The Effect of Temperature on the Precipitation of Cholesterol in an *in Vitro* System[1] (34941)

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The incubation of saturated solutions of cholesterol in triglycerides with certain dicarboxylic acids or with imidozole leads to the partial precipitation of cholesterol as a 1:1 clathrate with the precipitating agent. By this means the concentration of cholesterol in the triglyceride may be reduced to approximately one-half of saturation. No further precipitation can be brought about, even with large excesses of precipitating agent (1). This finding led to the conclusion that cholesterol in triglyceride solutions exists in two distinct "states of dispersion"; a less stable form that yields insoluble clathrates with appropriate dicarboxylic acid or imidazole, and a more stable form that does not yield clathrates (2). The results would suggest that, under the experimental conditions employed, the two forms are present at saturation in approximately equal amounts.

Parker and Bhaskar have now shown that cholesterol in carbon tetrachloride solution exists as a monomer at concentrations up to about 0.014 *M* (3). As the concentration is increased, cholesterol associates to form a dimer and at a concentration of $\sim$ 0.06 *M* a higher aggregate begins to form which becomes the predominant species at a concentration of $\sim$ 0.2 *M*. A marked temperature effect was observed with high temperature (53°) favoring the monomer and low temperature (5°) favoring the dimer and/or higher aggregates.

The findings with respect to the existence of cholesterol in various forms have been confirmed by Wright and Gaylor for cholesterol in triglyceride solution (4). In these studies, the first overtone of the fundamental OH-stretching frequency, rather than the fundamental itself, of cholesterol was followed in the near infrared region with a Cary recording spectrophotometer. Presumably, it is the dimer and/or higher forms of cholesterol that is/are precipitable with pimelic acid or imidazole while the monomer is not precipitable under the same conditions. The dimer and/or higher forms of cholesterol would correspond to the previously characterized "less stable" form of cholesterol while the monomer would correspond to the previously characterized "more stable" form of cholesterol.

The present studies are concerned with the precipitation of cholesterol by pimelic acid or imidazole at various temperatures. It was anticipated from the results of Parker and Bhaskar that cholesterol in triglycerides would be subjected to two opposing forces. At relatively high temperatures the solubility of cholesterol would be expected to increase, giving rise to relatively high concentrations of cholesterol where the dimer and/or higher forms would predominate solely as a result of the high concentration. On the other hand, high temperatures would lead to an equilibrium favoring the monomer. Conversely, at relatively low temperatures the solubility of cholesterol would be expected to decrease, giving rise to relatively low concentrations of cholesterol where the dimer and/or higher forms would not predominate as a result of the low concentration. Similarly, the low temperature would lead to an equilibrium favor-

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ing the dimer and/or higher forms. If this reasoning is correct, precipitation of cholesterol should be a maximum at some intermediate temperature where the temperature is high enough for large amounts of cholesterol to be dissolved with resulting increased amounts of dimer and/or higher forms to be present and, at the same time, low enough for the equilibrium to favor increased amounts of the dimer and/or higher forms. This situation has actually been found to exist with a maximum in the percentage of cholesterol precipitable with pimelic acid or imidazole occurring at about 40°, a temperature essentially synonomous with body temperature.

Materials and Methods. The cholesterol precipitation studies were carried out essentially as described previously in detail (1, 2). In brief, various amounts of cholesterol-4-^{14}C, sufficient to exactly saturate the triglycerides at the temperatures studied, as determined in preliminary experiments, were weighed out into 100 × 13 mm screw-capped Pyrex test tubes. The temperatures studied and the levels of cholesterol in milligrams per tube necessary to saturate corn oil at the temperatures studied are as follows: 5°, 30 mg; 22°, 50 mg; 38°, 64 mg; and 63°, 130 mg. For coconut oil, the values are as follows: 38°, 100 mg; 63°, 180 mg. Since coconut oil is a solid at both 5 and 22°, it was impossible to study the precipitation of cholesterol from coconut oil at these lower temperatures. Two milliliters of triglyceride were added to each tube. The tubes were then stoppered tightly and placed in a specially constructed apparatus that rotated the tubes at a rate of 60 rotations per minute. Rotation with incubation at the various temperatures was carried out for various lengths of time in an attempt to obtain complete equilibrium. In some experiments, after the initial incubation, the tubes were cooled at 5° for 18–24 hr to obviate possible instances of supersaturation, after which the tubes were subjected to an additional incubation period for 18–24 hr at the temperature studied. After incubation, the tubes were centrifuged for 10 min in a clinical centrifuge maintained at the particular incubation temperature studied. Approximately 1-ml aliquots of the supernatant solution from each tube were transferred by disposable pipettes to tared counting vials. After weighing, 10 ml of scintillation solution were added to each vial, and the solutions were counted in a liquid scintillation spectrometer. From the counts obtained and the weights of triglyceride solutions sampled, appropriate calculations of the percentage of cholesterol precipitated from saturated solutions at the various temperatures studied could be calculated. The cholesterol-4-^{14}C has an activity of about 200 dpm/mg (180 cpm/mg). The corn oil used was Mazola. The coconut oil was a commercial pharmaceutical preparation from either Magnus, Mabee & Reynard, Inc., or Gentry Corp.

