papers on methodology

A sensitive method for determination of cholesterol growth using model solutions of supersaturated bile

N. Busch, H. Tokumo, and R. T. Holzbach[1]

GI Research Unit and Department of Gastroenterology, Cleveland Clinic Foundation, One Clinic Center, 9500 Euclid Avenue, Cleveland, OH 44195-5218

Abstract We describe a novel, facile, and highly reproducible spectrophotometric technique to measure a cholesterol crystal growth curve. This method permits a quantitative estimate of the potency or comparative abundance of a given kinetic effector substance. In addition to several internal validation procedures, data are provided that support the usefulness of the technique in the assay of substances with either known promoting activity, i.e., human mucin glycoprotein, or those with known inhibitory activity, i.e., human apolipoprotein A-I.—**Busch, N., H. Tomuko, and R. T. Holzbach.** A sensitive method for determination of cholesterol crystal growth using model solutions of supersaturated bile. *J. Lipid Res.* 1990. **31:** 1903–1909.

Supplementary key words cholesterol crystal growth • kinetic effectors • inhibiting factors • promoting factors • apolipoprotein A-I • mucin

Important steps in the development of cholesterol gallstone disease are cholesterol nucleation and crystal growth resulting from the driving force of absolute cholesterol supersaturation, i.e., cholesterol saturation indices (CSI) > 1 (1–3). Although necessary, cholesterol supersaturation is not the sole determinant of formation of cholesterol crystals in bile. This is indicated by the fact that about 50% of biles from patients without gallstones are supersaturated in cholesterol (4). A better parameter than cholesterol supersaturation, for discrimination between biles from patients with (abnormals) and without (normals) gallstones, is the time for earliest detection of cholesterol monohydrate crystal measured by sequential light microscopy, which has come to be termed a "nucleation time" (5). In the past few years, strong evidence has been reported that gallbladder bile contains several factors capable of either promoting or inhibiting the formation of cholesterol crystals (6–8). Based on the nucleation time assay, these factors were termed pro- or anti-nucleators. Among the protein factors, gallbladder mucin showed a promoting effect and apoA-I and A-II an inhibiting effect; whereas albumin showed no significant effect (9–17).

Because of its terminological implications the so-called "nucleation time" came to be widely accepted as a measure of crystal "nucleation" as the earliest event in crystal formation. It should be pointed out, however, that resolution of light microscopy only permits detection of crystal sizes greater than 0.2 μm. There can be no question that crystals this large must be the product of previously undetected nucleation and crystal growth. The so-called nucleation time, therefore, represents essentially only one point on a crystal growth curve.

Using the conventional nucleation time assay principle, efforts to quantify an effect on cholesterol crystal formation have been based on determination of the greatest dilution of an effector-containing solution at which a significant difference in the time of initial crystal appearance in a model bile compared to a control could be observed (18). Other variants using the principle of this assay have included counting the number of crystals in an aliquot of model bile at fixed time intervals of crystal growth using a hemocytometer or by determining crystal mass using a Coulter Counter (12, 17, 19). An entirely different approach was reported in which radiochemically determined crystal formation was measured after either spontaneous nucleation or addition of seed crystals (20). In preliminary studies, however, our efforts to reproduce the reported data for the radiochemical method were unsuccessful. In part, this failure could be explained by a difference in model bile components, e.g., use of soybean-derived phospholipid, instead of conventionally used egg yolk phospholipid. Whatever the reasons, in our hands the radiochemical method showed little sensitivity and an unacceptably poor signal/noise ratio.

Abbreviations: STC, sodium taurocholate; STDC, sodium taurodeoxycholate; PL, phospholipid (egg lecithin); CH, cholesterol; CSI, cholesterol saturation index; TBS, Tris-buffered saline.

[1]To whom correspondence should be addressed.

The aim of the present work was to develop and validate a quantitative cholesterol crystal growth assay in model biles. We therefore systematically evaluated and standardized conditions under which cholesterol crystal mass in various model biles could be quantitatively determined by serial photometric turbidity measurements. These data can then be used to generate a crystal growth curve using the analysis presented here. To demonstrate the usefulness and sensitivity of the new assay procedure, we also report the influence on the crystal growth curve of two well-known effectors, having opposing effects, human gall bladder mucin and apoA-I (11–15).

