

# Measurement of cholesterol gallstone growth in vitro

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**Abstract** Methods to study growth of gallstones in the laboratory have not been reported. We here present such a method. Human cholesterol gallstones were harvested from patients with multiple nearly identical stones. The gallstones were washed and added to supersaturated model bile and the formation of cholesterol crystals and the increases in mass of human cholesterol gallstones were studied concurrently, over a period of weeks, using nephelometry and a microbalance, respectively. All stones incubated in model bile supersaturated with cholesterol increased in mass. Increases in the degree of supersaturation of cholesterol in the model bile resulted in increased growth of stones. The mass increases, the growth rates, and the spatial orientation of accreted crystalline cholesterol differed among various stone types. The kinetics and structures of stone growth were similar when the stones were incubated in supersaturated, native, human gallbladder bile. The structure of accreted cholesterol was the same as found on the surface of some human gallstones that were harvested during apparent active growth *in situ*. This simple method allows accurate measurements of stone growth *in vitro*, in patterns that mimic stone growth *in vivo*, and is useful for studies on the relationships of gallstone growth and the kinetics of cholesterol crystallization.—van den Berg, A. A., J. D. van Buul, J. D. Ostrow, and A. K. Groen. *Measurement of cholesterol gallstone growth in vitro*. *J. Lipid Res.* 2000. 41: 189–194.

**Supplementary key words** gallstone • growth • cholesterol crystals • bile

In human gallbladders containing supersaturated bile, cholesterol gallstones can form and grow by accretion of cholesterol crystals. By definition, over 70% of the mass of human cholesterol gallstones is cholesterol. In the development of cholesterol gallstones in bile, the first step appears to be the appearance of microscopic crystals of cholesterol monohydrate; this process is faster in bile from patients with cholesterol gallstone than in bile from patients without gallstones at similar levels of supersaturation with cholesterol (1). The crystals then grow into cholesterol plates, which are retained in the mucin layer and aggregate to form the stone nidus, wherein the cholesterol crystals are randomly ordered (2–4). Thereafter the stone grows by orderly accretion of crystalline cholesterol, forming the concentric rings of the body of the stone,

which consists of radially and/or horizontally ordered stacks of plates (5, 6).

There are few studies on stone growth and experimental models to investigate stone growth *ex vivo* are lacking. Consequently, insight into the kinetics and characteristics of the stone growth process has lagged behind understanding about the crystallization of cholesterol. We have, therefore, devised a simple method to study growth of cholesterol gallstones in supersaturated model bile and native human gallbladder bile.

## MATERIALS AND METHODS

### Chemicals and biles

Egg yolk lecithin (EYL), sodium taurocholate (TC), sodium taurodeoxycholate (TDC), and cholesterol were obtained from Sigma (St. Louis, MO). All other chemicals were reagent grade obtained from Merck (Darmstadt, Germany). After informed consent of the patients, sets of small human cholesterol gallstones were harvested from individual gallbladders within 1 h after elective cholecystectomy. After adherent bile was washed off with demineralized water, the stones were air dried and stored at  $-20^{\circ}\text{C}$ .

Model bile was prepared according to Kibe et al. (7), with modifications described by De Bruijn et al. (8). The EYL and cholesterol were dissolved in equimolar solutions of TC and TDC buffered to pH 7.0 with 25 mM PIPES containing 125 mM NaCl. In these simple systems total lipid contents were 10 to 12 g/dL and cholesterol saturation indices (CSI) varied from of 1.0 to 2.0 according to the critical tables of Carey (9).

### Measurements of increases in cholesterol gallstone mass and cholesterol crystal formation

Sets of matched cholesterol gallstones were thawed, vacuum-dried at room temperature for over 90 min, and weighed on a Mettler AG 204 Delta Range analytical balance (Mettler, Switzerland) with 0.1 mg accuracy. Dry weights of the individual stones

Abbreviations: CCMf, mass of cholesterol crystals in bile; CSI, cholesterol saturation index; EYL, egg yolk lecithin; PIPES, piperazine-N,N'-bis[2-ethanesulfonic acid]; TC, taurocholate; TDC, taurodeoxycholate.

