# Influence of a Plant Sterols Preparation on the Solubility of Cholesterol in Triglycerides.* (31510)

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Previous studies from this laboratory have shown that the solubility of cholesterol in a number of natural and synthetic triglycerides is decreased by the presence of certain dicarboxylic acids, imidazole, or a "β-sitosterol" preparation(1,2). The effect of a "β-sitosterol" preparation on the solubility of cholesterol in triglycerides had been observed previously by Wilkens and DeWitt(3). In the case of the dicarboxylic acids and imidazole, the decreases in solubility are accomplished by the formation of insoluble crystalline clathrates of cholesterol and dicarboxylic acid or imidazole involving a 1:1 ratio between cholesterol and clathrate-forming agent. Furthermore, these clathrates form only at cholesterol concentrations from one-half to full saturation(4). This observation led to the suggestion that cholesterol at saturation in triglycerides is present in two states of dispersion, one form present from one-half to full saturation that yields insoluble clathrates with appropriate dicarboxylic acid or imidazole and a second form present from zero to one-half saturation that does not yield clathrates.

The present studies are concerned with a more detailed study of the influence of a plant sterols preparation on the solubility of cholesterol in several triglycerides where the initial concentration of cholesterol was varied in increments up to full saturation.

*Materials and methods.* The cholesterol solubility studies were carried out essentially as described previously in detail(2). In brief, various amounts of cholesterol-4-$C^{14}$ and the plant sterols preparation were weighed out into 100 mm × 13 mm screw-capped pyrex test tubes. Two ml of tryglyceride 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 37° was carried out for approximately 18 hours. Following this incubation the tubes were centrifuged for 10 min in a clinical centrifuge maintained at 37°C. 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 counted in a liquid scintillation spectrometer. From the counts obtained and the weights of triglyceride solutions sampled, appropriate calculations of the solubility of the cholesterol in the various triglycerides were made.

The solubility of the plant sterols preparation in the various triglycerides was determined by essentially the same procedure used in the determination of cholesterol solubility except that following centrifugation of the tubes each tube was examined visually for the presence of undissolved material. Sufficient levels of the plant sterols preparation were studied so that the solubilities as determined by visual inspection are accurate to within 10%.

Cholesterol-4-$C^{14}$ of relatively low radioactivity was prepared by evaporating to dryness on the steam bath a solution prepared from unlabelled cholesterol (Distillation Products Industries, Rochester, N.Y.) and cholesterol-4-$C^{14}$ (benzene solution, New England Nuclear Corp., Boston, Mass.) in chloroform. The product was finely pulverized prior to use. The preparation of cholesterol-4-$C^{14}$ used in these studies had approximately 146 cpm/mg (292 dpm/mg).

The plant sterols preparation used was commercial material, labelled "β-sitosterol," from Aldrich Chemical Co., Inc., Milwaukee, Wis. This material contained 61% β-sitos-

* Supported by research grant G-3327 from National Science Foundation, by research grants AI-01891 and HE-04138 and career award KG-AM-16,612 from Nat. Inst. Health, and by funds made available through the State University of New York.

