

REMOVAL OF AN ATHEROGENIC FACTOR FROM THE HUMAN DIET

by 5411

INHI A. KIM CHUNG

Diplom, Justus Liebig Universität, Giessen, W. Germany, 1966

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Foods and Nutrition

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1968

Approved by:

E. Beth Fryer
Major Professor

20
210
74
1968
2-17-72
23

TABLE OF CONTENTS

INTRODUCTION.....	1
Rationale and Specific Aims of Investigation.....	1
Current Status.....	2
Review of Literature.....	3
Antisclerotic Effectiveness of Plant Sterols.....	3
Cholesterol Lowering Activity of Plant Sterols.....	3
Therapeutic Value of Sitosterol.....	4
Mechanism of Sitosterol's Cholesterol Lowering Effect.....	4
Intestinal Absorption of Sitosterol.....	6
Incorporation of Dietary Lipids in Eggs.....	7
EXPERIMENTAL DESIGN.....	8
Intestinal Absorption of Dietary Sitosterol.....	8
Control Period.....	9
Effect of Sitosterol Feeding on Egg Cholesterol Content.....	9
EXPERIMENTAL PROCEDURES.....	10
Treatment of Hens.....	10
Sitosterol.....	11
Purification of Commercial Sitosterol.....	11
Preparation of H ³ -Sitosterol.....	12
Determination of Purity and Specific Activity of the H ³ -Sitosterol Preparation.....	13
Sitosterol Used for Preparation of Diets.....	16
Experimental Diets.....	16
Preparation of Diets.....	16

Homogeneity Control of Diets.....	17
Specific Activity Measurement of Dietary Sitosterol.....	17
Analytical Procedures.....	18
Radioactivity Measurement.....	18
Color Reaction for Sterol Determination.....	19
Sterol Assay in Eggs.....	20
Sterol Assay in Plasma.....	22
Sterol Assay in Feces.....	22
Statistical Procedures.....	23
RESULTS AND CONCLUSIONS.....	24
Intestinal Absorption of Dietary Sitosterol.....	24
Radioactivity Measurements.....	24
Sterol Assay.....	26
Conclusion.....	26
Control Period.....	27
Productivity.....	27
Sterol Content of Eggs.....	27
Molting.....	29
Conclusions.....	29
Sitosterol Feeding Experiment.....	30
Total Sterol Level in Eggs.....	30
Sitosterol in Eggs.....	36
Cholesterol in Eggs.....	42
Miscellaneous Observations.....	42
Health of the Hens.....	42
Blood Sterols.....	42

Production and Appearance of Eggs.....	44
DISCUSSION.....	45
Sitosterol.....	46
Suitability of H ³ -sitosterol for Biological Experimentation.....	46
Lowering of the Cholesterol Levels in Eggs.....	47
Incorporation of Sitosterol into Eggs.....	49
Thoughts for Continued Research.....	50
SUMMARY.....	50
LITERATURE CITED.....	54
ACKNOWLEDGMENTS.....	58

INTRODUCTION

Rationale and Specific Aims of Investigation

The etiology of arteriosclerosis is largely unknown, but a vast body of experimental evidence indicates that the incidence of the lethal effects of this syndrome, namely thrombus and plaque formation, is reduced by proper nutrition. Various animal products, notably eggs, have obtained notorious status among atherogenic nutrients, most likely because of the abundance of certain lipids in these products. The beneficial values of milk, meat, and eggs, however, would argue against exclusion of these products from the diet. It would be desirable, therefore, to reduce the content of atherogenic lipids in foods of animal origin, while maintaining the beneficial properties of these products.

Although sitosterol metabolism in adult male animals has been extensively investigated, it has received little attention in female animals. The lipid composition of chicken eggs, especially that of the fatty acids, is known to pattern itself to a great extent after the lipid composition of the diets fed to the hens. Also, a small amount of plant sterols (obviously of dietary origin) has been found in eggs. Since, furthermore, the cholesterol-lowering effects of sitosterol in chickens resemble those observed in various mammalian species, it is expected that feeding laying hens diets supplemented with sitosterol (under the proper conditions for intestinal absorption

of the plant sterol) will result in a lower lipid concentration and in sitosterol being substituted for cholesterol in eggs-- factors beneficial to human nutrition.

Current Status

The antisclerotic activity of plant sterols is now well-documented. When sitosterol, the predominant plant sterol, is administered by mouth or parenterally to rabbits or chickens, it prevents the incidence of experimental atherosclerosis under influence of cholesterol feeding. It also reduces already existing atheromatous lesions in the aortas of these animals.

The efficiency with which plant sterols lower the levels of cholesterol and other atherogenic lipids in various tissues and body fluids of a large variety of animal species, including man, may be related to their antisclerotic activity. Sitosterol interferes with intestinal absorption of dietary and enterohepatically circulating cholesterol, and thus exerts its cholesterol lowering effects by promoting fecal excretion of sterols and their degradation products.

