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Cholesterol Particle Growth and Dissolution Rates in Aqueous Media

By WILLIAM I. HIGUCHI and HOSNY Y. SAAD

As part of the program on the transport, deposition, and dissolution of cholesterol in aqueous media, the growth and dissolution rates and the nucleation behavior of this compound in saline have been investigated. The Coulter counter was used in these studies to follow the particle size distribution changes of cholesterol suspensions with time. A comparison of the results with theory showed that the growth and dissolution rates were close to being diffusion controlled, despite the low rates (10 to 20 hours to grow to about 2-μ diameter or to dissolve a 2-μ diameter particle). Experiments at the higher supersaturation levels showed that a metastable phase preferentially nucleates, grows out rapidly, then redissolves as the more stable phase nucleates more slowly and grows out.

THE TRANSPORT, deposition, and dissolution mechanisms of cholesterol and cholesterol esters are of major importance to the eventual understanding of the diseased and the normal states of man (1, 2). A search of the literature indicates, however, that there has been relatively little quantitative work done on the physical chemistry of the kinetic aspects of these processes. It would appear that significant advances on the problems related to diseases and conditions characterized by excess cholesterol and lipid deposition can be assisted materially by such studies.

To initiate such studies, the growth of cholesterol particles in their supersaturated solutions and the dissolution of these particles in their undersaturated solutions are being investigated. The present report describes the results of the study of cholesterol behavior in their supersaturated and undersaturated saline solutions with particular emphasis on methodology. Other studies are now in progress on the application of present methods to evaluate the effects of additives on the growth and dissolution rates.

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EXPERIMENTAL

General Considerations.—Because the solubility of cholesterol in water is extremely low,[1] it was expected that both the growth rates and the dissolution rates would be low in water, even under the ideal diffusion-controlled situations. Therefore, it was decided to employ the Coulter counter because it had been shown recently (4, 5) that this instrument was suitable for growth and dissolution rate studies of sparingly soluble materials in their suspension or emulsion states.

To eliminate the possible importance of artifact effects in the growth studies, it was decided to try several methods for preparing the supersaturated solutions. Comparable results would then establish the general behavior more firmly.

Procedures for Growth Studies.—The sample of cholesterol monohydrate characterized in connec-

[1] About 2.5×10^{-8} Gm. ml.$^{-1}$. (See *Reference 3.*)

tion with the recent (3) solubility study was used in the present work. Supersaturated solutions for the growth experiments were prepared in the following manner.

Method A.—Approximately 1 Gm. of cholesterol was added to 3 L. of 0.90% NaCl solution[2] and boiled at 100° for 12 hours. Water was added from time to time to maintain the liquid level. At the end of this period, the suspension was filtered through a 0.22-μ filter[3] held in a water-jacketed filter holder maintained near 100° during the filtration. The first 300 ml. of the filtrate was rejected, and the remainder was transferred immediately to a 3-L. conical flask with a ground-glass stopper and placed in a water bath at 30 ± 0.1°. The solution was stirred slowly with a Teflon-coated magnet driven from below the bath. Aliquots then were removed periodically, and the suspension particles were sized directly and counted with the Coulter counter using the 50-μ aperture tube and calibrated with the 2.0-μ particle size latex particles (4, 5). Samples also were taken at different times, filtered, and the supernatant analyzed for cholesterol by the procedure described previously (3).

Method B.—This procedure was designed primarily to see whether minute impurities in the sample could be important. About 50 ml. of a 0.10 mg. ml.$^{-1}$ cholesterol solution in alcohol was added to 3 L. of the NaCl solution, and the resulting suspension was boiled for 5 hours. It then was filtered, and the procedure described in *Method A* was followed. The significant differences between methods *A* and *B* were then that in the latter case the solution was supersaturated by exposure to a smaller amount of cholesterol solid, and the temperature was kept at the boiling point for a shorter period.[4]

Method C.—In this procedure an alcohol solution of cholesterol was mixed rapidly with the aqueous NaCl solution to provide the supersaturation. Cholesterol solutions of different concentrations in a solvent mixture containing 60% ethanol and 40% of the 0.90% NaCl solution were prepared. In a given experiment, 40 ml. of the cholesterol solution was added to 160 ml. of the 0.90% NaCl solution in a 250-ml. water-jacketed beaker already positioned for counting with the Coulter counter. The solution was added by means of a constant-flow buret, and stirring was maintained constant during and after the mixing. Immediately after completing the addition, a timer was started, and counts were recorded for various threshold settings at different times. Samples also were taken periodically, filtered through the 0.22-μ filter, and analyzed for the solution cholesterol concentration.

