The Effect of Phosphatidyl Choline in Preventing the Precipitation of Cholesterol in an *In Vitro* System[1] (35087)

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The incubation of saturated solutions of cholesterol in triglycerides with certain dicarboxylic acids or with imidazole leads to the precipitation of cholesterol, presumably as a hydrogen-bonded complex or clathrate, involving the cholesterol and dicarboxylic acid or imidazole in a 1:1 ratio (1). Under these conditions, the level of cholesterol in solution may be reduced to approximately one-half that originally present at saturation despite the addition of excess amounts of clathrate-forming agent (2). The reason for this apparent anomalous behavior of cholesterol in triglyceride solution (2) appears clear as a result of spectroscopic studies of solutions of cholesterol in various solvents.

Parker and Bhaskar (3) have shown self-association of cholesterol and interaction of cholesterol with triglycerides by infrared measurements of the cholesterol OH-stretching frequency. At concentrations below 0.014 *M* in CCl_4, cholesterol exists only as a monomer. As the concentration is increased, cholesterol associates to form a dimer; at a concentration of ~0.06 *M*, a higher aggregate begins to form which becomes the predominant species at a concentration of ~0.2 *M*. Infrared spectra of mixed solutions of cholesterol and triglycerides gave evidence of the formation of a 1:1 hydrogen-bonded complex. Furthermore, when triglycerides are added to solutions of cholesterol in carbon tetrachloride, the cholesterol is hydrogen bonded to the esters. The results of Parker and Bhaskar have been confirmed and extended by Wright and Gaylor (4), who employed near infrared measurements of the first overtone of the fundamental OH-stretching frequency of cholesterol in the region of 1.380 to 1.500 μ, rather than in the actual infrared region.

Employing these procedures, the occurrence of higher aggregates of cholesterol as the predominant form in chloroform solution of concentrations of higher than ~0.2 *M*, as first described by Parker and Bhaskar, could readily be confirmed. In addition, hydrogen bonding of cholesterol to methyl palmitate could readily be observed by the loss of absorbancy at 1.400 to 1.420 μ. It would appear that the reason the concentration of cholesterol in triglyceride solution cannot be reduced at 37° below about 50% by any amount of dicarboxylic acid or imidazole is that it is only the higher aggregates of cholesterol that are involved in hydrogen bonding and precipitation with clathrate-forming agent (5). Since the aggregates do not form at low concentrations of cholesterol, it is apparent that the cholesterol concentration cannot be reduced below a certain level by the presence of dicarboxylic acid or imidazole. Studies on the precipitation of cholesterol by pimelic acid or imidazole as influenced by incubation temperature have shown the existence of a maximum in the percentage of cholesterol that may be precipitated (6). This maximum occurs at about 38–40°.

The suggestion has been made by Wright (2) that hydrogen bond formation between cholesterol and tissue components containing

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regularly spaced atoms capable of sharing the hydroxyl hydrogens of cholesterol, in the same way that dicarboxylic acids of appropriate size hydrogen bond with cholesterol to yield precipitates, may be the initiator of precipitation of cholesterol under certain pathological conditions, such as atherosclerosis. Similarly, Parker and Bhaskar (3) have suggested that the hydrogen-bonding properties of cholesterol may be factors in the mechanism of plaque formation in atherosclerosis. Accordingly, we have extended our studies of cholesterol precipitation by hydrogen-bonding agents with the expectation that these *in vitro* studies might shed light concerning the precipitation of cholesterol *in vivo*.

It has been observed in this laboratory that the presence of small amounts of a commercial brain fraction (Folch fraction no. 1) prevents the precipitation of cholesterol by dicarboxylic acids. Fractionation and characterization studies have led to the identification of phosphatidyl choline as the active component of the brain fraction. Phosphatidyl choline is exceedingly active in "protecting" cholesterol from hydrogen bonding with a dicarboxylic acid. Under the experimental conditions described, one part of phosphatidyl choline is sufficient to prevent or markedly delay precipitation in the presence of approximately 2000–4000 parts of cholesterol. These studies on the inhibition of cholesterol precipitation by phosphatidyl choline are described in this paper.

Experimental Methods. Cholesterol-4-^{14}C 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) of cholesterol-4-^{14}C (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).

The coconut oil used was a commercial pharmaceutical preparation from either Magnus, Mabee and Reynard, Inc., New York, N. Y., or Gentry Corporation, Fairlawn, N. J.