Because of its antisclerotic and cholesterol lowering properties, the use of sitosterol as a therapeutic agent has been proposed. No harmful effects of plant sterols have been observed even after prolonged administration of excessive amounts to humans and a variety of animal species.

When crystalline sitosterol is simply blended into a standard diet, the plant sterol is not absorbed from the intestines. To solve this problem, various workers resorted to the

addition of large amounts of lipid carriers to the sitosterol-containing diets. Under these circumstances, the plant sterol was absorbed. But the carrier lipids, besides having an irritating effect on the gut, in some cases offset the lipid lowering effects of sitosterol. Recently, efficient absorption of sitosterol has been reported following its dispersion on bulk material in a standard diet.

Review of Literature

Antisclerotic Effectiveness of Plant Sterols. Feeding of plant sterols reduced, or prevents, the incidence of experimental atherosclerosis in rabbits (1, 2) and fowls (3). Also, the injection of sitosterol, in combination with p-aminobenzoic acid, has strong antisclerotic effects in rabbits during prolonged cholesterol administration (4). The atheromatous lesions produced in cholesterol atherosclerosis do not regress spontaneously when animals are placed on a cholesterol-free diet (5), but under the influence of dietary sitosterol the aortas of fowls revert in appearance to that observed in the early stages of the disease (3)

Cholesterol Lowering Activity of Plant Sterols. Implicitly related to the above antisclerotic activity of plant sterols is their efficiency to lower tissue cholesterol levels. The plasma cholesterol lowering effect of sitosterol was discovered in 1951 (6), and has been confirmed by numerous workers. Lowering of the cholesterol levels in adrenals, liver lymphatics, aorta, and

aortic plaques have been clearly demonstrated (3, 7-10). Some experiments, however, failed to show such effects (11-13). This failure may have been due to the inclusion of large amounts of taurochelate and fatty acids in the sitosterol-containing diets. The results of a balance study on humans fed a sitosterol-containing diet showed that seven times more cholesterol was excreted in the feces than that which could be accounted for in the measured decrease in plasma cholesterol level (14).

Therapeutic Value of Sitosterol. Sitosterol is by far the most predominant plant sterol in the human diet. It has been estimated (15) that man ingests about 0.5 gram of sitosterol per day. Feeding diets with high concentrations of sitosterol over prolonged periods of time to humans (16, 17) and to various animal species is apparently without any harmful effects. Hence, the use of sitosterol as a therapeutic agent to decrease the concentrations of tissue cholesterol and lipids has been proposed (9).

Mechanism of Sitosterol's Cholesterol Lowering Effect. The chemical structure of sitosterol is very similar to that of cholesterol from which it differs only in having an ethyl group attached to the carbon atom in the 24-position located in the side-chain. The metabolism of sitosterol resembles that of cholesterol (10, 18*, 19): in the intestines it is partly

*These workers injected 0.34 mg of a crude mixture of C¹⁴-phytosterols, obtained from tobacco plant leaves grown in a labeled CO₂ atmosphere, intravenously into rats. Only quantitative differences between the metabolisms of cholesterol and the phytosterols were found. The plant sterols did enter various tissues, but were not converted to cholesterol to a detectable extent.

esterified, then transported via the lymphatics, and converted to bile acids, steroid hormones (15), etc. It is therefore conceivable that sitosterol would competitively inhibit several important steps in cholesterol metabolism.

It is well documented that sitosterol hampers the intestinal absorption of cholesterol (10, 20-24). A competitive mechanism has been proposed (10) since sitosterol causes increased cholesterol excretion in the feces (14, 25) while the plant sterol itself is absorbed (10, 26-28). Evidence has been presented (10, 21, 22, 28-30) that sitosterol interferes with the intestinal esterification of cholesterol, or with the formation of chylomicrons, or that the plant sterol competes with cholesterol for acceptor sites on lipoproteins that are involved in the transport of cholesterol from the lumen of the intestine into the lymphatics. Sitosterol may interfere with the intestinal absorption of cholesterol by the formation of mixed crystals which are undigestible (1, 2). Perhaps one of the major effects of sitosterol is to prevent reabsorption of endogenous cholesterol formed by the liver and excreted through the bile and perhaps that which is formed by the intestinal mucosa (24).

In addition to the inhibition of intestinal cholesterol absorption, sitosterol may have other mechanisms for lowering the cholesterol level in the body: when the intestinal step is by-passed by intraperitoneal administration of the plant sterol, increased excretion of cholesterol (10) and increased oxidative degradation of cholesterol (8, 9) are observed. Of course, these

"extra-intestinal" effects of sitosterol may be argued since some of the injected plant sterol is transported via the bile and thus may lower cholesterol levels by the intestinal mechanisms discussed.