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were 10 to 30 mg. Three matched stones from each set were placed in individual sterile plastic 20 mL tubes (Hospidex, France), each containing 2.0 mL of model bile. To assess stone growth, each stone was removed from its incubation, washed as above, vacuum-dried to constant weight at room temperature, weighed, and then replaced in its original tube and medium for further incubation. Increases in dry weights were expressed as percentage increase of the initial dry weight, to correct for the variation in the average size (see Table 1) of the various sets of stones.

The mass of free-floating cholesterol crystals formed (CCMf) was determined on duplicate samples of model biles, both by double light-scattering measurements (using the BN 100 nephelometer, Behring, Germany) (8) and by the enzymatic assay after harvesting the crystals via centrifugal filtration over a 0.22  $\mu$ m cellulose acetate filter and re-dissolution in isopropanol (using the cholesterol oxidase kit Sigma, St. Louis, MO) (10).

### Microscopy

Morphology of gallstones was studied at 10 to 200 times magnification under a Wild M8 stereomicroscope (Wild, Heerbrugg, Switzerland). The micromorphology of gallstone surfaces and the inner structure of fractured gallstones was studied by scanning electron microscopy of stones after shadow-casting with gold, using a Philips SEM 525 electron microscope (Eindhoven, The Netherlands).

### Statistics

For stone growth, the average of triplicate samples in each experiment was taken. Data are expressed as mean  $\pm$  SD. Statistical differences between groups were assessed by the Mann-Whitney U test. Correlation between parameters was measured by the Spearman rank correlation coefficient. Statistical significance was set at  $P < 0.05$ .

## RESULTS

Several sets of multiple, nearly identical, human gallstones were obtained. Two sets consisted of simple round stones with a macroscopic appearance of a smooth surface and one set consisted of semi-cubicle stones. A fourth set consisted of mulberry stones. After washing and drying, all sets of stones had a different color, indicating different amounts of biliary pigments incorporated into or under the outer surface. The average dry weight of selected sets of stones was between 15 and 21 mg, and all consisted of over 70% cholesterol by weight. Three sets of stones were used in experiments to study stone growth *ex vivo*. Relevant characteristics of the stones are given in Table 1.

When the various sets of stones were dried under high

TABLE 1. Morphological characteristics of sets of multiple human cholesterol gallstones used in stone growth studies

Variable	Patient		
	A	B	C
Mass percentage cholesterol	99	76	99
Structure	semi-cubicle	simple round	mulberry
Color	rusty red brown	brown	yellow
Average stone dry weight (mg)	$15.7 \pm 2.6$	$15.5 \pm 1.9$	$20.8 \pm 4.2$
Surface layer is shell-like	yes	yes	no

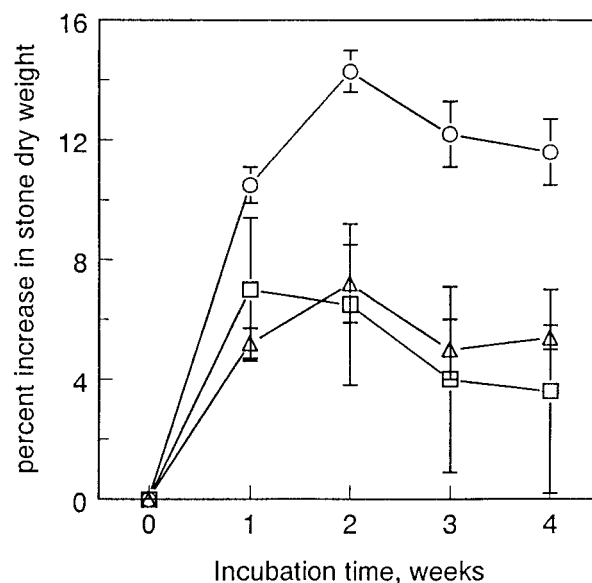
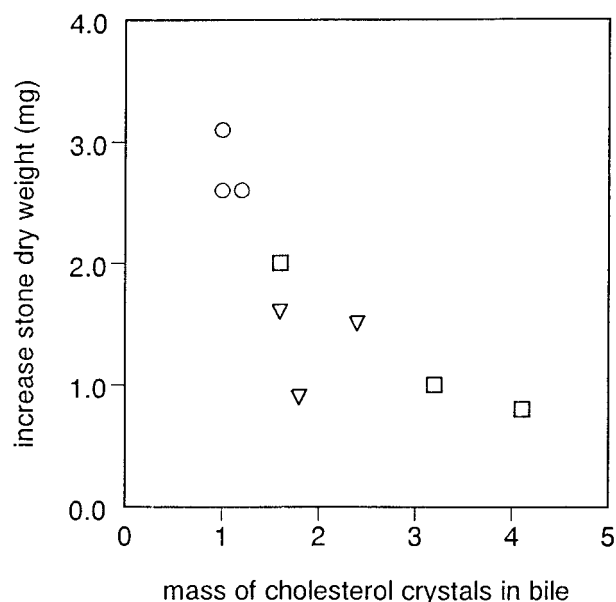