X-Ray Characterization of the Metastable Phase.—Because in *Method C* the growth and the subsequent dissolution of a metastable cholesterol phase was suspected, an experiment was performed to see whether the suspected new phase would be detectable by X-ray diffraction methods. For this purpose, 200 ml. of 0.10 mg. ml.$^{-1}$ 60% alcohol-saline solution of cholesterol was mixed rapidly with 800 ml. of the 0.90% NaCl solution. From the suspension that formed immediately, 100 ml. of sample was removed every 1000 seconds and quickly filtered through a 0.22-μ filter. The filter paper was quickly transferred to a vacuum desiccator and rapidly dried. The precipitate then was removed and mixed thoroughly with a smear of petrolatum on a glass slide. Then the X-ray diffraction patterns were obtained with the Siemens Crystalloflex IV X-ray diffractometer.

Procedure for Dissolution Rate Studies.—Suspensions of cholesterol obtained from the growth studies above were used in several dissolution rate experiments. In cases where suspensions from *Method C* were used, these were aged several days to

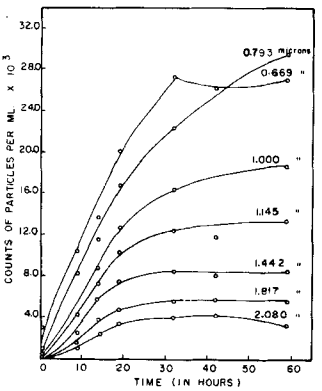

Fig. 1.—Coulter counter data showing the increases in cumulative counts with time at various threshold settings as cholesterol particles grow out of a supersaturated solution (*Method A*).

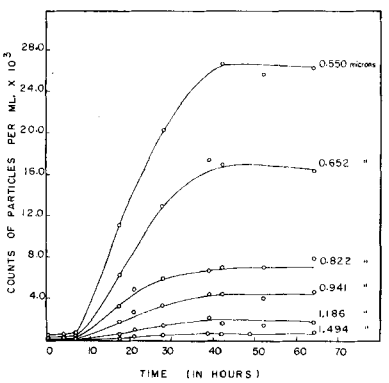

Fig. 2.—Coulter counter data showing increases in cumulative counts with time at various threshold settings for cholesterol growth experiment (*Method B*).

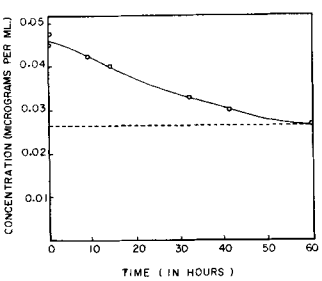

Fig. 3.—Solution cholesterol concentration *vs.* time for the experiment in Fig. 1, showing relief of supersaturation with time.

[2] All 0.90% NaCl solution used in this work was Normal Saline for Injection, Abbott Laboratories, North Chicago, Ill.
[3] Millipore Filter Corp., Bedford, Mass.
[4] An additional experiment was carried out that involved boiling only the supernatant solution for 5 hours. No change in cholesterol concentration was observed by this treatment.

insure the complete reversion of the cholesterol to the stable phase.

The suspensions were diluted into the 0.90% NaCl solution, and the changes in particle size distribution with time were recorded as before (5).

RESULTS AND DISCUSSION

Growth Studies with Methods A and B.—In Figs. 1 and 2 the Coulter counter data for the methods *A* and *B* growth experiments are presented. In Fig. 3, the supernatant cholesterol analysis data are given, showing the relief of supersaturation with time. In Figs. 4 and 5, the cumulative particle size distribution curves constructed from the data of Figs. 1 and 2 are shown.

These results, quite reproducible in most aspects, show that the growth rates and the amounts of growth obtained from *Method A* and *Method B* were approximately the same. The differences were primarily that in *Method A* there was the absence of an expected lag period that always occurred in *Method B*, and a plateau appeared in the *Method A* experiments in at least one instance (Fig. 4). The plateau is an indication that essentially all of the particles have grown to sizes that are large enough to be counted and sized.