Intestinal Absorption of Sitosterol. When sitosterol was fed, without further addition of carriers to the diet, to man (17) or rat (12), none was absorbed from the gastrointestinal tract and all plant sterols fed can be recovered unchanged from the feces. Addition of large amounts of oleic acid, with (12) or without (13) sodium taurocholate, resulted in absorption of about 23% of dietary sitosterol in rats. The presence of large amounts of oleic acid may have had an irritating effect on the intestines, and for this reason Schön and Engelhardt (31) substituted olive oil for the free fatty acid. Under these conditions sterol absorption in rats amounted to 56% when a 2% sitosterol-containing diet was fed, and to as much as 78% when sitosterol was presented in 4% concentration. It should be noted that in these rats the efficiency of plant sterol absorption increased with the higher sterol concentration in the diet. Of the 0.93% digitonin-precipitable sterols in corn oil (practically all sitosterol) 20% was absorbed by humans (14), and this may account to a large extent for the hypocholesterolemic effect of corn oil feeding (32).

The lipid carriers used in above feeding experiments may have facilitated plant sterol absorption merely by effecting a fine sterol dispersion in the intestine, as judged by the

observation (17) that daily doses of 5 to 10 grams of sitosterol, emulsified in carboxymethylcellulose, are absorbed in humans with 55% efficiency. This finding shows that emulsified plant sterol is absorbed with the same efficiency as cholesterol.

It is proper to speak here in terms of "probable absorption rates" since part of the plant sterols are metabolized in the intestine to substances that are not digitonin-precipitable, and then excreted. It has been estimated (10) that in rats this intestinal metabolism converts about 33% of the plant sterols absorbed. Even so, when a probable absorption efficiency of 78% (see above) is corrected for this factor, still more than 50% of the sitosterol included in the diet may become available to extra-intestinal tissues.

Incorporation of Dietary Lipids in Eggs. The literature discussed above, with the exception of the work by Betzien et al. (13) on aged hens, all dealt with adult male subjects. Information on sitosterol metabolism in ovulating females is limited to a recent paper (33) in which the effects of some dietary factors and drugs on cholesterol concentrations in eggs and plasma of hens are reported. Addition of 1% of sitosterol to a low-fat basal diet had no effect other than retarding the increase in plasma and egg cholesterol levels caused by dietary cholesterol. Addition of 1% of sitosterol to a diet containing 29% of safflower oil lowered the cholesterol level in blood and raised that in eggs. The same effects, however, were obtained with just the 29% safflower oil-containing diet. Addition of 5% of

lecithin as lipid carrier for sitosterol absorption to the low-fat basal diet containing 1% of sitosterol seemed to lower the cholesterol levels in plasma and eggs, but because of the small number of animals used and the large biological variations the authors did not attach significance to this finding. Though the authors could not detect sitosterol in eggs, they referred to a personal communication from Dr. T. A. Miettinen (Institute of Medical Chemistry, University of Helsinki, Helsinki, Finland) in which direct evidence was provided of plant sterol absorption in hens by gas chromatographic detection of campesterol, stigmasterol, and beta-sitosterol (to a sum of 1.2% of the total sterols present) in a batch of commercial eggs. Unfortunately, the authors did not present evidence for intestinal absorption of dietary sitosterol under the conditions of their experiment, nor did they look into effects on non-sterol lipids.

The lipid composition of eggs produced by hens fed diets rich in poly-unsaturated fatty acids is patterned after these diets; such eggs are commercially available.

EXPERIMENTAL DESIGN

Intestinal Absorption of Dietary Sitosterol

Standard chicken ration, to which 2% of labeled sitosterol had been added in a fine dispersion on carboxymethylcellulose, was fed to 2 laying and 2 non-laying hens over a 1-day period. From measurements of dietary intake and fecal sterol contents

during the subsequent 6 days an estimate of intestinal absorption of the plant sterol was obtained.

Control Period

Over a period of 2 months hens were kept in individual cages and fed standard chicken ration ad libitum. Several eggs were taken from each hen and analyzed for cholesterol. In this manner, estimates were obtained of both the day-to-day variation and the hen-to-hen variation in egg cholesterol levels. Control egg cholesterol levels were obtained for each hen, and hens were divided into groups of similar mean egg cholesterol content for the sitosterol feeding experiment.

Effect of Sitosterol Feeding on Egg Cholesterol Content

Experimental diets, containing the standard chicken ration and about 17% of a 3% carboxymethylcellulose solution with varying amounts of labeled sitosterol, were fed to groups of hens. The diet of the control group contained 17% of the carboxymethylcellulose solution without added sitosterol. Hens which--for various reasons--had not been included in the experimental groups were maintained on the standard ration so that they could serve as a double control in case the carboxymethylcellulose inclusion in the diet of the controls had an effect on the egg cholesterol level. The hens received the experimental diets over a 2-months period, after which they were refed the standard ration. Cholesterol and sitosterol contents of eggs collected during the periods of feeding sitosterol-containing diets and refeeding.

standard rations were measured, and occasional checks on the blood sterol levels of the hens were made.