Fig. 1. Growth of human cholesterol gallstones in model biles *in vitro*. Percent increases in dry weight (mean  $\pm$  SD) of three stones from each of three different patients, incubated for 4 weeks in model biles of CSI 1.6 and 12 g/dL total lipids. After reaching a maximum near 2 weeks, a phase of slow decrease in stone dry weight is observed. Squares and triangles: simple round cholesterol gallstones with smooth surfaces from two different patients A and B; circles: mulberry type of cholesterol gallstones from a single patient C.

vacuum, all achieved a constant mass within 90 min. Therefore, the drying was routinely performed for 90 min. When incubated in supersaturated model biles for 14 days, stones weighed only once at 14 days showed the same overall increase in dry weight as matched stones weighed at 3, 7, and 14 days. In the model bile system with CSI 1.7 and 10 g/dL total lipids, the mass of free floating cholesterol crystals (CCMf) in solution increased for up to 14 days, but not thereafter as has been described for this model bile system elsewhere (10). When incubated in model biles of initial CSI 1.7, stone masses increased most in the first 7 days and achieved maximum weights after 1 or 2 weeks (Fig. 1). After 2 weeks of incubating stones in model bile, the mass of CCMf in the model biles samples correlated inversely (correlation coefficient  $r = -0.81$ ,  $P < 0.01$ ) with the increases in the dry mass of the stones (Fig. 2). For most stones a slight decrease in stone dry weight was observed after the maximum value was reached. The arrest of growth was related to depletion of the supernatant of the excess cholesterol as it precipitated. In a separate experiment, after 8 weeks, one set of stones ( $n = 4$ ) that had increased  $8 \pm 2\%$  in dry weight was transferred to freshly prepared model biles of CSI 1.7 and 10 g/dL total lipids. The growth resumed immediately and another increase of  $6 \pm 3\%$  in dry weight was observed. In the simple lipid system of CSI 1.7, the mean masses of three stones obtained from three different patients A, B, and C had increased by 7, 7, and 15%, respectively, in 2 weeks. In simple model biles, with initial CSIs lower than 1.7, but with the total lipid content kept constant at 10 g/dL, both the plateau in CCMf and the maxi-