MATERIALS AND METHODS

Sodium salts of taurocholic acid (STC) and taurodeoxycholic acid (STDC) and apolipoprotein A-I (apoA-I) were purchased from Calbiochem (San Diego, CA). STC was twice recrystallized according to the method of Pope to achieve greater than 99% purity (20, 21). Egg lecithin (PL), grade I, was obtained from Lipid Products (S. Nutfield, Surrey, UK). Its purity was >99% by high performance liquid chromatography (22). Cholesterol (CH) (Eastman Kodak, Rochester, NY) was 99.8% pure as judged by differential scanning calorimetry. All other chemicals were ACS or reagent grade, and organic solvents were HPLC grade (Fisher Scientific Co., Pittsburgh, PA/Sigma, St. Louis, MO). Glassware was acid-washed and thoroughly rinsed with purified water prior to drying. Water was filtered, ion-exchanged, and glass-distilled (Corning Glass Works, Corning, NY). Solutions were filtered through 0.22-μm filters (Millipore Corp., Bedford, MA) and degasssed prior to use for model bile and growth assay.

Lipid analysis

Bile acid concentrations were measured enzymatically using 3α-hydroxysteroid dehydrogenase (E.C.1.1.1.50, Sigma, St. Louis, MO) (23). Lecithin concentrations were determined by the method of Bartlett (24, 25); cholesterol concentrations were assayed enzymatically based on cholesterol esterase (EC3.1.1.13) and cholesterol oxidase (EC1.1.3.6) using a commercially available assay kit (Boehringer Mannheim Corp. Indianapolis, IN) (26).

Model biles

Cholesterol saturation indices (CSI) were calculated based on Carey's critical tables (27). Concentrations of lipid stock solutions (STC in methanol, cholesterol in methanol, and phospholipid in choloform–methanol 2:1) were determined as described above, taking into account the expected dilutions caused by addition of seed crystals and effector solutions to the final model bile test solution. Appropriate aliquots of lipid stock solutions were mixed, flushed with nitrogen, and shaken at 37°C for 2 h. The mixtures were then evaporated to dryness under a stream of nitrogen, and shaken at 50°C with subsequent lyophilization for 6 h (8). The lyophilized lipid films were then resuspended in Tris-buffered saline (TBS) (25 mM Tris, 150 mM NaCl, pH 7.45) containing 3 mM NaN_3 as an antimicrobial agent. The suspension was then incubated in a G-24 Environmental Incubator/Shaker (New Brunswick Scientific, New Brunswick, NY) at 100 rpm and maintained at 55°C until microscopically isotropic. After 6 h, the clear solution was filtered through a preheated 0.22-μm Swinney-filter assembly (Millipore Corp., Bedford, MA), flushed with nitrogen, and incubated at 37°C for 15 min before use in the crystal growth assay.

Preparation of cholesterol monohydrate seed crystals

Cholesterol monohydrate crystals were prepared essentially as described by Igimi and Carey (28) with slight modifications. Five g of cholesterol was dissolved in 400 ml ethanol (95% v/v) and the solution was slowly cooled to room temperature. After 3 days large flat cholesterol crystals were harvested by filtration (0.45 μm, Millipore), washed with 100 ml H_2O, and resuspended in 40 ml 3 mM NaN_3 in water. To reduce crystal size, the suspension was sonicated six times for 60 sec each at 10-min intervals at a power output of 50 watts (Model W 140 Branson Co., Danbury, CT) keeping the sample in an ice-water bath. The suspension was then centrifuged at 1000 *g* for 5 min. The supernatant was collected, and the pellet was resuspended and again sonicated and centrifuged. The procedure was repeated until about 200 ml of supernatant was obtained. This supernatant suspension was then used for determination of a standard curve. Cholesterol crystals in the suspension were heterogeneous in size ranging to as large as 15 μm when measured by polarizing light microscopy. For preparation of seed crystals, the pooled cloudy supernatants were stored undisturbed at 4°C for 4 days. During this period, a small amount of sediment formed. The supernatant was carefully decanted and filtered through a 0.8-μm Millipore filter. The filtrate was then passed through a 0.22-μm Millipore filter and the concentrate was then resuspended in water/3 mM NaN_3, thus producing a seed crystal suspension with a crystal size ranging between 0.22 μm and 0.8 μm. The cholesterol concentration in the final seed crystal suspension was determined enzymatically after resolubilization in ethanol.

Preparation of a uniform cholesterol:phospholipid vesicle solution

Vesicle suspensions were prepared according to previously described methods (29, 30). Cholesterol (18 mM) and phospholipid (37 mM) were co-precipitated from stock solutions and dried under a stream of nitrogen at 50°C followed by lyophilization for 6 h. The residue was then resuspended in TBS, pH 7.45, shaken for 1 h at 300

rpm, 55°C, and sonicated at 37°C for 10 min in a water bath sonicator (Model 2200, Branson Co., Danbury, CT). To achieve a reduction in mean vesicular size, the suspension was filtered under pressure through a 0.22-μm Millipore filter and the cloudy filtrate was used as the vesicle stock solution for validation measurements.

