Cholesterol Particle Growth and Dissolution Rates II

Retardation Effects of Cholate

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The influence of cholate on the precipitation behavior of cholesterol in aqueous media has been investigated as a function of pH. The Coulter counter was employed to follow the particle size distribution changes with time in the supersaturated and undersaturated cholesterol suspension systems. It was found that in the high pH region (pH $\lesssim$ 6.5) the rates of growth and dissolution of the unstable phase and the rates of growth and dissolution of the stable phase were markedly retarded. At pH 8 and higher, the processes appeared to become completely inhibited. These observations are consistent with the mechanism involving the interfacial interaction of the cholate ion with the cholesterol crystal surface resulting in the formation of a barrier for interfacial transport of cholesterol from solution to solid. The data also suggest that at a low pH cholic acid catalyzes the deposition processes of cholesterol. The biological implications of these findings are pointed out.

THE IMPORTANCE of bile salts in the absorption and deposition of cholesterol was recognized long ago, but studies along more quantitative lines are relatively recent. The normal plasma cholesterol content appears (1) to be chiefly regulated by the liver, which manufactures and discharges cholesterol into plasma, then later removes and destroys or converts it into cholic acid and perhaps other substances. Pihl (2) studied the simultaneous effects of bile acids on absorption and liver deposition of cholesterol. He found that on a fat-free diet, ingested cholic acid did not increase cholesterol absorption but did increase the deposition of this sterol in the liver. Other investigators (3, 4) have reported that in mice and rabbits dietary cholic acid leads to liver deposition of cholesterol even in the absence of this sterol in the diet. Also pertinent are the findings of Endo (5) on the composition of gallstones removed from patients showing correlations between the amounts of cholesterol and bile acids in the stones.

These and other biological studies suggested that it may be of interest to explore the possible influence of cholate and related cholates on the *in vitro* physical chemical nucleation, growth, and dissolution behavior of cholesterol particles in aqueous media. The first phase of this study, presented and discussed in this communication, shows that even at very low concentrations in the high pH region cholate exerts marked inhibitory effects on the growth and dissolution processes. The magnitude of these effects is sufficiently great so that this mechanism of cholate inhibition should not be excluded from consideration wherever biological cholesterol deposition processes are being examined.

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EXPERIMENTAL

General Considerations.—The general aspects of the method of study involving the use of the Coulter counter have been described previously (6). In the present work cholesterol supersaturations for the growth studies were achieved by a mixing method similar to *Method C* described in the previous work. However, instead of employing alcohol cholesterol solutions, sodium cholate was used as a solubilizing agent for cholesterol, and these solutions were rapidly mixed with large volumes of aqueous saline solutions to provide the desired supersaturation levels.

Preparation of Sodium Cholate.—Sodium cholate was prepared by the reaction of cholic acid with reagent grade sodium hydroxide. The cholic acid used was reagent grade cholic acid recrystallized five times from 95% ethanol.

The sodium cholate was purified by extracting out the unreacted cholic acid with chloroform. The extraction step was repeated four times. The results of chemical analysis are shown in Table I.

TABLE I.—ELEMENTAL ANALYSIS OF SODIUM CHOLATE SAMPLE

Element	Theoret.	Found
C	66.98	66.94
H	9.18	9.26
Na	5.35	5.39

Preparation of Cholesterol-Sodium Cholate Stock Solution.—Purified cholesterol (6, 7) crystals were added in excess to a 2.0% solution of sodium cholate in saline (0.90% aqueous sodium chloride). The suspension was stirred at 25° for 2 to 3 weeks using a magnetic stirrer and a Teflon-coated stirring bar. Then the suspensions were filtered through a 0.22 μ pore size filter (Millipore) (6) and the filtrate analyzed (7) for cholesterol.

Procedure for Growth Studies.—Two different series of Tris[1] and phosphate buffer solutions (0.10 *M*)

[1] Tris (hydroxymethyl) amino methane. Supplied as Tham by the Fisher Scientific Co., Detroit, Mich.

