paper on methodology

A new method for the rapid measurement of cholesterol crystallization in model biles using a spectrophotometric microplate reader

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Abstract Measurements of the cholesterol crystal observation time, and particularly the crystal growth rate in model biles, are important in biliary pathophysiology. The aim of this study was to develop a semi-automated method permitting multiple, simultaneous, and precise measurements of the crystal growth rate in model biles. Incubated model biles were mixed with a high concentration of NaTDC to solubilize noncrystalline turbidity and spectrophotometric measurements were performed. In parallel, samples were observed by light microscopy. The absorbance correlated linearly with the crystal mass and permitted quantitation of the crystal growth rate. Polarized light microscopy was more sensitive than spectrophotometry for determining the initial crystal observation time, while spectrophotometry was more precise and quantitative for measuring the crystal growth rate.—**Sömjen, G. J., Y. Ringel, F. M. Konikoff, R. Rosenberg, and T. Gilat.** A new method for the rapid measurement of cholesterol crystallization in model biles using a spectrophotometric microplate reader. *J. Lipid Res.* 1997. **38:** 1048–1052.

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The crystal observation time (nucleation time) is presently the test of choice to discriminate between biles with and without cholesterol gallstones, as well as to characterize pro- and anti-nucleating factors and the propensity of biles to form crystals (1–4). The microscopic observation as performed today is subjective and permits only a rough estimate of the crystal growth rate. In view of the above, methods have been developed to measure the rate of crystal growth using spectrophotometric and ultracentrifugal methods (4–6). All these methods are labor intensive. The ultracentrifugal method (6), however, provides a more precise quantitation of crystal mass and allows the analysis of native biles.

The purpose of this study was to develop and validate a rapid and semiautomated method for the simultaneous measurement of cholesterol crystallization dynamics in multiple model bile samples. The data were presented previously (7).

METHODS

Materials

Cholesterol (Sigma, St. Louis, MO) was twice recrystallized from hot ethanol; Na-taurocholate (Sigma) was twice recrystallized from ethanol and ether (8); egg phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) was used without further purification. All lipids used in this study were pure by TLC standard. The composition of the three models used in this study is shown in **Table 1.** The lipids were dissolved in $CHCl_3/CH_3OH$ 2:1 (v/v), dried under N_2 at room temperature, lyophilized overnight, and kept at −20°C under argon. Models were prepared by suspending the dried lipids in 150 mM NaCl, 1.5 mM disodium EDTA, 50 mM Tris-HCl, pH 8.0 (NaTE), and incubating the suspension at 55°C for

Abbreviations: NaTC, sodium taurocholate; NaTDC, sodium taurodeoxycholate; E-PC, egg phosphatidylcholine; CSI, cholesterol saturation index; NaTE, sodium Tris EDTA buffer; COT, crystal observation time; CGR, crystal growth rate.

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TABLE 1. Composition of the model biles

Model	Cholesterol	E-PC	NaTC	TLC	CSI
		mM		*g/dl*	
I	18.5	33.6	120	9.2	160
II	15	30	150	10.3	134
III	10	20	100	6.8	146

E-PC, egg phosphatidylcholine; NaTC, sodium taurocholate; TLC, total lipid concentration; CSI, cholesterol saturation index.

1 h. Models were kept at 37°C under argon for over 12 days.

The two cholesterol crystal dispersions were prepared by incubating *a*) 30 mM cholesterol with 70 mM E-PC and 300 mM NaTC or *b*) 20 mM cholesterol, 30 mM E-PC, and 100 mM NaTC. After 2 months, crystals were harvested, washed, and suspended in NaTE buffer.

Crystal observation time (COT)

The COT of the model biles was determined by polarized light microscopy as described by Holan et al. (1). COT was defined as the time of first detection of at least three cholesterol crystals per microscopic field (100-fold magnification).

Measurement of crystal growth rate (CGR)

Aliquots (50 µl in triplicate) of models were mixed vigorously with equivalent volumes of 200 mM NaTDC in microplate wells. After 60 min at room temperature, they were shaken again in a spectrophotometric microplate reader (SPECTRA–STL, Austria) and the absorbance was measured (in duplicate). The slope of the curve of the daily absorbance measurements was determined by a linear regression fit to at least three measurements in the steepest region and defined as the CGR.