Preparation of gallbladder mucin

Human gallbladder mucin was prepared from abnormal gallbladder bile obtained at cholecystectomy for cholesterol gallstone disease. After ultracentrifugation at 100,000 *g* for 1 h. (Model L5-50, Beckman Instruments, Inc., Palo Alto, CA) bile samples were chromatographed on a 2.7 × 100 cm gel filtration column (Bio-Gel A-5m, Bio-Rad Corp., Richmond, CA). Mucin eluted as the high molecular weight fraction in the void volume. It was diafiltered with 10 mM ammonium bicarbonate and concentrated using a YM 10 ultrafiltration filter (Amicon Division, Danvers, MA) and then stored at −80°C. The content of phospholipid, bile acids, and cholesterol in the mucin fraction was below the detectable range of the respective assays. Mucin concentration was measured by the periodic acid-Schiff assay using bovine submaxillary gland mucin as a standard (31).

Crystal growth assay

Before the start of each crystal growth experiment, aliquots of filtered (0.22 μm) aqueous solutions of the effectors of interest were inserted into vials equipped with Teflon-lined screw caps, lyophilized, and resolubilized in 40 μl TBS. Control vials contained only 40 μl TBS. Vials were pre-equilibrated at 37°C. Aliquots (400–500 μl) of model bile, equilibrated at 37°C, were distributed to each vial 15 min after model bile filtration. The crystal growth assay was carried out in two versions: seeded and unseeded (spontaneous). At zero time, the crystal growth of one set of test solutions was initiated by adding a small amount of seed crystals (10–25 μl, to achieve 0.5–5 μg/ml of crystalline cholesterol in the final test solution). The second unseeded set of test solutions was adjusted for the volume of seed crystals by addition of the same volume (10–25 μl) of H_2O and subsequently allowed to initiate spontaneous nucleation and crystal growth.

Samples were flushed with N_2, incubated at 37°C, and shaken at 50 rpm. To determine the crystal concentration in the reaction mixture at a specific time, an aliquot (25 μl) of the model bile mixture was sampled and diluted with a TBS/10 mM STDC solution (dilution factor 15–50). After 20 min, absorbance at a single wavelength within the visible range (400–900 nm), was measured using a DU-50 Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) equipped with a semimicro quartz cell (10 mm pathway, 2 nm width). Measurements were made at different time intervals varying between 1 and 24 h depending on the crystal growth rate of the sample.

RESULTS

Specific measurement of crystal-related turbidity

Turbidity in the visible range that develops in filtered model bile is caused by light scattered from either vesicles or cholesterol crystals. To establish that the scattering contribution from vesicles can be eliminated by their dissolution with buffer/bile acid solution ($\geq$8-fold), absorbance spectra of stock solutions of vesicles (V), crystals (C), or mixtures of vesicles + crystals (V + C) were measured at identical dilutions (8-fold) with either TBS, pH 7.45, denoted as $(\ )_T$ or TBS + 10 mM STDC, pH 7.45 denoted as $(\ )_{T/S}$. Absorbance spectra of vesicles and crystals in TBS [$(V)_T$ and $(C)_T$] did not change within 3 h after dissolution (**Fig. 1**). The data show that the absorbance spectrum of the suspension containing crystals and vesicles is the summation of the absorbance spectra of suspensions containing only vesicles or those containing only crystals at identical dilutions in TBS [$O.D.(V + C)_T = O.D.(V)_T + O.D.(C)_T$]. Dissolving vesicles with bile acids resulted in a rapid time-dependent reduction in the absorbance spectrum for this suspension [$O.D.(V)_{T/S}$]. This approximates zero absorbance for $t > 15$ min and $\lambda > 500$ mm. For the suspension containing both vesicles and crystals, the absorbance spectrum [$O.D.(V + C)_{T/S}$] decreases at a similar rate and approximates the spectrum observed for the suspension containing only crystals in TBS/STD, [$O.D.(C)_{T/S}$] when observed at 3 h after dissolution.