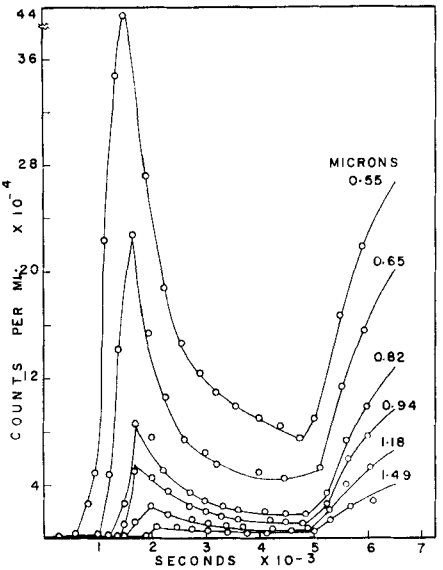

Fig. 1.—Coulter counter data showing the increases in cumulative counts with time at various threshold settings as cholesterol particles grow out of sodium cholate–cholesterol stock solution at pH 5.0. Sodium cholate concentration = 0.132 mg. ml.$^{-1}$. Cholesterol concentration = 0.66 mcg. ml.$^{-1}$.

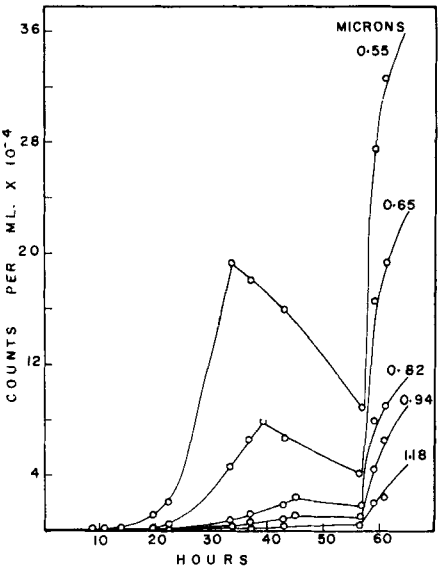

Fig. 2.—Coulter counter data showing the increases in cumulative counts of cholesterol particles with time at various threshold settings at pH 7.6. Sodium cholate concentration = 0.132 mg. ml.$^{-1}$. Cholesterol concentration = 0.66 mcg. ml.$^{-1}$.

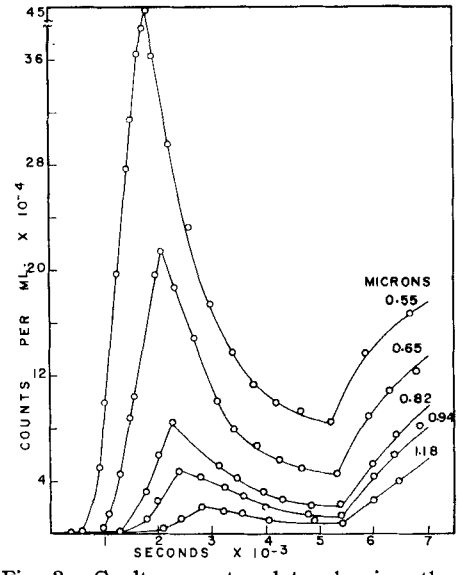

Fig. 3.—Coulter counter data showing the increases in cumulative counts of cholesterol particles with time at various threshold settings at pH 5.7. Sodium cholate concentration = 0.66 mg. ml.$^{-1}$. Cholesterol concentration = 0.66 mcg. ml.$^{-1}$

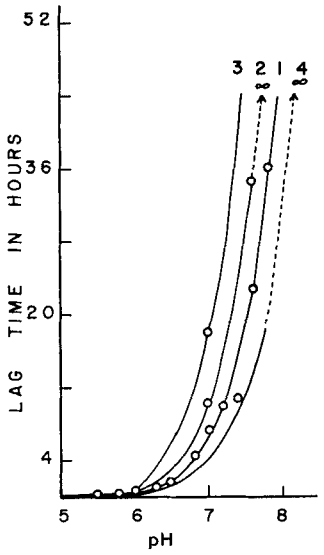

Fig. 4.—Lag time curves at various pH's constructed from the growth experiments. Key: 1, 0.132 mg. ml.$^{-1}$ sodium cholate; 0.66 mcg. ml.$^{-1}$ cholesterol. 2, 0.66 mg. ml.$^{-1}$ sodium cholate; 0.66 mcg. ml.$^{-1}$ cholesterol. 3, 0.66 mg. ml.$^{-1}$ sodium cholate; 3.30 mcg. ml.$^{-1}$ cholesterol. 4, 0.066 mg. ml.$^{-1}$ sodium cholate; 0.33 mcg. ml.$^{-1}$ cholesterol.