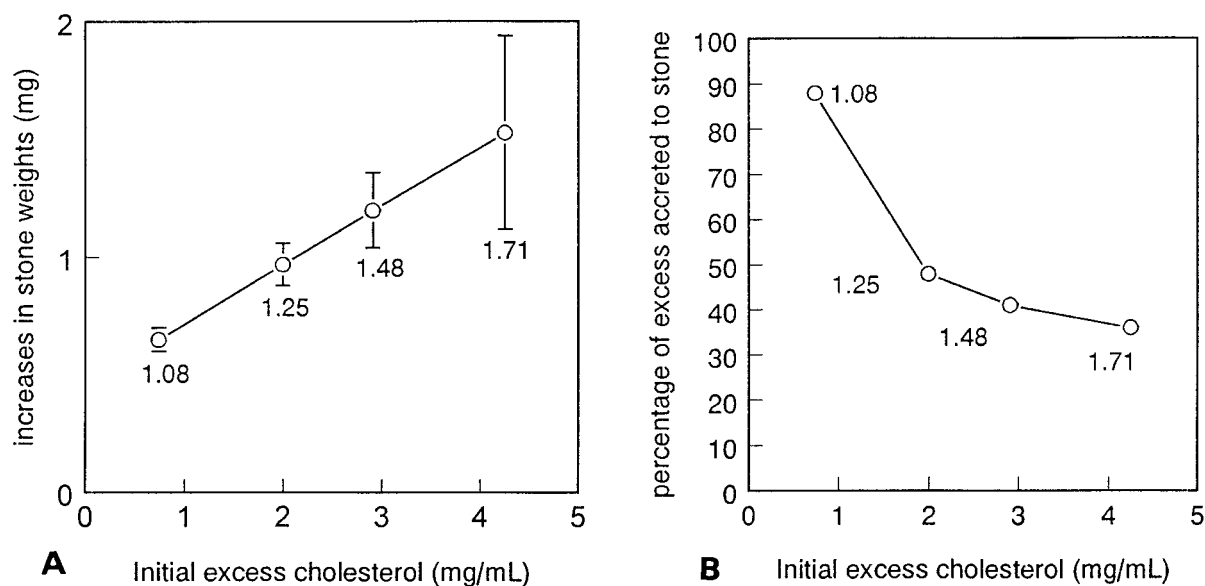


**Fig. 2.** The mass of free cholesterol crystals formed (CCMf) in supersaturated model bile is inversely related to the increases in dry weight of the gallstones. Small human cholesterol gallstones were incubated in 2 mL of model bile, CSI 1.6 and 12 g/dL total lipids, for 2 weeks. Squares and triangles: simple round cholesterol gallstones with smooth surfaces from two different patients A and B; circles: mulberry type of cholesterol gallstones from a single patient C. For all sets of stones  $n=3$  in this experiment. The mass of CCMf in the model bile samples correlated inversely with the increases in the dry mass of the stones. (The Spearman rank correlation coefficient  $r = -0.81$ ,  $P < 0.01$ .)

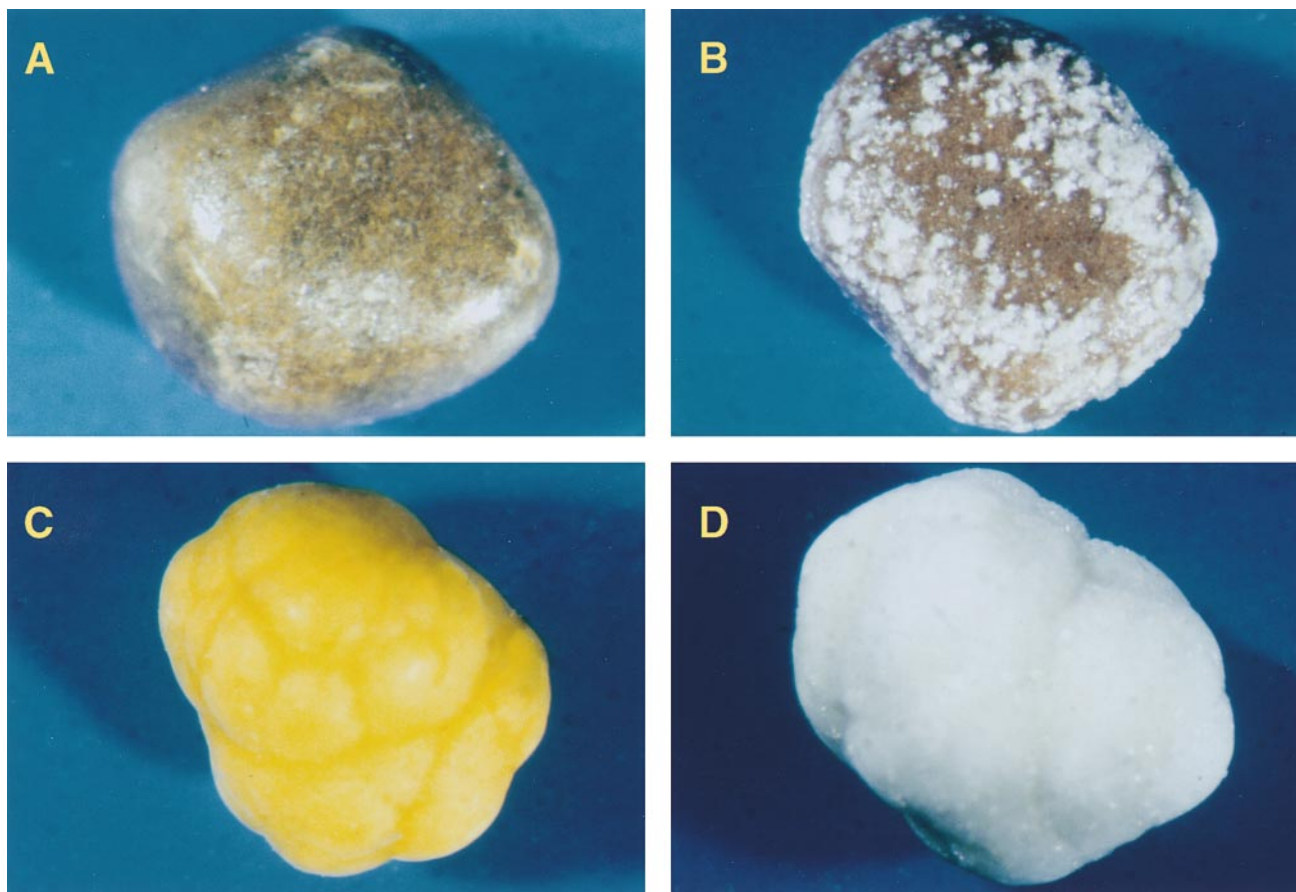
imum increases in stone dry weight were reached only after 3 weeks. After 3 weeks of incubation a linear relationship was found between the initial excess of cholesterol in the model bile and the overall increases in stone mass. (Fig. 3).