Chemical analysis

On the last day of the experiments, the samples were collected from the microwells, centrifuged in an Airfuge (Beckman) at 70000 rpm for 5 min, and supernatant solutions were separated. Chemical analyses of cholesterol and E-PC were performed (9) on the total samples (before centrifugation) as well as on the supernatant solutions (calculated recovery from E-PC was 75 to 105%). The amount of cholesterol in the pellets was calculated by subtracting the amounts in the supernatant solutions from the total. The crystalline character of the pellet was confirmed by polarized light microscopy.

Statistical analysis

Each lipid dispersion was prepared in triplicate and duplicate aliquots were measured from all samples. Mean values of optical density and standard errors were calculated. CGRs were calculated from linear regression analyses of the curves.

RESULTS

The spectrophotometric microplate reader performed 40 measurements of each sample along a diameter of each well with the light source on the bottom and the detector on the top. The high absorbance measured in the first and the last ten channels was probably due to light diffraction caused by the liquid meniscus. In order to avoid this artifact, only the ten central channels, which produced very similar results, were selected for calculation of the average OD of the sample in each well.

The kinetics of dissolution of turbidity after the addition of 200 mM NaTDC, caused by non-crystalline lipid particles such as uni- and multilamellar vesicles and stacked lamellae, are shown in **Fig. 1.** It is apparent that the OD not due to cholesterol crystals changed rapidly and a stable state was achieved within 20 min. In further studies, absorbance was routinely measured 60 min after addition of NaTDC. The OD of model I (Table 1) dispersions before and 60 min after mixing with 200 mM NaTDC was measured at 405, 620, and 750 nm. The difference in OD before and after dissolution was 73.2% ± 0.02 at 620 nm and 65.6% ± 0.01 at 750 nm as compared to the difference measured at 405 nm. Therefore, this latter wavelength was used in all subsequent experiments.

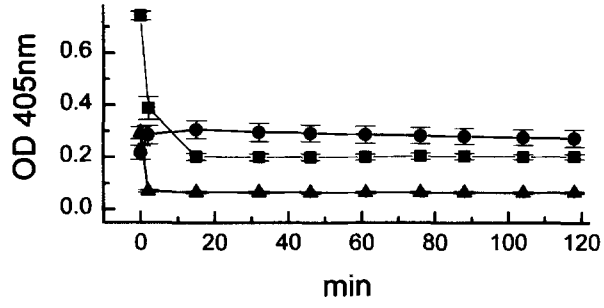

Fig. 1. Absorbance kinetics of the different model dispersions (Table 1). Model I (■), Model II (●) and Model III (▲). Measurements were performed for 120 min after the addition of 200 mM NaTDC. The symbols represent the means of six measurements; the bars represent the SEM.

TABLE 2. Crystallization properties of model biles

Model	COT	Crystal Growth Rate		
		Slope	R	P
	days			
I	1	0.053 ± 0.008	0.998	0.0449
II	1	0.095 ± 0.040	0.956	0.1889
III	4	0.028 ± 0.003	0.999	0.0268

COT, crystal observation time (nucleation time) was determined by microscopy. Crystal growth rate was determined by spectrophotometry, OD/day. R is the correlation coefficient between the measured data and the fitted line. P is the probability of error of R.

The amount of precipitable cholesterol in model dispersions at the end of the observation period (12 days) in the pellets was calculated (see Methods) and showed a linear correlation with the ΔOD of the dispersions (r = 0.8083 ± 0.0687, n = 15, P = 0.00027). The fluctuations in the results are due to the indirect method of determination of the crystalline cholesterol and the variability in recovery. Microscopic observations confirmed the presence of typical cholesterol monohydrate crystals in the pellet.

Two different dispersions (see Methods) of crystalline cholesterol were added in increasing amounts to a fixed volume of buffer solution. The OD increased linearly with the increasing concentration of cholesterol crystals in both dispersions (r_1 = 0.999 ± 0.273, n = 4; r_2 = 0.999 ± 0.737, n = 4). When 200 mM NaTDC was added, the OD was not appreciably altered. The daily measurements of the absorbance in the incubated dispersions yielded a sigmoidal curve. The slope of this curve was defined as the CGR = OD/day, (see Methods). Three dispersions with different crystallization dynamics were studied (**Table 2**). In model No. I, the COT was short and the CGR slow; in model No. II the COT was short and the CGR fast; In dispersion No. III the COT was long and the CGR slow. A comparison of the semiquantitative estimation of crystal numbers by light microscopy and the spectrophotometric measurements is shown in **Fig. 2.** The results indicate that the COT could be determined with a higher sensitivity by the microscopic method. The measurement of CGR was more accurate using the spectrophotometric method. The two methods produced grossly similar results.