EXPERIMENTAL PROCEDURES

Treatment of Hens

Thirty hens of a commercial strain, Hiline-934, hatched in May of 1966, weighed about 4.5 pounds each in August of 1967 when they were put in individual cages, provided with automatic water refilling systems, in an air-conditioned room. They were given an identification number and fed a standard chicken ration, KSU 16% commercial layer, ad libitum until October 20, 1967. They were refed this standard ration starting from December 17, 1967, after a 2-months period in which they received H^3 -sitosterol containing diets ad libitum. The radioactive feces was buried according to Atomic Energy Commission regulations, except when the intestinal sterol absorption was measured for which the feces had to be analyzed. A record of egg production was kept for each hen. Eggs were collected daily, labeled as to date and hen number, and stored in a special room maintained at 55 ± 0.5 °F.

Two-ml blood samples were taken from each hen on December 12, 1967 and again on January 12, 1968. The skin over the brachial vein in a wing was rubbed with 70% ethanol, some small feathers were removed, and blood was drawn via a 20-gauge sterile needle into a heparinized 2-ml syringe. Care was taken

to avoid development of hematomas. Egg production was not disturbed by the venapuncture.

Sitosterol

Purification of Commercial Sitosterol. Sitosterol was purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio. Commercial sitosterol is known (34) to contain a small proportion of dihydrositosterol. It has been shown (34) that in the labeling process of sitosterol by catalytic exchange with tritium the H atoms at carbon numbers 5 and 6 of dihydrositosterol exchange preferentially with tritium. Thus, a disproportionately large percentage of the label in a contaminated sitosterol preparation is found in the contaminant, dihydrositosterol. This contaminant was removed by combining the procedures of Werbin et al. (34) and of Marker and Lawsen (35), as follows.

Equal parts of commercial sitosterol and PCl_5 were ground in a mortar. The resulting paste was mixed with excess water heated on a steam bath for one hour. After cooling, the mixture was extracted with ethyl ether. The ether extract was washed with Na_2CO_3 solution, filtered, and evaporated to a small volume. Ethanol was added, and sitosteryl chloride crystallized out. After repeated recrystallizations from acetone, the sitosteryl chloride was mixed with 2 parts of anhydrous potassium acetate and refluxed in glacial acetic acid for 4 hours. By this method, the 3β -chloride group of sitosteryl chloride is converted to

the 3 β -acetate, while the 3 α -acetate is formed from the 3 β -dihyrositosteryl chloride (36). Upon addition of water, the sterol acetates were obtained in crystalline form and recrystallized several times from acetone-ethanol (1:1 v/v).

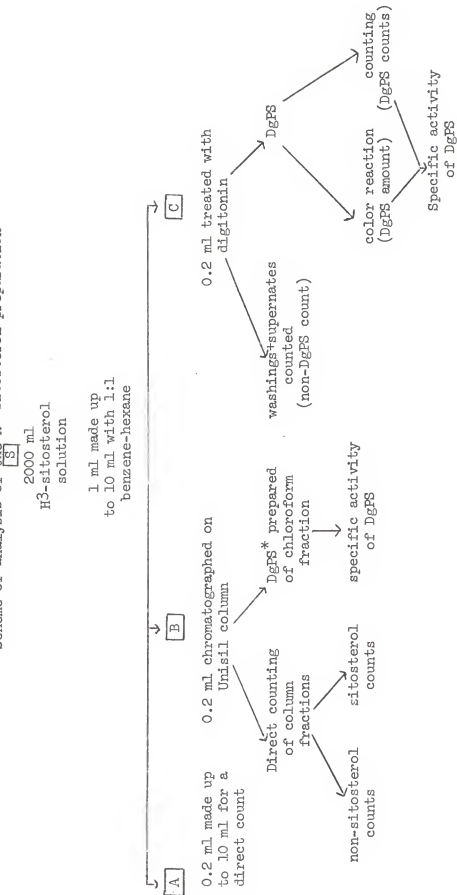
Preparation of H³-Sitosterol. One gram of the purified sitosterol was sent to the Nuclear Chicago Corporation, Des Plaines, Ill., for labeling with tritium according to the method of Gould (37). The acetate derivative of sitosterol was mixed with glacial acetic acid, prereduced platinum oxide catalyst, and 100 curies of tritiated water. The vessel containing this mixture was frozen in liquid nitrogen, evacuated, sealed, and shaken at 127°C for 3 days. Excess of tritiated water, catalyst, and acetic acid were removed, and a benzene solution containing the crude preparation of H³-sitosterol (2.13 curies) was returned. Benzene was evaporated from the preparation under reduced pressure, and the residual H³-sitosteryl acetate was refluxed 4 hours in 20% KOH solution in methanol (15). This procedure serves to hydrolyze the sterol acetate and to remove labile tritium from the compound. Water was added and the mixture extracted with petroleum ether (b. p. 40-60°C). The petroleum ether extract was washed with water until the water layer was free of radioactivity. The solvent was evaporated, and the sitosterol isolated as its digitonide, treated with pyridine, reisolated and recrystallized from acetone-ethanol, as described above. To increase the yields of the recrystallizations, unlabeled, purified sitosterol was added to the mother liquors. The yield was about 2000 ml of benzene solution