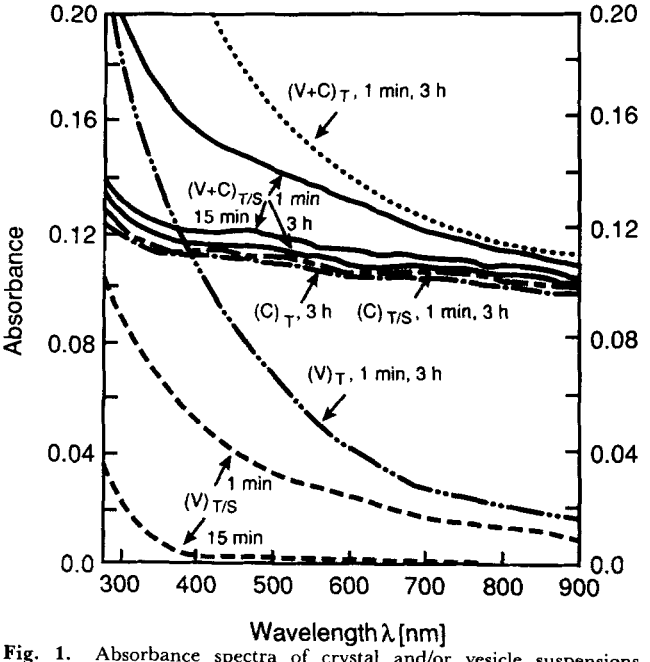

Fig. 1. Absorbance spectra of crystal and/or vesicle suspensions measured at various times after 8-fold dissolution with different solvents. A) Solvent TBS (25 mM Tris, 150 mM NaCl, pH 7.45): vesicles (-●●-) $(V)_T$; crystals (-●-) $(C)_T$; vesicles + crystals (●●●●) $(V + C)_T$; B) solvent TBS/10 mM STDC: crystals (– - –) $(C)_{T/S}$; vesicles (----) $(V)_{T/S}$; vesicles + crystals (——) $(V + C)_{T/S}$.

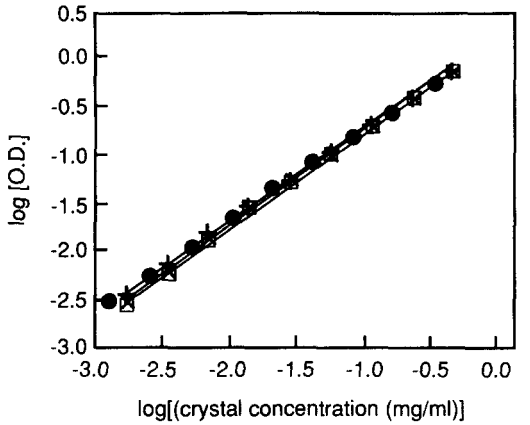

Fig. 2. Optical density of serially diluted crystal suspensions as a function of crystal concentration (mg cholesterol crystals/ml) and wavelength. Crystals from model bile: recorded at 500 nm (+), 700 nm (X), and 900 nm (□). Seed crystals recorded at 700 nm (●). Parameter of linear regression, see text.

Standard curve

Cholesterol crystal suspensions were prepared as described (seed crystals) or with crystals harvested on a 0.22-μm Millipore filter from supersaturated model bile after 1 week of incubation at 37°C. Cholesterol concentrations were measured after dissolving the crystals in ethanol. These values were in the range of 2 mg/ml. Crystal sizes after growth were up to 35 μm, as measured by polarizing light microscopy.

Suspensions were serially diluted in duplicate with TBS/10 mM STDC and the absorbance was found to be a linear function of the dilution for absorbancies < 0.9. This implies that the O. D. of these suspensions was a linear function of the crystal concentration when defined as mass of cholesterol in crystals per volume (mg cholesterol/ml). Correlation coefficients were > 0.999, and slopes were 1.77, 1.89, and 2.01 ml/mg, respectively, at λ = 900, 700, and 500 nm for suspensions of crystals harvested from the model bile. These correlations are plotted in **Fig. 2** in a double logarithmic form to enhance clarity for the relationships. In this representation, the slopes are 1.01, 1.02, and 1.00 and the Y intercepts are 0.25, 0.31, and 0.32 for λ = 900, 700, and 500 nm, respectively. Also shown in the figure is the standard curve of the other crystal suspension, prepared as described under seed crystal preparation, with absorbance measured at 700 nm. The standard curves for these two differently prepared sets of crystal suspension show nearly identical results.

Crystal growth curve

To establish the sensitivity and reproducibility of the assay, crystal growth curves were measured for three different model biles with CSI = 1.3; 1.4; 1.5 (total lipid 125 g/l, STC/PL = 4.4), prepared from identical stock solutions, with and without seeding. Mean values and standard deviations were calculated from the data of five test samples for each compositional set (n = 5). Seed crystal concentration was 1.5 μg/ml concentration crystals in each of the three different compositional sets.