As the stones grew, a whitish crystalline precipitate appeared on their surface. As shown in Fig. 4, in the case of the mulberry type of stone, this precipitate either covered the whole surface or was distributed multi-focally as was the case for the simple round stones. As shown in Fig. 5 scanning electron microscopy of the surfaces and the inner part of broken stones demonstrated that the orientation of newly deposited cholesterol crystals was related to the structure of the original outer surface. The mulberry type of stone had a fan-like orientation of crystals in the surface layer. New cholesterol was deposited in such a way that this structure was extended. The outer surface of the simple round stones was composed of concentric rings of horizontally placed crystals. Here, the accreted cholesterol was deposited as plates that had a perpendicular orientation towards the original surface. On top of these upright crystals, some tangential layering of cholesterol crystals was visible. The latter displayed trimmed edges and this probably indicated some dissolution because a small decrease in stone dry weight was observed in the later phase of the growth process as described above (Fig. 2).

On scanning electron microscopy, the whitish accretions on the surface of stones incubated in model bile were structurally quite similar to the deposits on the surface of some cholesterol gallstones harvested from human



**Fig. 3.** The growth of human cholesterol gallstones in simple model bile systems depends on the degree of supersaturation. Mean  $\pm$  SD of four simple round stones from patient B, incubated for 3 weeks in each of four model bile systems with different CSI and total lipids kept constant at 10 g/dL. All biles were made of the same stock solutions. Panel A, absolute increase in stone dry weight (mg) is plotted against the initial excess of cholesterol present in the model bile (mg). Panel B, the cholesterol that accreted to the cholesterol gallstones as a percentage of the initial excess of cholesterol present in the model bile. At lower CSI a larger percentage is deposited on the stone surface. The other part was free cholesterol crystals in solution. In both panels the exact CSIs of the model biles at the start of the incubations are given in the figures. The initial excess of cholesterol in mg/ml was calculated from the critical tables of Carey (9).



**Fig. 4.** Gross appearance of gallstones before and during growth in supersaturated model bile in vitro. A: Small simple round smooth-surfaced human cholesterol gallstone from patient B before incubation in model bile. B: A specimen of the same set of stones after 4 weeks of incubation showing focal deposits of cholesterol at its surface. C: Mulberry type of cholesterol gallstone before incubation. D: Mulberry type of stone with the whole surface covered by newly accreted cholesterol after incubation in model bile for 4 weeks. The actual maximum diameter of the stones is 4 to 6 mm. Incubations were performed in model bile with 12 g/dL total lipids, CSI 1.6.

gallbladders at cholecystectomy. The unit thickness, the spatial orientation and the dimensions of the spaces between crystals were very similar (Fig. 5, D and E).