at different pH values were prepared. From each buffer solution 150 ml. was measured and placed in a water-jacketed beaker previously positioned on the Coulter counter. Then a predetermined volume of the 2% sodium cholate–cholesterol was added, and the growth was followed as before (6). In all experiments the rate of addition of the mixing solution and stirring during mixing was maintained constant. During the growth phase, constant mild agitation was maintained by means of a propeller stirrer at 50 r.p.m.

Procedure for Dissolution Studies.—The stock suspensions of cholesterol particles were prepared by adding 5 ml. of a 1.0 mg. ml.$^{-1}$ alcoholic cholesterol solution to 1 L. of saline and stirring the resulting suspension for 4 days to ensure the complete con-

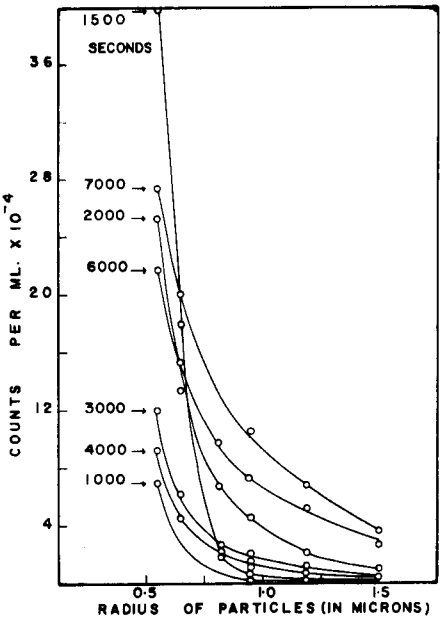

Fig. 5.—Cumulative particle size distribution curves constructed from data in Fig. 1.

version of any unstable phase to the stable phase of cholesterol (6). Then 10 ml. of this suspension was added to 150 ml. of each of the buffer solutions used in the growth studies. As was done previously (6), the dissolution rate was followed with the Coulter counter. The experiments were then repeated with the same buffers but with added cholate in the same concentration range as in the growth studies.

Solubility Determination of Cholesterol in the Presence of Cholate.—These experiments were designed to test the presence of (or absence of) any appreciable solution interaction between cholate and cholesterol in the solution concentration range of interest. About 50 mg. of cholesterol was added to 100 ml. of the above-mentioned buffers with and without added cholate (corresponding to 0.13 mg. $ml.^{-1}$) and placed in 125-ml. vials. These were sealed and rotated for 20 days in a water bath at 30°, after which time they were removed, the suspensions filtered, and the filtrates analyzed for cholesterol. The analytical procedure for cholesterol determination differed slightly from that previously described (7) in that filtrate was concentrated by flash evaporation to a volume of 1 ml. and then extracted with *n*-hexane. The hexane solution was then flash-evaporated to dryness, and the same procedure was followed beyond this point.

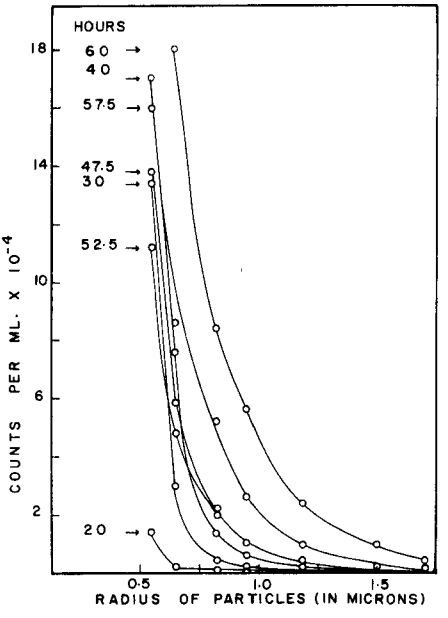

Fig. 6.—Cumulative particle size distribution curves constructed from data in Fig. 2.