All growth curves (**Fig. 3**) showed a sigmoidal pattern, describing the transition from a metastable model bile to a stable or pseudostable equilibrium state. As expected, with increasing CSI the crystal growth process begins significantly earlier and both growth rate (μg cholesterol crystal/ml · h) and final crystal concentrations are higher (**Table 1**). Seeding abolishes the influence of a nucleation event by initiating immediate crystal growth. Measured values for the final cholesterol crystal concentration are lower than the maximal theoretically possible values after spontaneous nucleation and crystal growth, and are higher in the seeded experiments. The crystal growth curve also varies with seed crystal concentration, as shown for a specific model bile (CSI 1.4, TL 125 g/l, BA/PL = 4.4) (**Fig. 4**). This indicates that, depending on the pathway of cholesterol crystal growth, different quasi-stable equilibria are reached. These equilibria are distinguishable because of the degree of reproducibility of the assay. Relaxation to the true equilibrium does not occur within the observation time. The discrepancy between observed crystal concentration in the unseeded experiment and the expected value, based on model bile parameters (27), may reflect the constraints of measurement accuracy mainly attributable to variation in model bile preparation. From a comparison of the crystal growth curves after seeding as shown in Figs. 3 and 4, it can be seen that a change in seed crystal concentration by as much as threefold yields an effect almost equivalent to a change in the CSI of a magnitude of only ± 0.1.

Effect of gallbladder mucin on cholesterol crystal growth in model bile

Gallbladder mucin was tested as an example of a crystal growth promoter. Crystal growth was measured at two

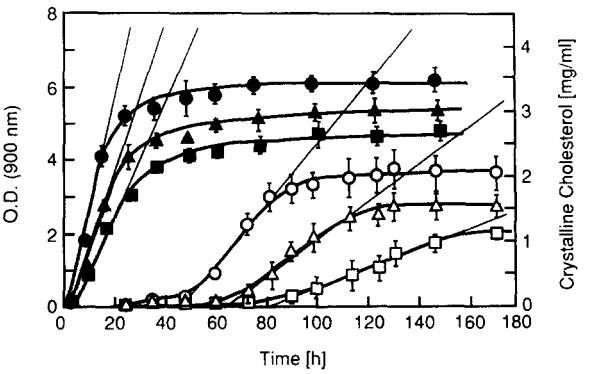

Fig. 3. Variations of cholesterol crystal growth curves in model bile (TL = 125 g/l. BA/PL = 4.4) with cholesterol saturation index (CSI) after spontaneous (open symbols) or induced crystal growth (closed symbols). Seed crystal concentration 1.5 μg/model bile. Mean values ± SD of five experiments each; (○/●) CSI = 1.5; (△/▲) CSI = 1.4; (□/■) CSI = 1.3. Derived parameters (see text).

TABLE 1. Summary of parameters derived from crystal growth curves (Fig. 3, Fig. 4)

CSI	Maximal Crystal Based on Model Bile	Seed Crystal	O.D. (900 nm)	"Measured" Crystal	Maximal Slope of Growth Curve	Extrapolated Onset Time
	mg/ml	*μg/ml*		*mg/ml*	*μg/ml·h*	*h*
1.3	1.65	0	2.01	1.14	15.5	80.6
		1.5	4.70	2.67	89.8	
1.4	2.20	0	2.76	1.57	28.6	62.1
		0.5	4.22	2.40	85.0	
		1.5	5.38	3.06	121.7	
		4.5	6.74	3.83	188.3	
1.5	2.76	0	3.61	2.05	46.3	44.6
		1.5	6.19	3.52	170.0	

different mucin concentrations (1.5 and 0.75 mg/ml) in the seeded and unseeded versions. Since model bile preparations vary slightly in composition with different batches, all results must be evaluated in relation to a control growth curve of an identical model bile prepared under identical conditions. Control curves were measured in triplicate. Upon addition of gallbladder mucin (**Fig. 5**) unseeded crystal growth begins distinctly earlier compared to control, in proportion to relative mucin concentrations, but reaches the same final values. The seeded curve also shows the same final values as the control experiment, but measurement sensitivity is insufficient to discriminate a possible growth rate inhibition. These results indicate that gallbladder mucin promotes nucleation in agreement with earlier studies (10–12, 32). In addition, however, mucin appears to retard cholesterol crystal growth rate.