The precipitation studies were carried out according to the following general procedure: 100-mg amounts of cholesterol-4-^{14}C were weighed out into 100 $\times$ 13-mm stoppered Pyrex test tubes. Forty mg of pimelic acid was added to each tube, except to those tubes carried through as controls involving no precipitation of cholesterol. Various amounts of the preparations or compounds to be studied with respect to influence on the precipitation of cholesterol were added to the tubes containing the dry cholesterol and pimelic acid. Two ml of freshly melted coconut oil 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/min. Rotation with incubation at 37° was continued, usually for approximately 18 hr or for an additional time, depending upon the purpose of the particular experiment. After incubation, the tubes were centrifuged for 10 min in a clinical centrifuge maintained at 37°. 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 [4 g of PPO (2, 3-diphenyloxazole) and 30 mg of POPOP (1,4-bis-2-[5-phenyloxazoyl]-benzene)/liter of toluene] were added to each vial and the solutions were counted in a Packard TriCarb Liquid Scintillation Spectrometer (Model 3375). Calculations were made of the amount of cholesterol remaining unprecipitated in the oil, making the assumption that the weighed aliquot taken was entirely coconut oil rather than a mixture of coconut oil, cholesterol, and, in most instances, material studied with respect to influence on precipitation. Since the solubility of cholesterol in coconut oil is in the order of 4% and the amounts of test materials used were insignificant, as far as weight is concerned, it is apparent that the results are not in error by more than about 4% by making this assumption. An experimental value in the order of 10,000 cpm/g indicates no precipitation of cholesterol, while a value in the order of 6000 cpm/g indicates

the maximum expected amount of precipitation. The experimental results have been characterized by an "all or none" effect. That is, under the experimental conditions described, cholesterol was usually either precipitated or not precipitated, and it was only occasionally that a stepwise, linear response was obtained.

The commercial bovine brain fraction studied was from either General Biochemicals, Inc., Chagrin Falls, O., or Sigma Chemical Co., St. Louis, Mo., and is described as Folch fraction no. 1 high in phosphatidyl inositol. Material from the two sources was indistinguishable as far as activity is concerned. The highly-purified egg and bovine phosphatidyl choline, lysophosphatidyl choline, phosphatidyl inositol, and sphingomyelin were from Supelco, Inc., Bellefonte, Pa. The synthetic L-α-phosphatidyl serine and L-α-phosphatidyl ethanol were from Nutritional Biochemicals Corp., Cleveland, O. Other preparations or compounds studied, which were all found to be inactive, were readily-available commercial preparations with little question concerning identity or purity.

Results. As indicated by the results of Expt. 1 (Table I), the addition of brain fraction no. 1 to an incubating mixture of

TABLE I. Summary of Experimental Findings.

Experiment	Pimelic acid	Material tested	Amount (mg/tube)	Duration of incubation (hr)	Triglyceride (epm/g)
1	—	None		20	9680
	+	None		20	5750
	+	Brain fraction #1	10.0	20	9900
2	—	None		22	9450
	+	None		22	5650
	+	Brain fraction #1	5.0	22	9980
	+	Brain fraction #1	10.0	22	9500
	+	Brain fraction #1	20.0	22	9500
	—	None		46	9600
	+	None		46	5460
	+	Brain fraction #1	5.0	46	10,100
	+	Brain fraction #1	10.0	46	9850
	+	Brain fraction #1	20.0	46	7300
	—	None		71	9980
	+	None		71	5575
	+	Brain fraction #1	5.0	71	10,300
	+	Brain fraction #1	10.0	71	10,400
	+	Brain fraction #1	20.0	71	5900
3	—	None		17	10,000
	+	None		17	6080
	+	Brain fraction #1	0.02	17	6150
	+	Brain fraction #1	0.04	17	6100
	+	Brain fraction #1	0.08	17	6600
	+	Brain fraction #1	0.20	17	10,350
	+	Egg phosphatidyl choline	0.025	17	6500
	+	Egg phosphatidyl choline	0.050	17	10,500
	+	Egg phosphatidyl choline	0.100	17	10,650
	+	Egg phosphatidyl choline	0.250	17	10,650
	+	Bovine phosphatidyl choline	0.025	17	6400
	+	Bovine phosphatidyl choline	0.050	17	10,100
	+	Bovine phosphatidyl choline	0.100	17	10,400
	+	Bovine phosphatidyl choline	0.250	17	10,350

TABLE I (continued)