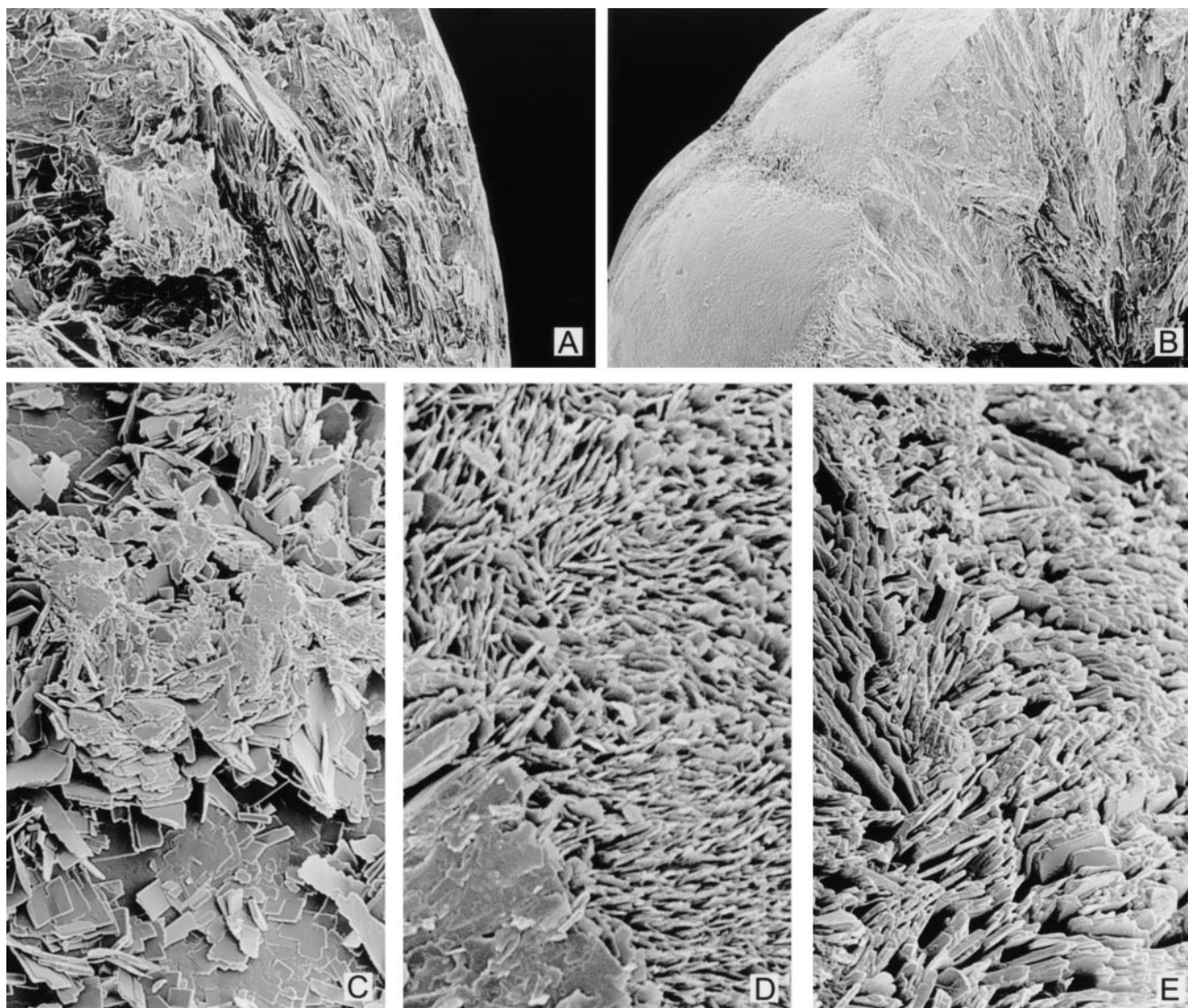
Biochemical assay of these accretions, scraped off the stone surface with a scalpel under a stereo microscope, showed that both the *in vitro* and *in vivo* accretions consisted of over 99% cholesterol by weight.