Effects of apoA-I on cholesterol crystal growth in model bile

The effect of apoA-I on crystal growth was tested in model bile at concentrations of 100 and 250 μg/ml, about 3–10 times the physiological concentration in bile (16). Addition of apoA-I strikingly inhibits crystal growth rate in both seeded and unseeded curves. It also reduces the final stable levels compared to control (**Fig. 6**). In addition, the time of first detectable crystal growth is delayed in the unseeded spontaneous curve. This indicates that apoA-I not only inhibits spontaneous nucleation as reported by Kibe et al. (15), but also is a powerful inhibitor of crystal growth rate. In addition, it reduces the maximum amount of cholesterol crystals that can be formed in a specific volume of model bile.

DISCUSSION

Until only recently, we and others have experimentally measured the time-point of initial appearance of microscopically detectable cholesterol crystals from an isotropic supersaturated native or model bile solution. This was taken as an indicator of what has come to be termed a "nucleation time." But clearly this oversimplified terminology must represent combined and overlapping processes involving several events. These include formation

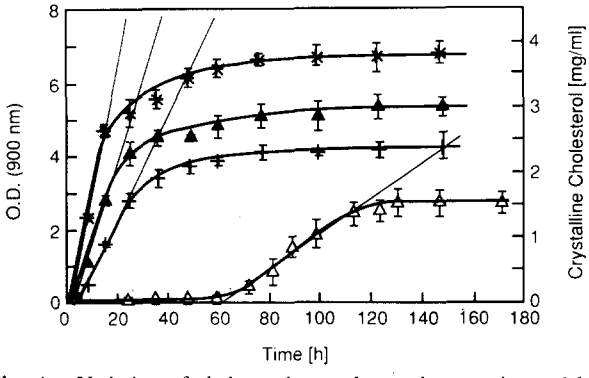

Fig. 4. Variation of cholesterol crystal growth curve in model bile (TL = 125 g/l, BA/PL = 4.4, CSI = 1.4) with seed crystal concentration. Mean values ± SD of five experiments each; (*) 4.5 μg seed crystal/ml model bile; (▲) 1.5 μg seed crystal/ml model bile; (+) 0.5 μg seed crystal/ml model bile; (△) no seeding.

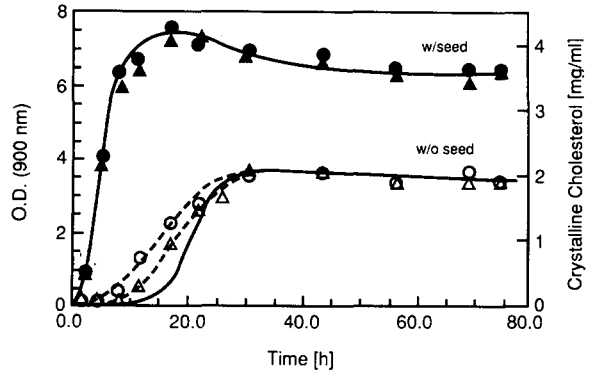

Fig. 5. Effect of gallbladder mucin on cholesterol crystal growth in model bile (CSI = 1.65, BA/PL = 4.4, TL = 100 g at two different concentrations. Control crystal growth curves are indicated by solid lines: closed symbols after seeding with 4.5 μg/ml; open symbols w/o seeding; (○/●) 1.5 mg GB-mucin/ml; (△/▲) 0.75 mg GB-mucin/ml.

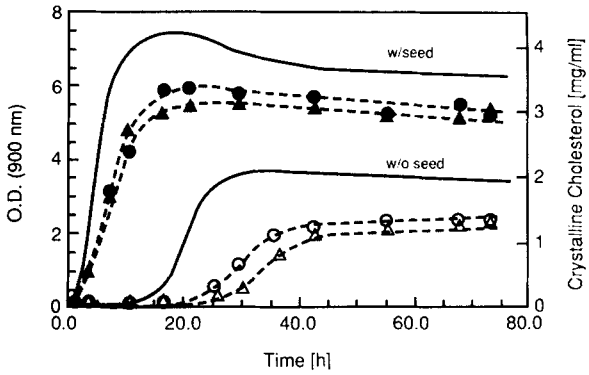

Fig. 6. Effect of apoA-I on cholesterol crystal growth in model bile (CSI = 1.65, BA/PL = 4.4, TL = 100 g/l). Control crystal growth curves are indicated by solid lines; closed symbols after seeding with 4.5 μg/ml; open symbols without seeding; (●/○) 100 μg apoA-I; (▲/△) 250 μg apoA-I/ml.

and growth of free cholesterol nuclei, formation of primitive microcrystals, and lastly, a certain amount of growth in size of these latter precipitate forms permitting them to reach the microscopically detectable range (ca. 2000 A). In addition to these limitations, the conventional nucleation time assay has proved in general to be too cumbersome, nonquantitative, and too insensitive in identifying the presence of and enrichment in protein and other putative effector substances potentially promoting or inhibiting cholesterol nucleation/crystal growth.