RESULTS

Growth Studies.—Figures 1 and 2 give the data obtained with the Coulter counter for two different pH values (pH = 5.0 and 7.6) when the cholesterol and cholate concentrations were the same. These may be considered as representative of the two extreme types with respect to the time dependence. In the low pH experiments with cholate, the rates of growth and dissolution of the first-appearing phase and the growth rate of the stable phase were always relatively rapid, approaching the behavior in plain saline (6). The same behavior was observed at all pH values when cholate was absent, *e.g.*, in the experiments involving the addition of an alcoholic cholesterol solution to buffer not containing cholate. However, at high pH and in the presence of cholate all three processes appeared to be markedly retarded and, in some cases, completely inhibited.

The general behavior was also found to be independent of the two buffers used at the same pH. Therefore, only the results with the Tris buffers will be discussed here. Also no pH changes were noted during the experiments whenever the buffers were used.

Figure 3 shows the results at pH 5.7 when the cholesterol concentration was the same as in Figs. 1 and 2 but when the total cholate concentration was 5 times greater. It is interesting to note that the behavior shown in Fig. 3 is much like that shown in Fig. 1. As the pKa for cholic acid is around 5.0 (8), the cholate ion concentration in the Fig. 3 experiment should have been considerably greater than in the Fig. 2 experiment. Therefore, the rate retardation phenomenon is not dependent on only the cholate ion concentration. This strongly suggests that the presence of cholic acid in solution is also an important factor, this species catalyzing the growth processes and therefore tending to offset the effects of the cholate ion.

In order to estimate the rates of the various processes, several methods for treating the data were employed. First, from the plots such as those given in Figs. 1–3, the lag time for a given experiment may be estimated by determining the times for appearance of appreciable counts at the lowest threshold setting on the instrument. This would correspond to the times at which the uppermost curves in Figs. 1–3 begin to build up— *viz.*, ~600 sec. in Fig. 1, ~24 hr. in Fig. 2, and ~600 sec. in Fig. 3.

The lag times determined in this manner are plotted in Fig. 4 for all of the experiments in the Tris buffers. It is apparent from these results that pH

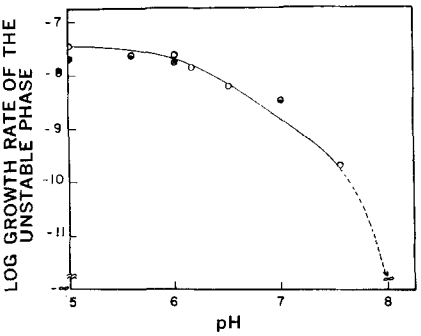

Fig. 7.—The log growth rates of the unstable cholesterol phase at various pH's and at various sodium cholate concentrations. Key: ○, 0.132 mg. ml.$^{-1}$ sodium cholate; 0.66 mcg. ml.$^{-1}$ cholesterol. ◒, 0.66 mg. ml.$^{-1}$ sodium cholate; 0.66 mcg. ml.$^{-1}$ cholesterol. ●, 0.066 mg. ml.$^{-1}$ sodium cholate; 0.33 mcg. ml.$^{-1}$ cholesterol. ⊗, 0.66 mg. ml.$^{-1}$ sodium cholate; 3.30 mcg. ml.$^{-1}$ cholesterol.

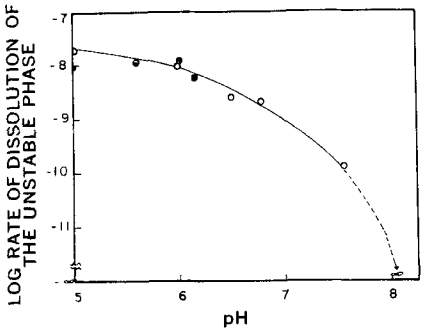

Fig. 8.—The log dissolution rates of the unstable cholesterol phase at various pH's and various sodium cholate concentrations. Key: ○, 0.132 mg. ml.$^{-1}$ sodium cholate; 0.66 mcg. ml.$^{-1}$ cholesterol. ◒, 0.66 mg. ml.$^{-1}$ sodium cholate; 0.66 mcg. ml.$^{-1}$ cholesterol. ●, 0.066 mg. ml.$^{-1}$ sodium cholate; 0.33 mcg. ml.$^{-1}$ cholesterol. ⊗, 0.66 mg. ml.$^{-1}$ sodium cholate; 3.30 mcg. ml.$^{-1}$ cholesterol.

is a very important factor influencing the time of appearance of the first phase during growth.