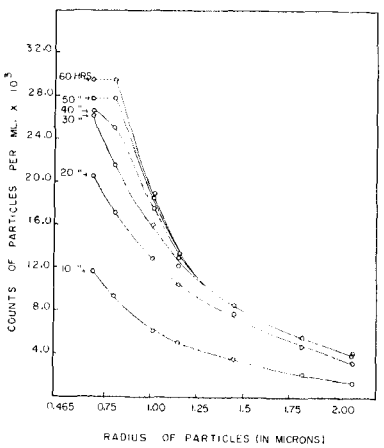

Fig. 4.—Cumulative particle size distribution curves constructed from data in Fig. 1.

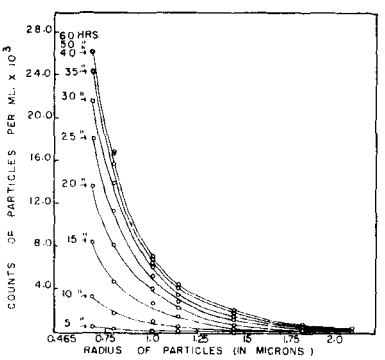

Fig. 5.—Cumulative particle size distribution curves constructed from data in Fig. 2.

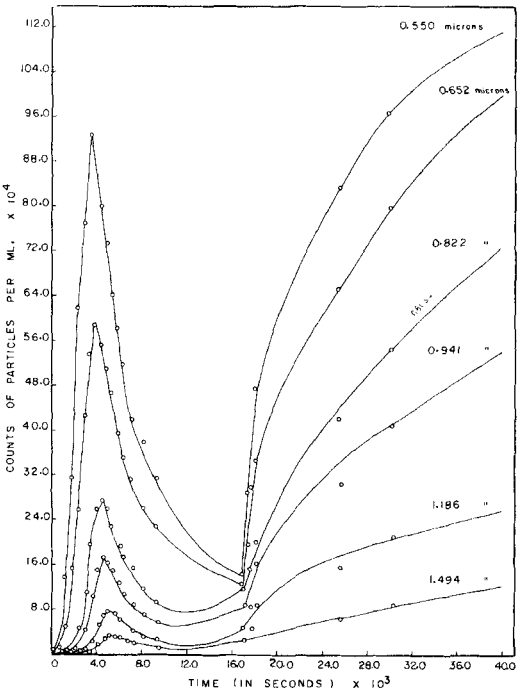

Fig. 6.—Coulter counter data showing time changes in cumulative counts at various threshold settings. The data show the appearance and disappearance of a metastable phase and the subsequent appearance of the stable phase (*Method C*).

It is important to point out that there were no significant changes in the particle size distribution curves after the growth had ceased. This permits one to assume safely that particle–particle aggregation rates and loss of particles to the walls were negligible and that therefore only molecular transport and deposition were involved.

From the results shown in Figs. 4 and 5, it can be seen that the rates of particle growth in the 10- to 20-hour period are about

$$\frac{da}{dt} = 1.4 \times 10^{-9} \text{ cm. sec.}^{-1} \pm 0.5 \times 10^{-9}$$

where da/dt is the rate of change in radius of the effective volume sphere based on the latex particle calibration (5). It is instructive to compare this value with that estimated from the diffusion controlled theory for the growth of a sphere of the same size. We may write (4, 5)

$$\frac{da}{dt} = \frac{D\Delta C}{\rho a} \qquad \text{(Eq. 1)}$$

where D is the diffusion coefficient for cholesterol in water, ΔC is the supersaturation, and ρ is the density. For the present situation, $D \simeq 4.3 \times 10^{-6}$ cm.^{2} second^{-1}, estimated from the Stokes-Einstein law; and $\Delta C \simeq 0.015$ mcg. ml.$^{-1}$, estimated from the 10- to 20-hour range in Fig. 3. Taking $a \simeq 1 \times 10^{-4}$ cm. and assuming $\rho \simeq$ unity, Eq. 1 gives

$$\frac{da}{dt} = 6.5 \times 10^{-10} \text{ cm. sec.}^{-1}$$

The relative close agreement suggests that the

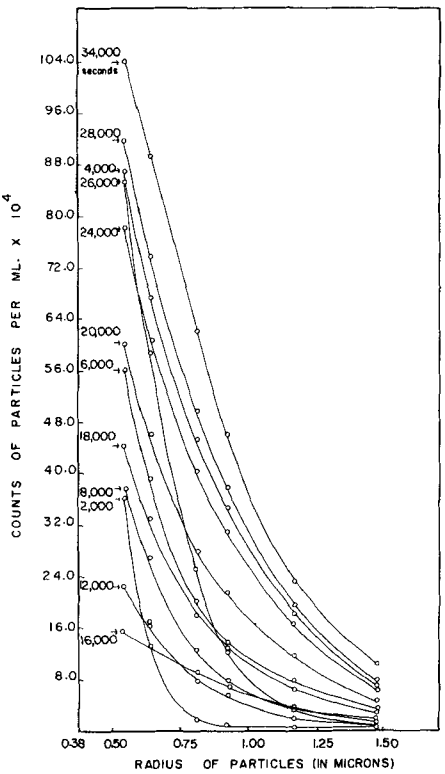

Fig. 7.—Cumulative particle size distribution curves constructed from data in Fig. 6.