Compared to the commonly measured nucleation time, determination of a crystal growth curve provides information, in addition to onset time, such as crystal growth rate and maximal final crystal concentration. The nucleation time, by contrast, represents only a single point on a crystal growth curve. Therefore, description of cholesterol crystallization by means of a crystal growth curve introduces some important new components to potential effector function analysis. An effector might influence only one, or two, or all three parameters. For example, a change in net cholesterol solubility resulting from the influence of an effector would initially result in a change in the apparent cholesterol saturation index. Thus, an increase would secondarily result in promotion of both nucleation and growth rate, as well as in an increase in final crystal concentration. Moreover, comparison of spontaneous growth curves with those after induced crystal growth can reveal indirect information concerning probable effects on the nucleation event.

We used simple photometric time-dependent turbidity measurements to quantitate cholesterol crystallization in model bile. A specific reading requires, however, that crystals must be the only scattering particles in the model bile. A specific reading requires, however, that crystals must be the only scattering particles in the model bile. This was assured in our studies by dilution of the test sample with bile acid/buffer solution. Since cholesterol monohydrate crystals are known to grow in a plate-like configuration, there can, unfortunately, be no simple theoretical deduction for the relation between optical cross-section and mean crystal volume that is proportional to crystal mass. Such would be the case for more ideal, i.e., spherical, scattering particles, but not for irregular cholesterol monohydrate crystals. We have shown empirically, however, for a given mixture of crystals (stock solution) that the absorbance is a linear function of dilution, i.e., crystal mass. Furthermore, there was only modest variation with crystal suspensions prepared by different techniques.

The results obtained for two effectors having known net promoting, i.e., mucin, and inhibiting effects, i.e., apoA-I, respectively, on crystal growth demonstrate the usefulness of the described method. It appears that mucin acts predominantly as a pronucleator while apoA-I seems mainly to inhibit crystal growth rate. Method resolution does not permit a decision as to whether apoA-I might also act as an antinucleator.

In conclusion, the new assay method yields information that can be acquired from a cholesterol crystal growth experiment using model bile. It has distinct advantages, especially in commonly encountered applications where mixtures containing antagonistic effectors need to be analyzed, for example, for subfractions in the separation of single kinetic effectors present in native bile.

