Additional Evidence Substantiating the Existence of a Readily Precipitable Form of Cholesterol in Triglyceride Solution[1] (35789)

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Evidence has been presented from these laboratories that cholesterol, at saturation in triglycerides, exists in two distinct "states of dispersion" (1). Part of the cholesterol, in the order of 40%, can be precipitated, presumably as a hydrogen-bonded clathrate, with certain dicarboxylic acids or with imidazole (2). This fraction of the total cholesterol was described as a "less stable" form. The remainder of the cholesterol, in the order of 60%, cannot be precipitated by any amount of dicarboxylic acid or imidazole. This fraction of the total cholesterol was described as a "more stable" form. Subsequently, Parker and Bhaskar (3) have shown by infrared spectrometry that cholesterol in solution may exist in two or more states of aggregation that are concentration and temperature dependent. These findings have been confirmed by Wright and Gaylor (4). According to Parker and Bhaskar (3), high temperatures and low concentrations favor the existence of a monomer of cholesterol, while low temperatures and high concentrations favor the existence of a dimer and/or higher form of cholesterol. Presumably it is the dimer and/or higher form of cholesterol that is "less stable" and precipitable with certain dicarboxylic acids or imidazole, while the monomeer is "more stable" and is not precipitable under these conditions.

While the above hypothesis is an attractive one, further substantiation is in order. It has been suggested that perhaps all the cholesterol in saturated solution in triglycerides, irrespective of its state of dispersion, forms clathrates with certain dicarboxylic acids or imidazole and that cholesterol appearing as a precipitate, represents "insoluble clathrate," while that cholesterol remaining in solution represents "soluble clathrate." According to this view, there need be no difference in reactivity between monomer and dimer and/or higher form with respect to the formation of clathrates with dicarboxylic acids or imidazole.

It is the purpose of this paper to show, with the aid of ^{14}C-pimelic acid and ^{14}C-imidazole, the essential absence of "soluble" clathrates of cholesterol in triglyceride solution in the presence of clathrate-forming agents, thus further substantiating evidence for the existence of a distinct form(s) of cholesterol readily susceptible to precipitation under the *in vitro* conditions of the present and prior studies.

Materials and Methods. The cholesterol precipitation studies were carried out essentially as described previously in detail (1, 2, 4). In brief, 100-mg amounts of either ^{14}C-labeled or unlabeled cholesterol (an amount sufficient to saturate 2 ml of coconut oil at 37°), 40-mg amounts of ^{14}C-labeled or unlabeled pimelic acid, and 40-mg amounts of ^{14}C-labeled or unlabeled imidazole, alone or in various appropriate combinations, were weighed out into 100 × 13-mm screw-capped Pyrex test tubes. All tubes then received 2 ml of freshly-melted coconut oil. The tubes were then stoppered tightly and placed in a specially constructed apparatus that rotated the tubes at a rate of 60 rotations/min at 37°. Following 18–24 hrs incubation, the tubes were centrifuged for 10 min in a clinical centrifuged maintained at 37°. Approximately 1-ml aliquots of the supernatant solution from each tube were transferred, by disposable

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pipettes, to tared counting vials. After weighing, 10 ml of scintillation solution was added to each vial, and the solutions were counted in a liquid scintillation spectrometer. From the counts obtained and a knowledge of the radioactivity of the cholesterol, the pimelic acid, and the imidazole used, it was possible to calculate the distribution of the radioactivity in solution, in undissolved material, and in material precipitated as a clathrate.

Cholesterol of relatively low radioactivity was prepared by evaporating to dryness on the steam bath a solution prepared from 100 g of cholesterol (Distillation Products Industries, Rochester, N.Y.) and 100 μg (10 μCi) 4-^{14}C-cholesterol (benzene solution, New England Nuclear Corp., Boston, Mass.) in 500 ml of chloroform. The product was pulverized in a laboratory mortar before use. The preparation used in these studies had an activity of 220 cpm/mg (250 dpm/mg). A test of purity by the method of solubility in coconut oil indicated a purity of about 95%.