growth process in the present cases is probably largely diffusion controlled. Because the shape of the particles and the importance of it have not been established[5] clearly, some uncertainty remains regarding the quantitative applicability of Eq. 1 to the present situation.

Growth Studies with Method C.—The growth behaviors in these experiments were quite different from those results obtained with methods *A* and *B*. Typical Coulter counter data from an experiment are shown in Figs. 6 and 7. In this particular run, a 10 mcg. $ml.^{-1}$ cholesterol solution in 60% alcohol–saline was diluted 1 to 5 in 0.90% NaCl solution. The plots show that during the first 3000 to 4000 seconds there is a rapid buildup in the number of particles. Then after 4000 seconds, the particle size distribution curves recede, and the particles almost disappear (16,000 seconds). There is then a reappearance of particles, and these continue to grow. While in this particular run the growth was followed for only about 10 hours, other runs showed that growth continued beyond 20 hours.

In Fig. 8, the corresponding supernatant cholesterol concentrations as a function of time are shown for the data in Figs. 6 and 7. There appears to be a plateau region between 1,000 and 10,000 seconds, after which the solution concentration decreases slowly. Such a plateau and the Coulter counter data of Figs. 6 and 7 are consistent with the following mechanism.

The first particles that appeared in these experiments were probably those of a metastable phase. These then reverted to the more stable phase. While the reversion may have involved direct solid-to-solid transformation to some extent, it appears that a considerable amount of the metastable phase redissolved during the growth of the stable phase at new sites, evidenced by the moving back of the particle size distribution curves (Fig. 7) to smaller sizes in this time period.

The X-ray results confirmed the above postulated mechanism. The diffraction patterns of the solids at early times differed significantly from those aged for longer times. Furthermore, the patterns changed with time over the 1,000 to 10,000 seconds period. Most significant in this connection was the simultaneous disappearance of one peak (1.6°) and the increase in the intensity of another (2.6°). This can be seen in Fig. 9, where the ratio of these two peak heights, with background subtracted, is

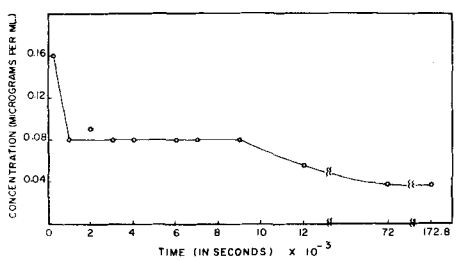

Fig. 8.—Solution cholesterol concentrations at different times for the experiment in Figs. 6 and 7.

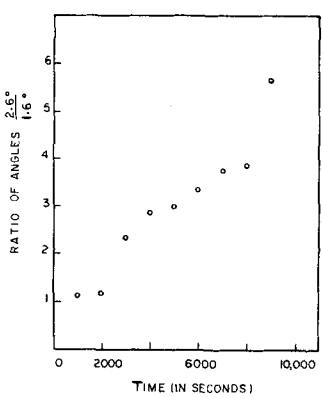

Fig. 9.—X-ray diffraction data showing the reversion of a metastable phase of cholesterol to the stable phase. The ordinate gives the ratio of the intensities of the 1.6° and the 2.6° peaks which are characteristic of the metastable and the stable phases, respectively. Gold standard used with Siemens Crystalloflex IV diffractometer.

[5] The matter of particle shape raises two questions. First, and perhaps most important, the response of the Coulter counter may not be proportional to the particle volume when particles are highly anisometric, particularly if they are very thin disk-shaped. Second, the meaning of Eq. 1 becomes less quantitative if the particle shapes deviate much from sphericity, although up to axial ratios of 10 (or 0.1) the effect is probably not greater than about a factor of 2. Several experiments have shown that based on the polystyrene latex spheres calibration, the volumes of cholesterol solids in suspension calculated from the Coulter counter data were two to three times greater than the known amounts introduced for growth following *Method C*. Microscopic observations of cholesterol suspension particles prepared by *Method C* and by similar methods involving less alcohol in the growth medium indicate that many of the particles of the stable phase grow out as thin plates. This problem is being examined in greater detail, and the results will be reported in a later communication.