containing about 32 mg of sitosterol and 55 μ c per ml. The solution was stored in a sealed bottle at -15°C. The free sterols were prepared from their acetate derivatives by refluxing for 2 hours with 4% KOH in methanol and subsequent addition of water. Digitonin was added to a solution of the free sterols in acetone-ethanol (see below) and the sitosteryl digitonide precipitate was washed repeatedly with acetone-ethanol to remove the α -dihydrositosterol. The sitosteryl digitonide was cleaved with pyridine. Upon addition of 20 volumes of ether per volume of pyridine used, the digitonin precipitate was removed by centrifugation. Free sitosterol was obtained by evaporation of the ether solution in a N₂ atmosphere. The sterol, recrystallized from a 1:1 (%) acetone-ethanol mixture had a melting point of 139-140°C.

Determination of Purity and Specific Activity of the H³-Sitosterol Preparation. An aliquot of the H³-sitosterol preparation was analyzed according to the scheme outlined in Fig. 1. Counting and digitonin procedures are given below.

By direct count (A; see fig. 1), 40 x 10⁷CPM per ml sitosterol solution (S) were found. The combined counting results of sample C showed the same number of total counts, but about 8.4 x 10⁶ CPM--21% of the total counts--were found in the washings and supernates of the DgPS. Analysis of sample C further showed that the sitosterol solution (S) contained 31.55 mg sterol per ml. From these data, a specific activity of 1.0 x 10⁶ CPM per mg sitosterol follows.

Fig. 1

Scheme of analysis of the H^3 -sitosterol preparation

*The abbreviation DgPS stands for digitonin-precipitable sterols.

Triplicate samples B (fig. 1) were chromatographed on 1-gram Unisil columns according to the method of Creech and Sewell (38). In a 5-ml graduated pipette, 1 g of Unisil (Clarkson Chemical Company, Inc., Williamsport, Pa.) was washed with 1:1 (v/v) benzene-hexane. The column was packed under 8 pounds of air pressure and a sample (not exceeding 10 mg of total lipids per gram of Unisil) was applied to the column. The column was eluted first with 15 ml of the 1:1 benzene-hexane mixture, and subsequently with 10 ml of 3:2 benzene-hexane, 20 ml of chloroform, and 10 ml of methanol. About 13% of the radioactivity applied to the column was recovered in the 1:1 benzene-hexane fraction. Since no sterol could be detected in this fraction with the color reaction (see below), these 13% were considered a non-sterol contamination of the H^3 -sitosterol. Small proportions of the label were found in the 3:2 benzene-hexane and methanol fractions in which the color reaction did reveal the presence of small quantities of sterols. About 74% of the radioactivity applied to the column (and about 90% of the amount of sterols chromatographed) were present in the chloroform fraction. Upon rechromatography of a portion of the chloroform fraction, no label was eluted with 1:1 benzene-hexane, and about 90% of the applied radioactivity reappeared in the chloroform fraction. The specific activity of sitosterol in the chloroform fraction amounted to 1.0×10^6 CPM per mg, the same value as found in sample C (fig. 1).

Sitosterol Used for Preparation of Diets. For the preparation of diets containing H³-sitosterol, portions of the H³-sitosterol stock solution were chromatographed on Unisil, and aliquots of the chloroform fractions were used. Unlabeled sitosterol included in the diets was purchased from Nutritional Biochemicals Corporation and used as such.

Experimental Diets

Preparation of Diets. First, 8500 ml of 3% carboxymethylcellulose (CMC) in water was prepared by blending 255 grams of CMC into 8500 ml water, care being taken to avoid formation of lumps. To 3750 ml of the gelatinous CMC preparation 750 grams of unlabeled sitosterol and 1.5×10^8 CPM of chromatographically purified H³-sitosterol were added. A creamy emulsion of this mixture was produced with a Stein mill (Fred Stein Mill Laboratories, Atchison, Kansas). A liquid-solid blender (Patterson-Kelley Company, East Stroudsburg, Pa.) was employed to mix 10 kg quantities of the standard chicken ration with various amounts of the 3% CMC and emulsified H³-sitosterol preparations, as follows:

- (i) for the 1% - sitosterol diet, 500 g of emulsion and 1500 g of 3% CMC;
- (ii) for the 2% - sitosterol diet, 1000 g emulsion and 1000 g of 3% CMC;
- (iii) for the 4% - sitosterol diet, 200 g of emulsion;
- (iv) for the control diet, 200 g of 3% CMC.