In order to estimate the growth and the dissolution rates of the first phase and the growth rate of the stable phase, the data in Figs. 1 and 2 have been replotted as cumulative particle size distribution curves in Figs. 5 and 6. From plots such as these, all three rates may be easily estimated when sufficient data were taken in a given experiment. The results of the determinations of these rates for growth and dissolution of the first phase and growth for the stable phase are shown in Figs. 7–9.

The uncertainties in the rate data in Figs. 7–9 are as large as about 50% in many instances because of the different estimated rates for different places along the size distribution curves. However, because ranges in rates of several orders of magnitude are under consideration here, these uncertainties do not alter the conclusions that will be presented.

In the experiments at the highest pH (pH 8.0), the precipitation of cholesterol was completely inhibited up to over 72 hr., even though in some cases the supersaturation was more than 100 times greater than the solubility. The $-\infty$ points in Figs. 7–9 correspond to these experiments.

It is noteworthy that the amount of cholesterol present during growth was of secondary importance as far as growth rates are concerned. The results with 0.33 mcg. ml.$^{-1}$ and 0.66 mcg. ml.$^{-1}$ cholesterol appear to fall nearly on the same curves in Figs. 7–9. It should be mentioned, however, that the number of particles at any given time was approximately proportional to the total cholesterol concentration when all other factors were the same.

With regard to pH dependence, the results shown in Figs. 7–9 appear to parallel each other. The rates for all processes become reduced with increasing pH. Furthermore, at low pH the rates

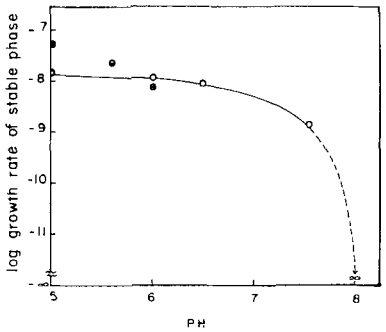

Fig. 9.—The log growth rates of the stable cholesterol phase at various pH's and various sodium cholate concentrations. Key: ○, 0.132 mg. ml.$^{-1}$ sodium cholate; 0.66 mcg. ml.$^{-1}$ cholesterol. ◒, 0.66 mg. ml.$^{-1}$ sodium cholate; 0.66 mcg. ml.$^{-1}$ cholesterol. ●, 0.066 mg. ml.$^{-1}$ sodium cholate; 0.33 mcg. ml.$^{-1}$ cholesterol. ⊗, 0.66 mg. ml.$^{-1}$ sodium cholate; 3.30 mcg. ml.$^{-1}$ cholesterol.

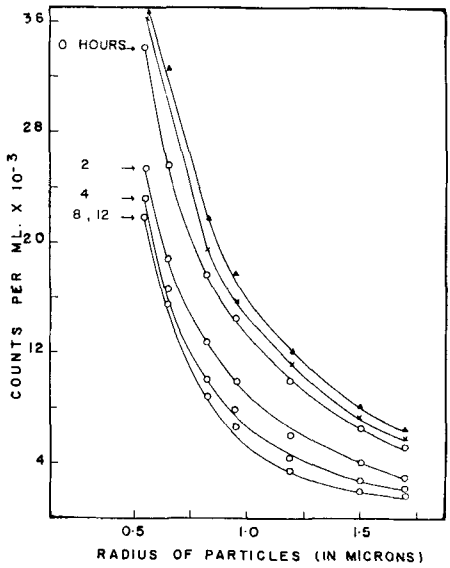

Fig. 10.—Cumulative particle size distribution curves for the dissolution of cholesterol particles at pH 8.0 in the presence and in the absence of sodium cholate. Key: ○, no sodium cholate present (blank); △, in the presence of 0.132 mg. ml.$^{-1}$ sodium cholate at 0 hr.; ×, in the presence of 0.132 mg. ml.$^{-1}$ sodium cholate after 26 hr.