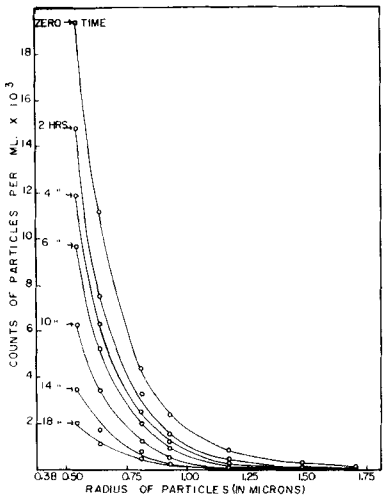

Fig. 10.—Cumulative particle size distribution curves showing dissolution of cholesterol particles prepared by *Method C* in saline. Ten-milliliter run. (See *text*.)

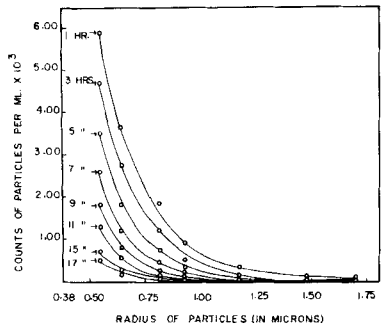

Fig. 11.—Cumulative particle size distribution curves showing dissolution of cholesterol particles in saline. Three-milliliter run. (See *text*.)

plotted as a function of time. As the X-ray pattern for the aged suspension corresponded to that for the thermodynamically stable monohydrate, these results confirmed the Coulter counter evidence that a metastable intermediate first grows out.

Qualitatively, the same behavior was observed with other cholesterol concentrations to where a 2.5 mg. ml.$^{-1}$ solution was diluted 1 to 5 in saline. The first peak counts occurred at about 2,000 to 4,000 seconds, dissolution followed, then the growth of the stable phase was observed at a later time.

Other studies (6) have indicated that this unstable intermediate phase formation may occur in cholesterol growth out of sodium cholate solutions. Thus, this phenomenon may prove to be a general one observable at relatively high initial supersaturation ratios.

Let us now consider the quantitative aspects of the results given in Fig. 7. From the 2,000 and 4,000 seconds curves, the growth rate of the metastable phase in this time period is estimated to be about $da/dt = 1.5 \times 10^{-8}$ cm. second^{-1} which is about 10 times greater than the growth rates for the stable phase.

From the growth data at later times ($t \gtrsim 24{,}000$ seconds), the growth rate of the stable phase is about $da/dt = 1.9 \times 10^{-9}$ cm. second^{-1}. Considering the probable somewhat greater supersaturation with respect to the stable phase (see Fig. 8), this value for the rate of growth of the stable phase compares well with that (1.4×10^{-9} cm. second^{-1}) obtained from methods *A* and *B* and with that predicted by Eq. 1.

DISSOLUTION RATE STUDIES

In Figs. 10 and 11, results of dissolution rate experiments are presented. In these runs, 10 to 3 ml., respectively, of a cholesterol suspension prepared by *Method C* was added to 150 ml. of a 0.90% NaCl solution. The rate in the first 5 to 7 hours is estimated from the curves to be about $-da/dt = 1.0 \times 10^{-9}$ cm. second^{-1}; the rate is somewhat greater for 3 ml., as one would expect. This value agrees well with the theoretical prediction of about 1.1×10^{-9} cm. second^{-1}, based on Eq. 1 with $\Delta C \simeq 2.5 \times 10^{-8}$.

Similar results, but somewhat higher (up to about 50%), were obtained with suspensions prepared by methods *A* and *B*. The suspensions prepared by methods *A* and *B* were not so suitable because the particle concentrations were already low; therefore, with dilution the uncertainties were always greater.

ADDITIONAL COMMENTS

These studies have shown that the growth of cholesterol in 0.90% NaCl, and therefore presumably in water, is close to being diffusion controlled. However, the three-dimensional nucleation of the stable phase may involve an appreciable energy barrier because the studies show that at high initial supersaturation ratios, a metastable phase preferentially nucleates.

The methods described here should provide convenient ways of evaluating the role of agents that may alter the nucleation, growth, or dissolution behavior of cholesterol and related low solubility materials in aqueous environments.

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