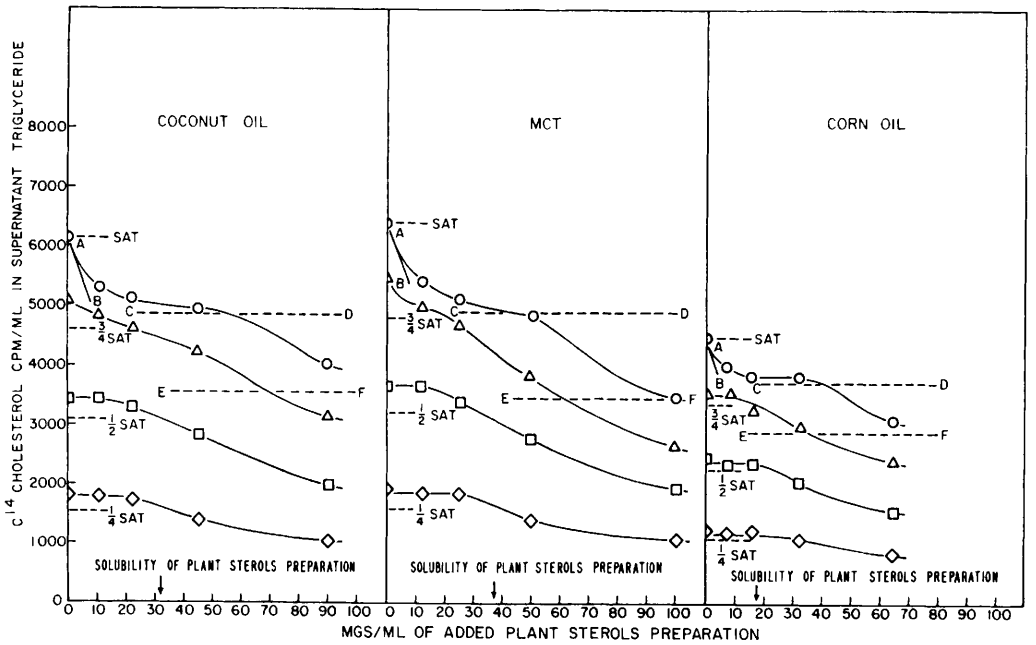


FIG. 1. Influence of a plant sterols preparation on solubility of cholesterol in coconut oil, MCT, and corn oil. Open diamond, intial cholesterol concentration, one-quarter seturated; □—□, initial cholesterol concentration, one-half saturated; △—△, initial cholesterol concentration, three-quarters saturated; ○—○, initial cholesterol concentration, fully saturated.

terol (24$\beta$-ethylcholest-5-en-3$\beta$-ol), 32% of the very closely related campesterol (24$\alpha$-methylcholest-5-en-3$\beta$-ol), and 7% of an unidentified material as determined by gas-liquid chromatography.

The coconut oil used was N.F. IX grade obtained from Magnus, Mabee and Reynard, Inc., New York. The corn oil was a commercial preparation of 'Mazola' from Best Foods, New York. The medium chain triglycerides (henceforth referred to as MCT) were a gift from Drew Chemical Corp., New York.

*Results and discussion.* The results obtained in several experiments involving the effect of the plant sterols preparation on the solubility of cholesterol in coconut oil, MCT, and corn oil where the initial concentration of cholesterol was in the order of one-quarter, one-half, three-quarters or full saturation are summarized in Fig. 1. The curves are complex but appear to be susceptible to interpretation.

With all the triglycerides studied, at cholesterol concentrations of full saturation the presence of increasing increments of the plant sterols preparation reduced the amount of cholesterol in solution. At very low levels of the plant sterols for every molecule of the plant sterols going into solution one molecule of cholesterol comes out of solution. Theoretical lines illustrating this effect are indicated in the graph by the lines A-B. As the level of added plant sterols preparation is increased so that both cholesterol and the plant sterols are present in excess of saturation, cholesterol and the plant sterols, if they compete for the same solubility sites, should be present in amounts proportional to their respective solubilities. Using the determined solubilities of cholesterol and the plant sterols, the level of cholesterol in solution when the levels of cholesterol and the plant sterols are in excess of saturation are shown by the lines E-F in the graph. It is apparent that cholesterol is more soluble than would be predicted on the basis of calculations involving the solubilities of cholesterol and the plant sterols if the assumption is made that the two compounds compete for the same solubility sites.

Accordingly, calculations have been made of the amount of cholesterol in solution if plant sterols compete with only one-half of the cholesterol present at saturation. This level of cholesterol in solution for each of the triglycerides studied is given by the lines C-D in the graph. It is apparent that, within experimental error, as the level of plant sterols is increased to the point where both cholesterol and plant sterols are present in amounts in excess of saturation, the level of cholesterol in solution is that expected if plant sterols compete with one-half of the cholesterol that is present in a fully-saturated solution.

It was next of interest to determine more definitively, in additional studies, the lower limit of cholesterol concentration where competition between cholesterol and plant sterols for solubility sites exists. Accordingly, with MCT as an example, since MCT is a synthetic preparation and free from the complication of endogenous sterols, a condition that does not exist with either coconut oil or corn oil, the effect of a small amount of plant sterols (12.5 mg/ml of triglyceride) on the solubility of a wide range of cholesterol concentrations up to full saturation was studied. Using the data obtained, the difference between the cholesterol concentration in the absence and in the presence of the plant sterols has been plotted in Fig. 2 as a function of initial cholesterol concentration in the absence of the plant sterols. These data when plotted on log-log paper yield a straight line of the type $\log y = m \log x + \log c$. By calculating the slope of this line to give m and substituting values of x and y for a point on the straight line to yield c, a curve fitting closely the experimental points of Fig. 2 is given by $y = 0.0000000315\ x^{6.4}$. On differentiating this equation to yield the slope at any value of x, one obtains $dy/dx = 0.000000200\ x^{5.4}$. To find the value of x (concentration of cholesterol in mg/ml of triglyceride) at which the effect of the plant sterols becomes appreciable, the value of x where the curve turns upward at an arbitrary inclination of 10° (slope = 3.4) has been calculated. This value turns out to be approximately 22 mg/ml, a value very close to a concentration equal to one-half the solubility of cholesterol in the triglyceride.