Experiment	Pimelic acid	Material tested	Amount (mg/tube)	Duration of incubation (hr)	Triglyceride (cpm/g)
4	—	None		17	10,100
	+	None		17	6250
	+	Brain fraction #1	0.02	17	5850
	+	Brain fraction #1	0.04	17	5950
	+	Brain fraction #1	0.08	17	9980
	+	Brain fraction #1	0.20	17	10100
	+	Synth-L-α-phosphatidyl choline	0.025	17	5980
	+	Synth-L-α-phosphatidyl choline	0.050	17	6120
	+	Synth-L-α-phosphatidyl choline	0.100	17	6400
	+	Synth-L-α-phosphatidyl choline	0.250	17	6480
	+	Synth-L-α-phosphatidyl choline	0.500	17	9650
	+	Synth-L-α-phosphatidyl choline	1.0	17	10,600
	+	Synth-L-α-phosphatidyl choline	2.0	17	9860
	+	Synth-L-α-phosphatidyl choline	5.0	17	9950
	+	Synth-L-α-phosphatidyl choline	10.0	17	10,300
	+	Synth-L-α-phosphatidyl choline	20.0	17	10,000
5	—	None		22	9860
	+	None		22	5740
	+	Synth-L-α-phosphatidyl choline	5.0	22	5740
	+	Synth-L-α-phosphatidyl choline	10.0	22	5950
	+	Synth-L-α-phosphatidyl choline	20.0	22	6070
	+	Synth-L-α-phosphatidyl choline	40.0	22	6100
6	—	None		41	10,150
	+	None		41	6000
	+	Synth-L-α-phosphatidyl choline	0.250	41	5900
	+	Synth-L-α-phosphatidyl choline	0.500	41	6040
	+	Synth-L-α-phosphatidyl choline	1.0	41	6020
	+	Synth-L-α-phosphatidyl choline	2.0	41	10,300
	+	Synth-L-α-phosphatidyl choline	5.0	41	10,000
	+	Synth-L-α-phosphatidyl choline	10.0	41	5950
	+	Synth-L-α-phosphatidyl choline	20.0	41	6020

cholesterol and pimelic acid in coconut oil prevents the precipitation of approximately one-half of the cholesterol by insoluble clathrate formation involving hydrogen bonding between the cholesterol and pimelic acid. There appears to be a fairly wide range of concentrations over which the brain fraction is active. Detectable activity is observed at 0.08–0.20 mg/tube (Expt. 3), while at 20 mg or more/tube, particularly after incubation times of several days (Expt. 2), precipitation of cholesterol occurs in the presence of high levels of brain fraction no. 1. In a preliminary survey of possible compounds that might be active in preventing cholesterol precipitation, no compound, including a sample of synthetic L-α-phosphatidyl choline with palmitic acid as the sole fatty acid, was found to be active. It was concluded, therefore, that identification of the compound or compounds responsible for preventing the precipitation of cholesterol might have to be arrived at by isolation and characterization according to classical procedures. Accordingly, a sample of 400 mg of brain fraction no. 1 was subjected to a 100-tube countercurrent distribution according to the procedure of Cole *et al.* (7) involving aqueous methanol and carbon tetrachloride as the two phases. The entire contents of the even-numbered

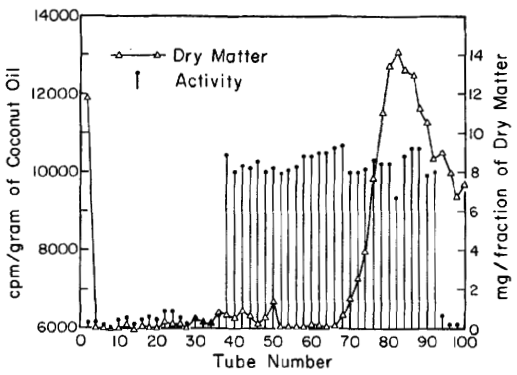

FIG. 1. Countercurrent distribution of Folch fraction No. 1.

tubes were taken to dryness *in vacuo* at 40°. Dry weight and activity determinations were made on each fraction. The results of these determinations are summarized in Fig. 1. It is apparent that activity was quite widely dispersed and did not coincide with phosphatidyl inositol (the main component of brain fraction no. 1). It is also apparent that many of the fractions were quite active, showing a maximal effect in preventing cholesterol precipitation while showing no weighable dry matter.

The mass spectrum of countercurrent distribution fraction no. 55 revealed the presence of compounds exhibiting intense peaks as high as *m/e* 604. The high mass ions, complex nature of the spectrum, and the known occurrence of phosphatidyl choline in brain tissue prompted the determination of the mass spectrum of commercial bovine brain phosphatidyl choline. Its spectrum was almost identical with that of fraction no. 55. This very empirical approach is not being supplemented by further work on the characterization of the countercurrent fractions and the bovine brain phosphatidyl choline sample.