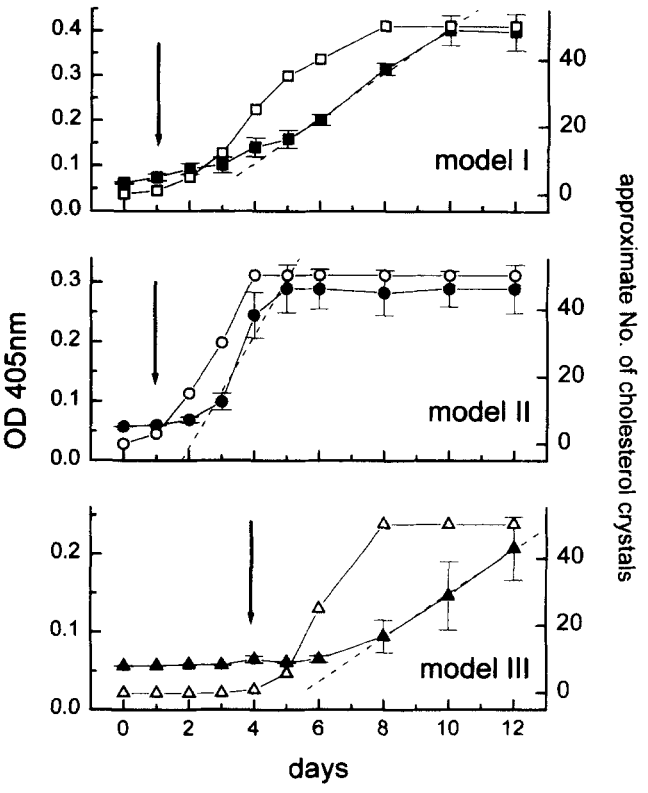

Fig. 2. Cholesterol CGR curves of three different model biles (see Table 1) as measured spectrophotometrically (black symbols) and microscopically (open symbols). All symbols represent the means of six measurements; the bars represent the SEM. The dashed lines represent the linear regression fits (Table 2) to the spectrophotometric measurements (see Methods). The cholesterol COT as monitored by polarized light microscopy is indicated by the arrows, which show the day of first appearance of at least three crystals. The approximate number of cholesterol crystals was counted per microscopic field at a magnification of 100-fold.

DISCUSSION

The nucleation time or COT tests are important not only in the discrimination between lithogenic and non-lithogenic biles, but also in the study of pro- and antinucleating factors and the effect of various therapeutic agents on the kinetics of cholesterol crystallization. In order to facilitate the study of model bile solutions and to measure the CGR in a quantitative manner, Busch, Tokumo, and Holzbach (4) introduced a spectrophotometric method instead of the more subjective light microscopic method. Model biles were diluted 8- to 50-fold with 10 mM NaTDC. Presumably this dissolved the various vesicular-lamellar compounds that are the major source of turbidity. Thereafter, a light beam (400 to 900 nm) was passed horizontally across the previously agitated cuvette; absorption was measured and was presumed to be due to cholesterol crystals. Repeated measurements over time indicate the rate of growth of the cholesterol crystal mass. The method has, however, several drawbacks. Cholesterol crystals tend to precipitate

and, despite previous agitation of the cuvette, the horizontal beam will not measure crystals that have settled at the bottom of the tube. Also, in our experience, the final bile salt concentration after dilution is not always sufficient to dissolve the noncrystalline compounds (vesicles, multilamellar vesicles, stacked lamellae, and liquid crystals) in model biles. As these particles absorb much more light than cholesterol crystals, the persistence of even a fraction of these particles will severely impair the measurement of crystal mass. Light absorption is measured in one cuvette at a time, which makes it labor intensive.

Various investigators used 10 and 65 mM TDC (final concentrations) in addition to the endogenous bile salts (10). The higher the final concentration the more rapid and reliable is the dissolution of the noncrystalline particles. Therefore, we used the final concentration of 100 mM TDC. Marked dilution (up to 50-fold), as used by some investigators (11), will not affect the crystals but may markedly alter the interrelationship of other cholesterol complexes in bile (10), which might be of investigational interest.

The recently described method of Corradini et al. (6) uses ultracentrifugation on a NaBr gradient and measures the separated crystal fraction chemically. Holan et al. (1) used the pellet after centrifugation for microscopic estimation of the crystal number. Konikoff and Carey (12) used sucrose density gradients to monitor cholesterol crystallization.