Pimelic acid of relatively high radioactivity was prepared by crystallizing from water, after Norit treatment, a few milligrams of 1, 7-^{14}C-pimelic acid (2.75 mCi/mmole, Orlando Research, Inc., Orlando, Fla.) and 10 g of unlabeled pimelic acid (Nutritional Biochemicals Corp., Cleveland, O.). This preparation had an activity of 56,000 cpm/mg. Material of relatively low activity, suitable for the present studies, was prepared by recrystallizing from water, following Norit treatment, a mixture of 1 g of the high potency labeled pimelic acid and 10 g of unlabeled pimelic acid. The low potency pimelic acid had an activity of 5500 cpm/mg (6250 dpm/mg). A test of purity by the method of solubility in coconut oil indicated a purity of about 98%.

Imidazole of relatively low activity was prepared by the combined methods of Milas and Terry (5) and of Synder *et al.* (6), starting with 2,3-^{14}C-fumaric acid. The low potency material had an activity of about 560 cpm/mg (635 dpm/mg). A test of purity by the method of solubility in coconut oil indicated a purity of about 93%. I am indebted to Dr. Helen G. Oien, Merck Institute for Therapeutic Research, Rahway, New Jersey, for the labeled imidazole.

The coconut oil used in these studies was from Morgan Products Corp., New York, N.Y.

Quantitation of the various ^{14}C-labeled compounds following the incubations has been done in terms of coconut oil (cpm/g) from which actual amounts of the compounds could be calculated from specific activities.

Results and Discussion. The cholesterol precipitation studies with either pimelic acid or imidazole were designed primarily to determine the amount of clathrate-forming agent soluble in coconut oil alone, as compared with the amount of clathrate-forming agent encountered in the coconut oil in the presence of cholesterol precipitate. The difference in the amount of clathrate-forming agent encountered under the two conditions would represent the amount of clathrate-forming agent present as "soluble" clathrate. If this difference were large, it would indicate the existence of significant amounts of "soluble" clathrate and would be evidence for the existence of uniformly-reacting cholesterol at saturation in coconut oil, where part of the cholesterol clathrate would be soluble and part would be insoluble. On the other hand, if the difference were small or negligible, it would indicate the absence of significant amounts of "soluble" clathrate and would be evidence for the existence of two or more species of cholesterol at saturation in coconut oil, one species of which precipitates with clathrate-forming agent, while the other species does not. Suitable controls have been included, demonstrating once again the phenomenon of cholesterol precipitation by clathrate-forming agents.

Typical protocols are summarized in Table I. With respect to experiments involving pimelic acid, 100 mg of cholesterol is sufficient to saturate 2 ml of coconut oil and provide a slight excess. Forty mg of pimelic acid is theoretically sufficient to precipitate 96 mg of cholesterol or 240% of that capable of being precipitated by any level of pimelic acid. In the presence of ^{14}C-cholesterol as the sole addition to coconut oil, 9650 cpm/g were found in the supernatant solution following incubation. This indicates a solubility of 4.40% for cholesterol in coconut oil. In the

TABLE I. Summary of Experiments.

Tube no.	Cholesterol (mg) ^{14}C-Labeled	Cholesterol (mg) Unlabeled	Pimelic acid (mg) ^{14}C-Labeled[a]	Pimelic acid (mg) Unlabeled	Coconut oil (g)	^{14}C-Activity in supernatant solution (cpm/g)	
Pimelic acid experiment							
1	100	—	—	—	2	9740	9650
2	100	—	—	—	2	9560	
3	100	—	—	40	2	5850	5825
4	100	—	—	40	2	5800	
5	—	—	40	—	2	5400	5440
6	—	—	40	—	2	5480	
7	—	100	40	—	2	8670	8645
8	—	100	40	—	2	8620	
			Imidazole (mg)				
Imidazole experiment			^{14}C-Labeled[b]	Unlabeled			
1	100	—	—	—	2	9520	9575
2	100	—	—	—	2	9630	
3	100	—	—	40	2	5500	5575
4	100	—	—	40	2	5650	
5	—	—	40	—	2	2030	2040
6	—	—	40	—	2	2050	
7	—	100	40	—	2	2410	2590
8	—	100	40	—	2	2770	