Accordingly, some natural, highly-purified phospholipids were studied in the *in vitro* precipitation system. It was found that egg or bovine phosphatidyl choline is very active in preventing cholesterol precipitation. At a level of about 25–50 μg/tube of phosphatidyl choline, no precipitation of cholesterol present at a level of 100,000 μg/tube occurs. Thus, the ratio of phosphatidyl choline to cholesterol to prevent the initiation of precipitation is about 1 to 2000–4000. Reexamination of synthetic phosphatidyl choline showed detectable but low activity in experiments of short duration. In experiments of longer duration, synthetic phosphatidyl choline showed either no activity at all or activity at a fairly high level with no activity at both lower and higher levels (Table I, Expts. 5 and 6).

It can be concluded that there is some structural specificity for activity with respect to the fatty acids of phosphatidyl choline. A phosphatidyl choline with a "natural" distribution of fatty acids is much superior to that of a synthetic phosphatidyl choline containing palmitic acid as the only fatty acid. It may be argued that the activity of highly-purified egg or bovine phosphatidyl choline is due to an unknown contaminant in these "natural" preparations. This seems most unlikely, due to the high purity and high activity of these preparations.

Although the list of known compounds or preparations tested is not exhaustive, none of the following materials has shown any activity: alginic acid; γ-aminobutyric acid; butylated hydroxytoluene; caffeine; camphor gum; carnitine; Celite; synth-L-α-cephalin; diaminopimelic acid; *N,N'*-diphenyl-*p*-phenylenediamine; egg albumin; farnesyl phosphate; glyceryl phosphate; inositol; lauric acid; lysophosphatidyl choline; lysophosphatidyl serine; *l*-menthol; *trans, trans*-muconic acid; myristic acid; nicotinic acid; phosphatidyl ethanol; phosphatidyl inositol; L-proline; β-sitosterol; sodium polethylenesulfonate; sorbic acid; spermidine phosphate; spermine phosphate; stearic acid; tiglic acid; tryptone; Tween 80; water; yeast extract.

It is significant that compounds as closely related to phosphatidyl choline as phosphatidyl inositol, phosphatidyl ethanol, phosphatidyl serine, lysophosphatidyl choline, and lysophosphatidyl serine showed no detectable activity.

Discussion. These studies have shown that the precipitation of cholestrol from triglyceride solution by hydrogen bonding with

pimelic acid may be prevented by the presence of very small amounts of phosphatidyl choline. In the order of one part of phosphatidyl choline in the presence of 2000–4000 parts of cholesterol is sufficient to prevent the initiation of precipitation. A glib explanation would suggest that one molecule of phosphatidyl choline "coats" a micelle containing 2000–4000 molecules of cholesterol in such a way that the OH groups of cholesterol are not accessible for hydrogen bonding with the O groups of the dicarboxylic acid. This explanation seems hardly possible, since a micelle containing, for example, 3000 molecules of cholesterol would have a mass of over 1,000,000; a mass of this size would not be expected to remain in true solution in the triglyceride. For the present, it must be concluded that the phenomenon observed is not readily explainable.

The present experiments were carried out in a completely lipid environment. It is appreciated that in living systems, while there may be isolated compartments with a completely lipid environment, the majority of living processes take place in the presence of aqueous phases. If there is any analogy between the *in vitro* studies described here and the fate of cholesterol *in vivo,* it is suggested that in a more aqueous environment the lipoproteins function in an analogous way to the phospholipid in the present lipid environment. Indeed, the lipoproteins have long been held responsible for the transport and solubilization of cholesterol.

Assuming that a compound that would prevent the precipitation of cholesterol as a consequence of hydrogen bonding with a component of tissue might have utility in the treatment of diseases involving cholesterol deposition, the procedures described in this paper might find utility in the screening for such a compound. An ideal compound should be one unaffected by digestive and tissue enzymes and should have both lipid and aqueous solubility.

Summary. The precipitation of cholesterol from saturated solution in coconut oil by pimelic acid is prevented or markedly inhibited by trace amounts of highly purified phosphatidyl choline. A number of other phospholipids, as well as a number of other miscellaneous compounds, were found to be inactive in preventing the precipitation of cholesterol in the *in vitro* system. The possible implications with respect to the precipitation of cholesterol *in vivo* are discussed.

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