to assess potential nucleating proteins in nucleation assays with model biles. Unfortunately, due to the presence of biliary pigments, this method cannot be utilized for nucleation assays with native gallbladder biles.

We thank Dr. Steven Strasberg for supplying the human bile samples and critical reading of the manuscript.

*Manuscript received 19 December 1994 and in revised form 1 June 1995.*

## REFERENCES

1. Holzbach, R. T., M. Marsh, M. Olszewski, and K. R. Holan. 1973. Cholesterol solubility in bile. Evidence that supersaturated bile is frequent in healthy man. *J. Clin. Invest.* **52:** 1467–1479.
2. Holan, K. R., R. T. Holzbach, R. E. Hermann, A. M. Cooperman, and W. J. Claffey. 1979. Nucleation time: a key factor in the pathogenesis of cholesterol gallstone disease. *Gastroenterology.* **77:** 611–617.
3. Sedaghat, A., and S. M. Grundy. 1980. Cholesterol crystals and the formation of cholesterol gallstones. *N. Engl. J. Med.* **302:** 1274–1277.
4. Gollish, S. H., M. J. Burnstein, R. G. Ilson, C. N. Petrunka, and S. M. Strasberg. 1983. Nucleation of cholesterol monohydrate crystals from hepatic and gall-bladder bile of patients with cholesterol gallstones. *Gut.* **24:** 836–844.
5. Levy, P. F., B. F. Smith, and J. T. LaMont. 1984. Human gallbladder mucin accelerates nucleation of cholesterol in artificial bile. *Gastroenterology.* **87:** 270–275.
6. Groen, A. K., C. Noordam, J. A. G. Drapers, P. Egbers, P. L. M. Jansen, and G. N. J. Tytgat. 1990. Isolation of a potent nucleation-promoting activity from human gallbladder bile: role in the pathogenesis of gallstone disease. *Hepatology.* **11:** 525–533.
7. Upadhya, G., P. Harvey, and S. Strasberg. 1993. Effect of biliary immunoglobulins on the nucleation of cholesterol. *J. Biol. Chem.* **268:** 5193–5200.
8. Harvey, P. R. C., G. A. Upadhya, and S. M. Strasberg. 1991. Immunoglobulins as nucleating proteins in the gallbladder bile of patients with cholesterol gallstones. *J. Biol. Chem.* **266:** 13996–14003.
9. Abei, M., H. Nuutinen, P. Kawczak, J. Schwarzendrube, S. Pillay, and R. T. Holzbach. 1994. Identification of human biliary alpha-1 glycoprotein as a cholesterol crystallization promoter. *Gastroenterology.* **106:** 231–238.
10. Núñez, L., L. Amigo, A. Rigotti, L. Puglielli, G. Mingrone, A. V. Greco, and F. Nervi. 1993. Cholesterol crystallization-promoting activity of aminopeptidase-N isolated from the vesicular carrier of biliary lipids. *FEBS Lett.* **329:** 84–88.
11. Offner, G. D., D. Gong, and N. H. Afdhal. 1994. Identification of a 130-kilodalton human biliary concanavalin A binding protein as aminopeptidase N. *Gastroenterology.* **106:** 755–762.
12. Kibe, A., R. T. Holzbach, N. F. LaRusso, and S. J. T. Mao. 1984. Inhibition of cholesterol crystal formation by apolipoproteins in supersaturated model bile. *Science.* **225:** 514–516.
13. Ohya, T., J. Schwarzendrube, N. Busch, S. Gresky, K. Chandler, A. Takabayashi, H. Igimi, K. Egami, and R. T. Holzbach. 1993. Isolation of a human biliary glycoprotein inhibitor of cholesterol crystallization. *Gastroenterology.* **104:** 527–538.
14. Busch, N., H. Tokumo, and R. T. Holzbach. 1990. A sensitive method for determination of cholesterol crystal growth using model solutions of supersaturated bile. *J. Lipid Res.* **31:** 1903–1909.
15. Kibe, A., M. A. Dudley, Z. Halpern, M. P. Lynn, A. C. Breuer, and R. T. Holzbach. 1985. Factors affecting cholesterol monohydrate crystal nucleation time in model systems of supersaturated bile. *J. Lipid Res.* **26:** 1102–1111.
16. Konikoff, F. M., D. S. Chung, J. M. Donovan, D. M. Small, and M. C. Carey. 1992. Filamentous, helical, and tubular microstructure during cholesterol crystallization from bile. Evidence that cholesterol does not nucleate classic monohydrate plates. *J. Clin. Invest.* **90:** 1155–1160.