[a] 40 mg contained 220,000 cpm/2 g or 110,000 cpm/g of coconut oil.
[b] 40 mg contained 22,400 cpm/2 g or 11,200 cpm/g of coconut oil.

presence of ^{14}C-cholesterol and unlabeled pimelic acid addition to coconut oil, 5825 cpm/g found in the supernatant solution following incubation. This indicates a precipitation of 40% of the cholesterol. In the presence of ^{14}C-pimelic acid as the sole addition to coconut oil, 5440 cpm/g were found in the supernatant solution following incubation. This indicates a solubility of 0.10% for pimelic acid in coconut oil. In the presence of ^{14}C-pimelic acid and unlabeled cholesterol addition to coconut oil, 8645 cpm/g were found in the supernatant solution following incubation. The 20 mg/g of ^{14}C-pimelic acid provided 110,000 cpm/g. From the counts obtained in the control tubes involving ^{14}C-labeled cholesterol and unlabeled pimelic acid, it may be calculated that, under similar conditions involving unlabeled cholesterol and ^{14}C-pimelic acid, 45,100 cpm/g would be in the cholesterol–pimelic acid precipitate. Thus, the partition of ^{14}C-radioactivity from pimelic acid in tubes containing unlabeled cholesterol, ^{14}C-pimelic acid, and coconut oil is as follows:

8645 cpm/g in solution

$$\text{or } \frac{8645}{110{,}000} \times 100 = 7.9\% \text{ in solution,}$$

45,100 cpm/g in cholesterol–pimelic acid precipitate

$$\text{or } \frac{45{,}100}{110{,}000} \times 100 = 41.0\% \text{ in precipitate,}$$

110,000 − (8645 + 45,100) = 56,255 cpm/g in solid pimelic acid

$$\text{or } \frac{56{,}255}{110{,}000} \times 100 = 51.1\% \text{ in solid.}$$

Total 100.0%

The difference between 8645 cpm/g (the amount of radioactivity from ^{14}C-pimelic acid found in the presence of unlabeled cholesterol and ^{14}C-pimelic acid) and 5,440 cpm/g (the amount of radioactivity from

^{14}C-pimelic acid found in the presence of ^{14}C-pimelic acid alone) is 3205 cpm/g. This value is sufficient to account for only 1.4 mg/g out of 50 mg/g (2.8%) of cholesterol as "soluble" clathrate. Calculated another way, the partition of cholesterol following incubation of a saturating level of cholesterol in coconut oil with an excess of pimelic acid is as follows:

	(mg/g)
Cholesterol in solution	27.0
Cholesterol that conceivably might be present as "soluble" clathrate	1.4
Cholesterol present as insoluble cholesterol–pimelic acid clathrate	21.6
Total	50.0

Thus, it may be concluded that when cholesterol and excess pimelic acid are incubated with coconut oil, approximately 40% of the cholesterol is precipitated as an insoluble cholesterol–pimelic acid clathrate, but *there is essentially no soluble cholesterol–pimelic acid clathrate*. This finding is consistent with the previous conclusions that, at saturation of cholesterol in triglycerides, a "less stable" (dimer and/or higher form?) form of cholesterol precipitable by clathrate-forming agents and a "more stable" (monomer?) form of cholesterol not precipitable by clathrate-forming agents coexist in the approximate proportions of 40 to 60%, or roughly half and half.