In this manner, 12-kg quantities of the different diets were

prepared each time when needed. All four diets contained 0.5% of CMC and, in the sequence listed above, 0.82%, 1.67%, 3.33%, and 0% sitosterol. To prevent yeasting, the diets had to be stored exposed to air.

Homogeneity Control of Diets. From each diet prepared, eight 250-mg portions sampled at random were weighed out in prepared counting vials. The samples were extracted with 0.6 ml of methanol and 10 ml of a solution of scintillators in toluene (see below) for 12 hours at room temperature with occasional shaking, and then counted. Corrections for quenching due to methanol and diet in the samples were made by internal standardization (see below). The radioactivity measurements ranged within 5% around the means of groups of replicate samples. This indicated a satisfactory degree of homogeneity of the diets.

Specific Activity Measurement of Dietary Sitosterol. From each diet, 6 samples were extracted with 1:1 (v/v) acetone-ethanol. Portions of the extracts were treated with digitonin, and radioactivity and mass determinations of the sterol digitonides were made. The specific activity of sterols in the various batches of diets made up in the course of the entire experiment ranged from about 185 to 195 CPM per mg. The average specific activity, which amounted to 188 CPM per mg sterol, will be used for calculation of the sitosterol contents of eggs and blood. Neither the specific activity, nor the CPM per gram diet, was variable upon storage of the diets.

For the study of intestinal absorption of dietary sitosterol a 2% sitosterol diet with higher specific activity, 385 CPM per mg sterol, was used.

Analytical Procedures

Radioactivity Measurement. A packard TRI-CARB liquid scintillation spectrometer (Packard Instrument Company, Inc., La Grange, Ill.) was used. With a tritium standard solution of known radioactivity content, the proper dial settings on the instrument for tritium counting were found. At the settings whereby CPM²/background reached its peak value, a counting efficiency of 33% was registered.

A solution containing 3.0 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter toluene was kept in brown bottles for periods not exceeding 6 weeks. For measurement of radioactivity, an aliquot of a sample was evaporated in a counting vial, 10 ml of the toluene solution was added, and the sample was counted. When the H³ of a sample not soluble in toluene had to be assayed, e.g., sterol digitonides, an aliquot of a methanol solution of the sample was mixed in the counting vial with 10 ml of the scintillator solution in toluene. In the latter event, and also when colored or non-homogeneous solutions had to be counted, a correction had to be made for the fact that methanol, or color, or solid particles, absorb some of the radiation emitted in the counting vial and thereby decrease the counting efficiency.

Correction for quenching was routinely made by the method of Internal Standardization: a sample and a blank containing only the scintillator solution and no radioactivity were counted. Then an accurately measured volume of a highly labeled solution was added to both sample and blank, and the vials were recounted. The difference in counting rate between blank plus labeled solution and blank per se indicates how much radioactivity was added to the blank and, hence, to the sample. When a sample contains quenching material, its increase in counting rate (after addition of the labeled material) will be smaller than the increase measured in the blank. The ratio between the increases of sample counting rate and blank counting rate indicates by which fraction of the blank's efficiency the sample was counted. Multiplication of a sample's original count by the reciprocal of above ratio, the "quench factor", standardizes the countings obtained on samples containing different amounts of quenching materials.

Routinely, at least three aliquots were taken from each sample, and each aliquot was counted until more than 2500 counts were collected. The probable (95%) error in the average of replicate aliquots was about 2%. Due to higher counting rates, quench factors could be determined within 1% probable error.

Color Reaction for Sterol Determination. The sterol content in methanol solutions of the digitonides was measured according to the method of Zlatkis et al. (39). At least triplicate aliquots from each sample were pipetted into 30-ml test tubes

and evaporated. From a cholesterol standard solution, triplicate samples were taken of 10, 20, 30, 40, 50, and 60 μ g per tube and evaporated, and empty test tubes were included as blanks. Three ml of glacial acetic acid were added to each tube, and from a solution, containing 1 ml of 10% FeCl_3 in glacial acetic acid per 100 ml concentrated sulfuric acid, 2 ml was squirted forcibly into the glacial acetic acid solutions of sterols. Immediately after addition of the color solution, the tubes were shaken and left in the dark for 45 to 90 minutes. Light absorbancies due to the violet colors were read at 560 $m\mu$ in a spectrophotometer against the glacial acetic-color solution blanks. The formula of the straight standard line was calculated, and the sterol contents of the unknowns were derived.