Results and Discussion. The data obtained are presented in Tables I and II and by Fig. 1. With corn oil as the triglyceride and pimelic acid as the precipitating agent, no precipitation of cholsterol takes place at 5° and only a small amount of precipitation

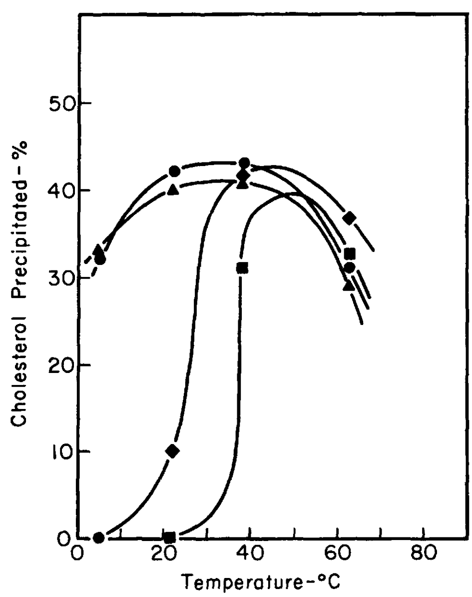

FIG. 1. The effect of temperature on the precipitation of cholesterol by pimelic acid or imidazole from solution in corn oil. Pimelic acid, no cooling period (■—■); pimelic acid, cooling period (♦—♦); imidazole, no cooling period (▲—▲); imidazole, cooling period (●—●).

TABLE I. The Effect of Temperature on the Precipitation of Cholesterol by Pimelic Acid from Solution in Corn Oil or Coconut Oil.

			Cholesterol in solution	
Tri-glyceride	Temper-ature (°)	Incu-bation (hr)	Without pimelic acid (cpm/g)	With pimelic acid (cpm/g)
Without an intervening cooling period				
Corn oil	5	21½	2900	3000 (0)[a]
	5	91½	3000	3060 (0)
	22	29½	4900	5120 (0)
	22	53	5100	5250 (0)
	38	24	6680	5640 (15)
	38	47½	6890	4760 (31)
	63	52	12,400	8320 (33)
	63	72	12,350	8720 (30)
Coconut oil	38	15½	10,100	6100 (40)
	38	19	10,000	6085 (39)
With an intervening cooling period				
Corn oil	5	72	2980	3110 (0)
	22	72	5280	4770 (10)
	38	72	7540	4770 (37)
	63	72	12,750	8040 (37)
Coconut oil	38	48	9000	4940 (45)
	63	48	17,100	11,050 (35)

[a] The figures in parentheses indicate the percentage of cholesterol precipitated by the presence of pimelic acid. These figures are arrived at by dividing the amount of cholesterol in solution after incubation with pimelic acid by the amount of cholesterol in saturated solution after incubation without pimelic acid and multiplying this value by 100 to give the percentage of cholesterol remaining in solution after incubation with pimelic acid which, after subtracting from 100, yields the percentage of cholesterol actually precipitated from saturated solution by incubation with pimelic acid.