With respect to experiments involving imidazole, similar controls were prepared involving 100 mg of ^{14}C-cholesterol, 40 mg of imidazole, and 2 ml of coconut oil. Forty mg of imidazole is theoretically sufficient to precipitate 227 mg of cholesterol or 570% of that capable of being precipitated by any level of imidazole. In the presence of ^{14}C-cholesterol as the sole addition to coconut oil, 9575 cpm/g were found in the supernatant solution following incubation. This indicates a solubility of 4.35% for cholesterol in coconut oil. In the presence of ^{14}C-cholesterol and unlabeled imidazole addition to coconut oil, 5575 cpm/g were found in the supernatant solution following incubation. This indicates a precipitation of 42% of the cholesterol. In the presence of ^{14}C-imidazole as the sole addition to coconut oil, 2040 cpm/g were found in the supernatant solution following incubation. This indicates a solubility of 0.36% for imidazole in coconut oil. In the presence of ^{14}C-imidazole and unlabeled cholesterol addition to coconut oil, 2590 cpm/g were found in the supernatant solution following incubation. The 20 mg/g of ^{14}C-imidazole provided 11,200 cpm/g. From the counts obtained in the control tubes involving ^{14}C-cholesterol and unlabeled imidazole, it may be calculated that under similar conditions involving unlabeled cholesterol and ^{14}C-imidazole, 2072 cpm are in the cholesterol-imidazole precipitate. Thus, the partition of ^{14}C-radioactivity from imidazole in tubes containing unlabeled cholesterol, ^{14}C-imidazole, and coconut oil is as follows:

2590 cpm/g in solution

$$\text{or } \frac{2590}{11{,}200} \times 100 = 23.2\% \text{ in solution,}$$

2072 cpm/g in cholesterol–imidazole precipitate

$$\text{or } \frac{2072}{11{,}200} \times 100 = 18.4\% \text{ in precipitate,}$$

$11{,}200 - (2590 + 2072) = 6538$ cpm/g in solid imidazole

$$\text{or } \frac{6538}{11{,}200} \times 100 = 58.4\% \text{ in solid.}$$

Total 100.0%

The difference between 2590 cpm/g (the amount of radioactivity from ^{14}C-imidazole found in the presence of unlabeled cholesterol and ^{14}C-imidazole) and 2040 cpm/g (the amount of radioactivity from ^{14}C-imidazole found in the presence of ^{14}C-imidazole alone) is 550 cpm/g. This value is sufficient to account for only 5.7 mg/g out of 50 mg/g (11%) of cholesterol as possible "soluble" clathrate. Calculated another way, the partition of cholesterol following incubation of a saturating level of cholesterol in coconut oil with an excess of imidazole is as follows:

	(mg/g)
Cholesterol in solution	25.0
Cholesterol that conceivably might be present as "soluble" clathrate	5.7
Cholesterol present as insoluble cholesterol–imidazole clathrate	19.3
Total	50.0

Thus, here with imidazole as with pimelic acid, it may be concluded that when cholesterol and excess imidazole are incubated with coconut oil, approximately 42% of the cholesterol is precipitated as an insoluble cholesterol–imidazole clathrate, but *there is essentially no soluble cholesterol–imidazole clathrate*. The conclusion from the imidazole experiments is the same as that drawn from the pimelic acid experiments; namely, that at saturation of cholesterol in triglycerides, a "less stable" form of cholesterol precipitable by clathrate-forming agents and a "more stable" form of cholesterol not precipitable by clathrate-forming agents coexist in triglycerides.

Summary. Experiments involving the precipitation of cholesterol from saturated solution in coconut oil by excess amounts of either ^{14}C-pimelic acid or ^{14}C-imidazole demonstrate that at equilibrium in either case there is essentially no more ^{14}C in solution than that due to the inherent solubility of the ^{14}C-pimelic acid or ^{14}C-imidazole in coconut oil. These findings are interpreted as indicating the absence of "soluble" clathrates of cholesterol and pimelic acid or imidazole and are consistent with the hypothesis that cholesterol at saturation in triglycerides is present both as a "less stable" (dimeric and/or higher form?) and as a "more stable" (monomeric?) form, where only the dimeric and/or higher form precipitates under the *in vitro* experimental conditions.

1. Wright, L. D., Proc. Soc. Exp. Biol. Med. **121**, 265 (1966).

2. Wright, L. D., and Presberg, J., Proc. Soc. Exp. Biol. Med. **115**, 497 (1964).

3. Parker, F. S., and Bhaskar, K. R., Biochemistry **7**, 1286 (1968).

4. Wright, L. D., and Gaylor, J. L., Lipids **4**, 526 (1969).

5. Milas, N. A., and Terry, E. M., J. Amer. Chem. Soc. **47**, 1412 (1925).

6. Snyder, H. R., Handrick, R. G., and Brooks, L. A., Org. Syn. **22**, 65.

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