Preliminary trials established that cholesterol and sitosterol have the same chromogeneity, and that their colors have maximum light absorbancy at the same wave length. Hence, the sum of these sterols was found by the color reaction. However, the sitosterol was labeled, and its specific activity known. Dividing the observed radioactivity in a sample by this specific activity yielded the amount of sitosterol present. By subtraction of this amount from the total sterol content measured with the color reaction, the cholesterol level was found.

Sterol Assay in Eggs. An egg which had been stored in a cool room at 55°F was allowed to equilibrate with the laboratory temperature. The yolk was isolated, quickly blotted with filter paper, and weighed in a rectangular plastic tray. The

yolk was pierced, a sample of 1-2 grams was poured from a corner of the tray into the tube of a Potter-Elvehjem homogenizer containing about 15 ml of a 2:1 chloroform-methanol mixture, the tray with the remainder of the yolk was rapidly weighed, and a second 1-2 gram yolk sample was taken immediately. The yolk samples were homogenized in the solvent mixture and the homogenates were left overnight at room temperature. Then the protein precipitate was removed by centrifugation and twice re-extracted with the 2:1 chloroform-methanol mixture on a steam bath. After the 3 extractions the protein precipitate was hydrolyzed with NaOH and analyzed for sterols; digitonin-precipitable sterols were absent and no radioactivity was found. The extracts were combined and made up to 50 ml. From both sample extracts duplicate 3-ml samples were transferred into 15-ml centrifuge tubes. The solvents were removed under N_2 atmosphere, 4 ml of 1:1 acetone-ethanol and 4 drops of 30% NaOH in water were added and the samples were kept in a tube heater at $60^\circ C$ for 30 minutes under a N_2 atmosphere with occasional stirring. Sterol esters are hydrolyzed by this procedure. The samples were neutralized with a few drops of acetic acid (phenolphthalein end-point), 2 ml of a 0.5% solution of digitonin in 50% ethanol were added, and the samples were stirred, sealed with parafilm, and left overnight for complete precipitation of the sterols (40). The sterol digitonides were centrifuged out; the supernatant was discarded after testing with additional digitonin solution showed that no more digitonin-precipitable

sterols were present. The digitonides were washed once with 1:2 acetone-ether and once with ether, dried, and dissolved in 6 ml of methanol. From the 4 methanol solutions obtained from each egg, replicate 0.6 ml aliquots were taken for counting and quench corrections, and replicate 0.2 ml aliquots were used for the color reaction.

Sterol Assay in Plasma. Two-ml blood samples were obtained from a brachial vein in a heparinized syringe and transferred into 5-ml centrifuge tubes. The tubes were centrifuged for 15 minutes at 2500 x g. Duplicate 0.2-ml plasma samples were saponified with 3 drops of 30% NaOH at 60°C for 30 minutes; 2 ml of 1:1 acetone-ethanol were added, the sample was stirred and left at room temperature for 2 hours. A precipitate obtained by centrifugation was re-extracted two times each with 1 ml of the acetone-ethanol mixture at 60°C. The extracts were combined, treated with digitonin, and processed further as described above.

Sterol Assay in Feces. For the study of intestinal absorption of dietary sitosterol, feces were collected daily from metal trays placed under the hens. The feces were weighed, homogenized in 10% NaOH and heated to saponify sterol esters and to stop bacterial degradation of sterols. The samples were diluted with water and stored in closed containers at room temperature until analyzed. A convenient aliquot of the known total volume of feces was extracted three times with petroleum ether (b.p. 40-60°C). The combined petroleum ether extracts were washed three times with water and then evaporated to dryness.

The residue was made up to known volume with 1:1 acetone-ethanol; aliquots of this solution were taken for direct counting, and additional aliquots were treated with digitonin and analyzed as described above.

Statistical Procedures

Application of the t-test for comparison of mean values at more than one interval in a time-course study does not take into account the fact that each mean value is dependent on the mean values of preceding time points. Nevertheless, the t-test was applied, at the 5% level of significance, to decide whether the mean values for experimental hens differed from those observed in control animals after certain experimental feeding periods, because treatment of the data by the more precise analysis of covariance was precluded by the difficulty in expressing, by a mathematical formula, the relation between experimental values (e.g., total sterol contents or CPM) and the time of sampling.

Before applying the t-tests on various groups of observations, Bartlett's test (41, page 179) was applied to test that the variances of the different normally distributed populations were equal. Such was found to be the case with all experimental parameters tested.

RESULTS AND CONCLUSIONS

Intestinal Absorption of Dietary Sitosterol

Results of the absorption experiment are given in table 1. The 2% sitosterol diet was found to contain 7000 CPM and 18.2 mg sitosterol per gram. Measured amounts of the diets were presented to the hens in metal containers that fitted inside the feeding trough. Metal partitions were placed between the diet containers in the trough to prevent neighbouring hens from sharing each other's meal. Hen no. 2 turned one of the partitions over its food container, and, hence, had no access to food during the first 22 hours. Feeding of the labeled diet was therefore continued, to hens 1 and 2 only, for an additional 18 hours. After the feeding periods, the containers with residual labeled diet were weighed, and the hens were fed their standard ration. Feces collected daily on the hour at which the H^3 -sitosterol feeding had started, were pooled as indicated in the table.