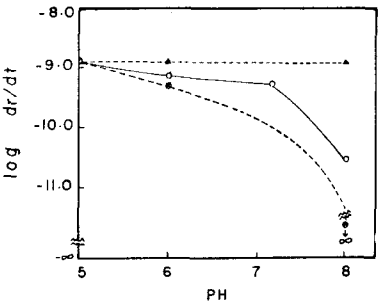

Fig. 11.—The log rates of dissolution of cholesterol particles at various pH's in the presence and in the absence of sodium cholate. Key: △, no sodium cholate present (blank); ○, in the presence of 0.132 mg. ml.$^{-1}$ sodium cholate; ⊗, in the presence of 0.66 mg. ml.$^{-1}$ sodium cholate.

appear to be independent of the cholate ion concentration. This means that at low pH, even when the cholate ion concentration was high enough for inhibition at the higher pH, no significant retardation was observed. This again is consistent with the idea of cholic acid catalysis.

Dissolution Rate Studies.—When no cholate was present or at low pH when cholate was present, the dissolution behavior of the cholesterol particles was much like that in plain saline (6). These relatively rapid rates correspond to essentially diffusion controlled rates.

However, with cholate in the higher pH region, considerable retardation of rates was observed. For example, Fig. 10 shows that the presence of 0.133 mg. ml.$^{-1}$ cholate drastically reduced the dissolution rate at pH 8.

In Fig. 11, the initial rates of dissolution are presented. As was done previously (6), these rates were estimated from the initial rates of translation of the particle size distribution curves such as those given in Fig. 10. It was necessary to consider only the initial rates because in these experiments the suspension concentrations were relatively high; consequently, the solution build-up rate of cholesterol was relatively rapid.

The results given in Fig. 11 again point out the importance of pH when cholate was present. There is marked reduction in rates over the diffusion controlled rates at high pH.

Solubility Determinations.—The cholesterol solubility study yielded values ranging from about 0.022 to 0.026 mcg. ml.$^{-1}$ for all pH values with and without 0.132 mg. ml.$^{-1}$ cholate. It appears therefore that solution interaction of cholesterol and cholate was not an important factor in the present study.

DISCUSSION

All of the present findings appear to be consistent with the idea that an interfacial phenomenon involving the cholate ion is responsible for the retardation and inhibition of the growth and dissolution of both phases of cholesterol. At high pH, where the effects of the additive are most important, the cholate ion is the predominant species.

Other mechanisms seem to be unimportant in the present situation. From the solubility study it has been found that little tendency exists for a cholate–cholesterol solution interaction in the concentration ranges of interest. Even if some solubilization tendency were observed, the rate results are always in the direction opposite to that predicted by any equilibrium interaction. Also solution desolubilization (or decomplexation) kinetics must be ruled out because, when an additional experiment was carried out in which an alcoholic cholesterol solution was added to a large amount of buffered saline containing cholate, the same effects were observed.

It is somewhat surprising that cholate, a relatively rigid ion, would adsorb at such low concentrations so strongly causing effects of such magnitude. Furthermore, it is also surprising that it appears to be the cholate ion rather than cholic acid or a mixture of the two species that is responsible for the inhibitory effects.

The proposed mechanism should be applicable to both cholesterol phases, although the retardation of rates appears to begin at somewhat lower pH values for the unstable phase (see Figs. 7 and 8), suggesting that there may be some quantitative differences. Therefore, the mechanism may be a relatively nonspecific one to the extent that other compounds with the basic four-ring structure characteristic of cholesterol and cholate may show similar effects. Such compounds as taurocholate and glycocholate are now being investigated in our laboratories.

While the inhibitory effects of the cholate ion, as discussed above, are extremely interesting, the catalytic role of cholic acid on the rates is very noteworthy. This catalytic effect may be some kind of a competitive adsorption involving the cholic acid molecule and the cholate ion, resulting in the reduction of the effectiveness of the barrier responsible for the retardation and inhibition of cholesterol growth and dissolution. More studies on both aspects are necessary for a more complete characterization of this problem from the physical chemical standpoint.

Because these effects are important in the physiological pH range and because they occur at very low cholate levels, the possible biological importance of these findings is apparent. It is likely that the present observations and the results of future studies along these same lines will contribute considerably toward the eventual understanding of the complex biological aspects of the deposition of cholesterol and of other substances in the body.

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