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REFERENCES

1. Admirand, W. H., and D. M. Small. 1968. The physicochemical basis of cholesterol gallstone formation in man. *J. Clin. Invest.* **47:** 1043–1052.
2. Carey, M. C., and D. M. Small. 1978. The physical chemistry of cholesterol solubility in bile. *J. Clin. Invest.* **61:** 998–1026.
3. Small, D. M. 1980. Cholesterol nucleation and growth in gallstone formation. *N. Engl. J. Med.* **302:** 1305–1307.
4. Holzbach, R. T., M. Marsh, M. Olszewski, and K. Holan. 1973. Cholesterol solubility in bile. Evidence that supersaturated bile is frequent in healthy man. *J. Clin. Invest.* **52:** 1467–1479.
5. Holan, K., 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.
6. Holzbach, R. T., A. Kibe, E. Thiel, J. H. Howell, M. Marsh, and R. E. Hermann. 1984. Biliary proteins: unique inhibitors of cholesterol crystal nucleation in human gallbladder bile. *J. Clin. Invest.* **73:** 35–45.
7. Burnstein, J. M., R. G. Ilson, C. N. Petrunka, and S. M. Strasberg. 1983. Evidence for a potent nucleation factor in gallbladder bile of patients with cholesterol gallstones. *Gastroenterology.* **85:** 801–807.
8. 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.
9. Gallinger, S., P. R. C. Harvey, C. N. Petrunka, R. G. Ilson, and S. M. Strasberg. 1987. Biliary proteins and the nucleation defect in cholesterol cholelithiasis. *Gastroenterology.* **92:** 867–875.
10. Lee, S. P., J. T. LaMont, and M. C. Carey. 1981. Role of gallbladder mucus hypersecretion in the evolution of cholesterol gallstones. *J. Clin. Invest.* **67:** 1712–1723.
11. Gallinger, S., R. D. Taylor, P. R. C. Harvey, and S. M. Strasberg. 1985. Effect of mucous glycoprotein on nucleation time of human bile. *Gastroenterology.* **89:** 648–658.
12. Levy, P. F., B. F. Smith, and J. T. LaMont. 1984. Human gallbladder mucin accelerates in vitro nucleation of cholesterol in artificial bile. *Gastroenterology.* **87:** 270–275.
13. Smith, B. F., and J. T. LaMont. 1986. The central issue of cholesterol gallstones. *Hepatology.* **6:** 529–531.
14. Lee, T. J., and B. F. Smith. 1989. Bovine gallbladder mucin promotes cholesterol crystal nucleation from cholesterol-transporting vesicles in supersaturated model bile. *J. Lipid Res.* **30:** 491–498.
15. Kibe, A., R. T. Holzbach, N. F. LaRusso, and S. J. Mao. 1984. Inhibition of cholesterol crystal formation of apolipoproteins A-I and A-II in model systems of supersaturated bile: implications for gallstone pathogenesis in man. *Science.* **225:** 514–516.
16. Sewell, R. B., S. J. Mao, T. Kawamoto, and N. F. LaRusso. 1983. Apoproteins of high, low, and very low density lipoproteins in human bile. *J. Lipid Res.* **24:** 391–401.
17. Whiting, M. J., and J. McK. Watts. 1985. Cholesterol gallstone pathogenesis: a study of potential nucleating agents for cholesterol crystal formation in bile. *Clin. Sci.* **68:** 589–596.
18. Drapers, J. A. G., A. K. Groen, J. P. J. Stout, F. J. Hoek, P. L. M. Jansen, and G. N. J. Tytgat. 1987. Quantification of cholesterol nucleation influencing activity in human gallbladder bile. *Clin. Chim. Acta.* **165:** 295–302.
19. Toor, E. N., D. F. Evans, and E. L. Cussler. 1978. Cholesterol monohydrate growth in model bile solutions. *Proc. Natl. Acad. Sci. USA.* **75:** 6230–6234.
20. Whiting, M. J., and J. McK. Watts. 1983. Cholesterol crystal formation and growth in model bile solutions. *J. Lipid Res.* **24:** 861–868.
21. Pope. J. L. 1967. Crystallization of sodium taurocholate. *J. Lipid Res.* **8:** 146–147.
22. Patton, G. M., S. J. Robins, J. M. Fasulo, and S. Bennett Clark. 1985. Influence of lecithin acyl chain composition on the kinetics of exchange between chylomicrons and high density lipoproteins. *J. Lipid Res.* **26:** 1285–1293.
23. Turley, S. D., and J. M. Dietschy. 1978. Re-evaluation of the 3α-hydroxysteroid dehydrogenase assay for total bile acids in bile. *J. Lipid Res.* **19:** 924–928.
24. Fiske, C. H., and Y. SubbaRow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66:** 375–400.
25. Bartlett, G. R. 1959. Phosphorus assay by column chromatography. *J. Biol. Chem.* **234:** 466–468.
26. Allain, C. C., L. S. Poon, C. S. Chan, W. Richmond, and P. C. Fu. 1974. Enzymatic determination of total serum cholesterol. *J. Clin. Chem.* **20:** 470–475.
27. Carey, M. C. 1978. Critical tables for calculating the cholesterol saturation of native bile. *J. Lipid Res.* **19:** 945–955.
28. Igimi, H., and M. C. Carey. 1981. Cholesterol gallstone dissolution in bile: dissolution kinetics of crystalline (anhydrate and monohydrate) cholesterol with chenodeoxycholate ursodeoxycholate, and their glycine and taurine conjugates. *J. Lipid Res.* **22:** 254–270.
29. Mabrey, S., P. L. Mateo, and J. M. Sturtevant. 1978. High-sensitivity scanning calorimetric study of mixtures of cholesterol with dimyristoyl- and dipalmitoylphosphatidylcholines. *Biochemistry.* **17:** 2464–2468.
30. Hamilton, R. L., Jr., J. Goerke, L. S. S. Guo, M. C. Williams, and R. J. Havel. 1980. Unilamellar liposomes made with the French pressure cell: a simple preparative and semiquantitative technique. *J. Lipid Res.* **21:** 981–992.
31. Mantle, M., and A. Allen. 1978. A colorimetric assay for glycoproteins based on the periodic acid/Schiff stain. *Biochem. Soc. Trans.* **6:** 607–609.
32. Smith, B. F. 1987. Human gallbladder mucin binds biliary lipids and promotes cholesterol crystal nucleation in model bile. *J. Lipid Res.* **28:** 1088–1097.