## DISCUSSION

The present work is the first study on growth of human cholesterol gallstones in biles supersaturated with cholesterol *in vitro*. Repeated drying and weighing of the stones did not disturb the growth process. Stone growth, arrested after several weeks when the biles became depleted of excess cholesterol, was resumed promptly when the stones were transferred to supersaturated, fresh biles. A clear correlation between the initial amounts of excess cholesterol present in model biles and the maximal increases in stone mass were observed. Moreover, the size and structure of the cholesterol crystals accreted to the stones in model biles closely resembled the crystals on the surface

of gallstones harvested from a patient in whom the stones appeared to be in a phase of growth. Furthermore, the structures we observed here were also described for other samples of human gallstones that were studied after harvest and without prior incubation *ex vivo* both by Osuga et al. (6) and Wolpers and Hofmann (5). We therefore feel this simple *in vitro* method suitably mimics the process of stone growth *in vivo*.

The random orientation of cholesterol crystals in the center of human stones (5, 6) suggests that stone nidation may involve compaction of preformed crystals into a particle of minimal size to accomplish retention in the gallbladder. Stone nidation has been suggested to result from temporary dysfunction of the gallbladder leading to stasis and/or an increase in pH with precipitation of calcium salts (2, 11) leading to formation of concrements of both calcium salts and cholesterol crystals. Entrapment of cholesterol crystals and calcium salt precipitates in the mucin gel layer that lines the gallbladder wall would facilitate stone nidation (14). After nidation of the stone, the subsequent growth of stones around the center proceeds by progressive accretion of crystalline cholesterol from supersaturated bile. We here showed that growth of the mass of



**Fig. 5.** Scanning electron microscopy of the surface of the stones. Original surface and structure of the outer layer of cholesterol crystals in human cholesterol gallstones used in the study of growth of stones in model bile. A: Simple round type (from patient B); B: mulberry type (from patient C). C, D, E: High magnification of cholesterol crystals deposited on the surface of human cholesterol gallstones during incubation in bile in vitro; C: Simple round type (from patient B); D: mulberry type (from patient C); E: cholesterol crystals deposited on the surface of a human gallstone in vivo. Note that the cholesterol crystals at the surface of the stones either after growth in model bile (D) or after harvest from native bile (E), are plates with similar thickness, similar spacing, and similar structure of the edges. Original magnifications: (A) 40 $\times$ ; (B) 60 $\times$ ; (C) 250 $\times$ ; (D) 800 $\times$ ; (E) 1000 $\times$ .

cholesterol crystals can take place in the absence of biliary mucins and calcium salts. However, in the same model bile system, the patterns of growth differed among sets of stones from three patients. Thus, the mulberry stones from patient C accreted the whitish precipitate diffusely over the stone surface, whereas the simple round stones from patients A and B accreted multi-focal precipitates (Fig. 4). Though this indicates that the pattern of deposition of cholesterol during stone growth is governed, at least in part, by the nature of the underlying surface, it is not yet clear which characteristics of the surfaces determine pattern and rate of further growth.

Growth of a single or multiple gallstone(s) into any appreciable mass (grams) is probably a process of years (2, 3,

5, 6). In our in vitro systems, stone weight increased by up to 15% within 1 week. The growth rate seems to depend on the lipid contents and the CSI of the biles, as well as on the characteristics of the stone surface. Most stones harvested from patients had a smooth pigmented surface. Only few sets of stones displayed whitish precipitates on such a surface at the time of harvest. These observations all support the concept that growth in vivo is probably a discontinuous process, most likely a balance between accretion and dissolution. The success of complete therapeutic dissolution is limited to relatively pure cholesterol gallstones (13). This might indicate the importance of intermittent episodes of calcium salt precipitation during growth, because calcium/pigment shells may encapsulate

the cholesterol mass between episodes of growth and limit the dissolution. This might be the reason for the presence of concentric calcium salt deposits between layers of cholesterol crystals in the body of multi-layered stones (12).

Because virtually all cholesterol gallstones have a matrix of mucus glycoproteins (14) and/or calcium salts (15), and because we recently showed that mucins and calcium salt precipitates additively stimulate the crystallization of cholesterol from model bile (16), we will use our methods to define further the relationships between bile components that regulate the crystallization of cholesterol and growth of cholesterol gallstones in bile. ■■

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