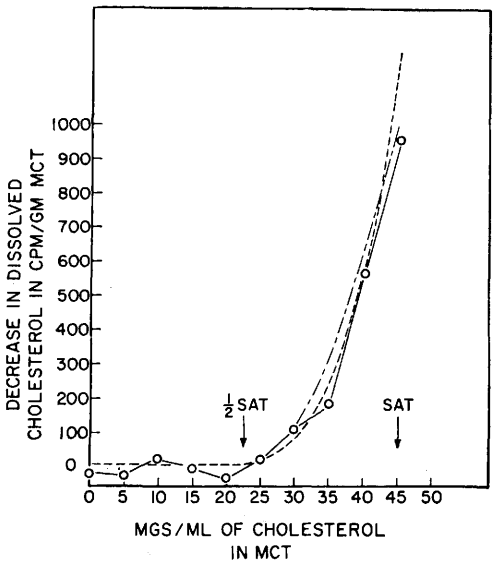


FIG. 2. Reductions in solubility of cholesterol in MCT accomplished by the presence of a relatively small amount of the plant sterols preparation. ———, experimentally determined values; - - - - - - -, curve for the equation $y = 0.0000000315\ x^{6.4}$; — — — —, curve for the equation $y = 1.88(x-22)^2$.

Thus, from this experiment, the plant sterols compete with cholesterol only when the concentration of cholesterol is greater than one-half of saturation. If it be accepted that the plant sterols begin to compete with cholesterol for solubility in MCT when the initial concentration of cholesterol is one-half of saturation or approximately 22 mg/ml, a less unwieldly equation can be calculated for the data of Fig. 2 involving one-half of a parabola whose origin is displaced from 0 to 22 on the x-axis. This curve is given by $y = 1.88\ (x - 22)^2$ and is illustrated by the dashed line in Fig. 2. Thus, either curve $y = 0.0000000315\ x^{6.4}$ or $y = 1.88\ (x - 22)^2$, shows that significant competition between the plant sterols and cholesterol for solubility sites begins at a level of cholesterol concentration of approximately one-half of saturation.

With respect to the effect of the plant sterols on the solubility of cholesterol at initial concentrations of cholesterol of one-half or less of saturation, there is no effect of the added plant sterols until solid plant sterols are present. In the presence of increasing amounts of solid plant sterols, cholesterol comes out of solution. It should be pointed out, however,

that the amount of cholesterol coming out of solution with a given level of the plant sterols is a *direct* function of the level of cholesterol present. This is exactly opposite to what would be expected if cholesterol and the plant sterols were in competition for solubility sites. The data obtained are those expected if dissolved cholesterol were adsorbed on the solid plant sterols or if cholesterol and the plant sterols formed mixed crystals. It is not possible with the present data to decide between these two, or possibly other, alternatives. Mixed crystals of cholesterol and β-sitosterol have been observed by Davis when a mixture consisting of equimolar amounts of cholesterol and a β-sitosterol preparation is crystallized from methanol(5).

*Summary.* The effect of a plant sterols preparation on the solubility of cholesterol in various triglycerides at several levels of cholesterol concentration was studied. At cholesterol levels between one-half and full saturation cholesterol and the plant sterols preparation compete for solubility sites in the triglycerides. At cholesterol levels below one-half of saturation the dissolved plant sterols are without effect on the solubility of cholesterol but in the presence of excess, undissolved plant sterols preparation the concentration of cholesterol in solution is reduced, perhaps by adsorption on the undissolved plant sterols or by the formation of mixed crystals of cholesterol and the plant sterols. These studies furnish further evidence for the suggestion, previously made, that cholesterol at saturation in triglycerides is present in two states of dispersion; one form from one-half to full saturation that yields insoluble clathrates with appropriate dicarboxylic acid or imidazole and now appears to compete with the plant sterols preparation for solubility sites, and a second form from zero to one-half saturation that does not yield clathrates and does not appear to compete with the plant sterols.

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Received May 6, 1966. P.S.E.B.M., 1966, v123.

## Urolithiasis in the Rat. V. *In vivo* Dissolution of Calculi.* (31511)

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Calculi formation in the urinary tract of a number of experimental animals can be induced by dietary means such as deficiencies of vit. A or $B_6$, magnesium or phosphate, excessive vit. D, or an imbalance between protein and mineral intakes(1). Over 50% of weanling rats fed diets with an imbalance of protein and mineral form uroliths within 2 or 3 weeks (2), but higher dietary protein levels protect against urolithiasis. The calculi-inducing properties of the imbalanced diet are thought to be related to its low content of precursors of acidic ions, particularly sulfate, and its high carbonate content. The present study was designed to ascertain whether rats with calculi formed in this manner could be freed of them by altering the dietary regimen.

*Materials and methods.* Male, weanling rats of the NMRI-D strain were used in all experiments. The basal calculogenic diet (15P4) had the following percentage com-

* Bureau of Medicine and Surgery, Navy Department, Research Task MR005.06.0003. The opinions in this paper are those of the author and do not necessarily reflect the views of the Navy Department or the naval service at large. This research was supported by the Advanced Research Projects Agency (Project AGILE) and was monitored by Nutrition Section, Office of International Research, National Inst. Health, under ARPA Order 580 plan number 298.