takes place at 22°. This lack of significant precipitation at these lower temperatures is probably attributable to the very low solubility of pimelic acid in corn oil at these temperatures such that a "solubility product" between cholesterol and pimelic acid is not exceeded. Precipitation of cholesterol by pimelic acid occurs at 38°, particularly if an intervening cooling period to favor crystallization is carried out. Precipitation at 63° also occurs, but the amount of cholesterol precipitated at 63° is less than that precipitated at 38°, so that a maximum in the percentage of cholesterol precipitated is observed at around 40–42°. With corn oil as the triglyceride and imidazole as the precipitating agent, precipitation of cholesterol occurs at all temperatures studied with a definite maximum in the percentage of cholesterol precipitated occurring at around 38–40°. The precipitation of cholesterol by imidazole at 5 and 22°, in contrast to the results obtained at these temperatures with pimelic acid, is probably attributable to the much greater solubility of imidazole as compared with pimelic acid so that a "solubility product" is readily exceeded. With coconut oil as the triglyceride and either pimelic acid or imidazole as the precipitating agent, the percentage of cholesterol precipitated at 38° is greater than that precipitated at 63°.

TABLE II. The Effect of Temperature on the Precipitation of Cholesterol by Imidazole from Solution in Corn Oil or Coconut Oil.

			Cholesterol in solution	
Tri-glyceride	Temper-ature (°)	Incu-bation (hr)	Without imidazole (cpm/g)	With imidazole (cpm/g)
Without an intervening cooling period				
Corn oil	5	19¼	2720	2080 (23)[a]
	5	41½	3000	2020 (32)
	22	26	5120	3020 (41)
	22	50	5100	3070 (40)
	38	24	6680	3950 (41)
	38	47½	6890	4120 (40)
	63	52	12,400	8300 (33)
	63	72	12,350	8700 (30)
Coconut oil	38	48	10,000	5600 (44)
With an intervening cooling period				
Corn oil	5	72	2980	2030 (32)
	22	72	5280	3050 (42)
	38	72	7540	4330 (43)
	63	72	12,750	8780 (31)
Coconut oil	38	48	9070	5080 (44)
	63	48	17,100	11,000 (36)

[a] Figures in parentheses indicate the percentage of cholesterol precipitated by the presence of imidazole. See Table I for the method of calculation.

Although other possible interpretations have not been ruled out, the present results are compatible with the following explanation: Pimelic acid, imidazole, and a few other related compounds precipitate cholesterol from solution in triglyceride by forming an insoluble clathrate with a dimer and/or higher aggregate of cholesterol. No precipitation occurs with the monomer of cholesterol. Higher temperatures favor increased solubility of cholesterol with an attendant increased proportion of concentration-dependent dimer and/or higher aggregate. At the same time, the higher temperatures are associated with a change in equilibrium favoring the monomeric form of cholesterol. Lower temperatures favor decreased solubility of cholesterol with an attendant decreased proportion of concentration-dependent dimer and/or higher aggregate. At the same time, the lower temperatures are associated with a change in equilibrium favoring the dimeric or higher form of cholesterol. At an intermediate temperature, a maximum occurs in the proportion of dimer and/or higher aggregate of cholesterol so that maximum precipitation by clathrate-forming agent occurs. It happens that this maximum is in the order of 38–42°.

If the above explanation has any merit, it would appear that at a given temperature and with a given precipitating agent, cholesterol is precipitated until the concentration of cholesterol is reduced to the point where there is insufficient dimer and/or higher aggregate to exceed the "solubility product" of the clathrate. In previously reported studies, involving a variety of triglycerides and several precipitating agents and a single temperature of 38°, the amount of cholesterol remaining in solution in the presence of an excess of precipitating agent averaged about 57%. In other words, about 43% or approximately one half of the cholesterol was precipitated from saturated solution leading to the conclusion that cholesterol is present in oils in two separate states of dispersion which, at saturation at the temperature used, are approximately equal in amount.

In vitro studies of the precipitation of cholesterol by hydrogen bonding with various agents may or may not have any analogy with the deposition of cholestrol *in vivo*. The possibility has been suggested, however, a number of times (2–4) and additional studies on the behavior of cholesterol under *in vitro* conditions seem warranted.

Summary. A maximum exists at approximately body temperature in the amount of cholesterol that may be precipitated by pimelic acid or imidazole from saturated solutions of cholesterol in triglycerides.

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