Ultracentrifugation methods are laborious and do not permit the simultaneous analysis of a large number of samples. The presently described method was meant to overcome some of the above-mentioned difficulties. It allows the simultaneous measurement of multiple samples and thereby automates and shortens the procedure. The light beam traverses the solution vertically and therefore always includes the bottom of the well where cholesterol crystals may have sedimented despite previous agitation. The high initial and final (100 mM) bile salt concentration facilitates the dissolution of noncrystalline particles. However, in our experience, even under these conditions there are exceptions and noncrystalline particles sometimes persist. The present method measures (rapidly) the turbidity in ten different points across the center of the well and is not limited to a single measurement. There is also an advantage to simultaneous measurement (controls and various concentrations of test substances) of all models in the study, instead of measuring single models sequentially. In cases of rapid nucleation, the time lag between the measurements of various samples may become significant.

Nevertheless, several limitations remain. The initial COT, when very few crystals are present, is probably earlier determined visually than spectrophotometrically. On the other hand, the observation of one to two cholesterol crystals per low power microscopic field is also unreliable and the next day no crystals may be observed. We therefore defined the COT as the first observation of at least three crystals. The present method like the original method of Busch et al. (4) is suitable only for defined model biles. These methods cannot be used in native biles, where noncrystalline (and also non-lipid) compounds cannot be reliably dissolved by the addition of NaTDC.

An increase in the OD can be caused by an increase in the number of crystals, in the size of crystals, or changes in the shape of crystals, or their relative position to the incident light beam. The relative effects of all these factors are not easily separable or measurable. However, the progressive addition of cholesterol crystals from a given dispersion resulted in a linear increase in the OD. Therefore, when a given cholesterol dispersion is manipulated, the change in the OD seems to provide a good estimate of the crystal mass. Recently, Harvey and Upaydhya (13) have described a spectrophotometric method measuring turbidity in microtiter plates. It can, however, be used only in a limited number of cases, as one defined bile model dispersion that does not show non-crystalline turbidity is used. They propose to evaluate the influence of pro- and antinucleating factors exclusively in this defined model dispersion.

All the above-mentioned methods will contribute to a more quantitative and rapid measurement of cholesterol crystal growth in model bile dispersions and thus facilitate studies of cholesterol crystallization.

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REFERENCES

1. 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.
2. Small, D. M. 1980. Cholesterol nucleation and growth in gallstone formation. *N. Engl. J. Med.* **302:** 1305–1307.
3. 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.
4. 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.

5. Groen, A. K., J. A. G. Drapers, and G. N. J. Tytgat. 1988. Cholesterol nucleation and gallstone formation. *J. Hepatol.* **6:** 383–387.
6. Corradini, S. G., A. Cantafora, L. Capocaccia, P. Dellaguardia, L. Giacomelli, and M. Angelico. 1994. Development and validation of a quantitative assay for cholesterol crystal growth in human gallbladder bile. *Biochim. Biophys. Acta.* **1214:** 63–72.
7. Sömjen, G. J., R. Rosenberg, O. Warshavskaya, Y. Ringel, F. Konikoff, and T. Gilat. 1995. A semi-automated method for quantitation of cholesterol crystallization in model biles. XIIIth International Bile Acid Meeting, San Diego, CA.
8. Pope, J. L. 1967. Crystallization of sodium taurocholate. *J. Lipid Res.* **8:** 146–147.
9. Sömjen, G. J., and T. Gilat. 1983. A non-micellar mode of cholesterol transport in human bile. *FEBS Lett.* **156:** 265–268.
10. de Bruijn M. A. C., C. Noordam, B. G. Goldhoorn, G. N. J. Tytgat, and A. K. Groen. 1992. The validity of cholesterol nucleation assay. *Biochim. Biophys. Acta.* **1138:** 41–45.
11. Busch, N., N. Matiuck, S. Sahlin, S. P. Pillay, and R. T. Holzbach. 1991. Inhibition and promotion of cholesterol crystallization by protein fractions from normal human gallbladder bile. *J. Lipid Res.* **32:** 695–702.
12. Konikoff F. M., and M. C. Carey. 1994. Cholesterol crystallization from a dilute bile salt-rich model bile. *J. Crystal Growth* **144:** 79–86.
13. Harvey, P. R. C., and G. A. Upaydhya. 1995. A rapid, simple high capacity cholesterol crystal growth assay. *J. Lipid Res.* **36:** 2054–2058.