Radioactivity Measurements

Aliquots of the feces collected were extracted for total lipids. Measured portions of the extracts were evaporated in counting vials and counted. Recoveries on days 5 and 6 were insignificant compared to what the hens had eaten and, hence, collection of feces was discontinued after the 6th day. About 36% of the ingested radioactivity was recovered in the feces of the laying hens over the 6-day period after feeding of the labeled diet, and about 13% in the feces of the non-layers. The smaller absorption percentages found in the laying hens.

TABLE 1

Recovery of H³-sitosterol in the feces of hens fed 2% H³-sitosterol diet

The 2% sitosterol diet, containing 7,000 CPM of rechromatographed H³-sitosterol and 18.2 mg total sitosterol per gram diet was fed for a period of either 22 hours (hens 3 and 4) or 40 hours (hens 1 and 2) ad libitum. Hen 2 did not eat during the first 22 hours. Feces were collected quantitatively after each 24-hours interval starting from the beginning of the first feeding period. Feces collections of several days were pooled as indicated

Hen no.	Intake of labeled diet		Radioactivity in feces ¹			Total % of ingested H ³ recovered			Mg DgPS in feces ²			Total % of ingested sterol recovered
	g	g	Day 2-4	Day 5-6	Day 5-6	%	mg	mg	mg	%		
1	45	45	39,400	198,000	1,700	38.0	104	596	6.0	43.2		
2	0	70	0	168,000	1,900	34.7	1.8	471	3.7	37.5		
3	31	-	9,400	11,000	500	9.7	35	37	1.9	13.2		
4	29	-	26,300	6,900	600	16.6	74	13.3	2.8	17.0		

¹Direct counting of the total lipid extract.

²Assays of digitonin precipitates prepared from aliquots of the total lipid extract.

compared to the non-layers, may indicate that the intestinal absorption capacity had been exceeded by the larger sterol consumption of the layers.

It should be pointed out that labeled metabolites formed from absorbed H^3 -sitosterol may have been present in the total lipid extracts of feces. The H^3 recoveries listed in table 1 thus represent maximum estimates of unabsorbed H^3 -sitosterol.

Sterol Assay

Digitonin precipitable sterols (DgPS) were isolated from the total lipid extracts and analyzed. The recoveries of DgPS, expressed as percentages of the amounts of sitosterol eaten by the various hens, agree well with the radioactivity data (table 1). The consistent finding of a slightly higher recovery of DgPS than that of H^3 in the feces may have 2 causes: 1) the standard chicken feed contained a small amount of plant sterols; and 2) some of the cholesterol produced by the hen was excreted in the feces via the enterophepatic route. An estimate of the amount of fecal DgPS attributable to these causes may be obtained from the results of hen no. 2: while fasting on the first day, she excreted 1.81 mg of DgPS; over a 2-day period, on days 5 and 6, she excreted 3.69 mg of DgPS.

Conclusion

The finding that more than 60% of the sitosterol contained in the 2% sitosterol diet was absorbed by the laying hens lead

to the acceptance of the method of sitosterol dispersion on carboxymethylcellulose as an effective means of administering the plant sterol to hens.

Control Period

During a period of about 2.5 months prior to the sitosterol feeding experiment the hens were kept in individual cages and fed standard ration. A record was kept of egg production, and 2-6 eggs of each hen were analyzed for their sterol contents. The results of these samplings are presented in table 2. In this table, the hens have been grouped (on the basis of productivity and egg sterol content) according to the diets administered to them during the subsequent sitosterol feeding experiment.

Productivity

Only hens that were actively producing during October were used for the sitosterol feeding experiment.

Sterol Content of Eggs

On the average, eggs contained 14-15 mg of sterols per g yolk during the control period. The day-to-day variation in sterol content of eggs obtained from a given hen was remarkably small, and not significant. Several hens, however, had a significantly ($P < 0.05$) higher egg sterol level than other hens (compare, for instance, hen no. 2 with hen no. 20). Because

TABLE 2

Egg production and egg sterol levels recorded during the control period from August 1 until October 20, 1967; division of hens into groups for the subsequent sitosterol feeding experiment

Hen no.	Number of eggs produced in			Mean egg sterol level ¹	Experimental diet fed to group ²	Group's mean egg sterol content
	Aug.	Sept.	Oct. 1-20			
				mg sterol per g egg yolk		
					mg sterol per g egg yolk	
6	21	21	14	15.18±0.10 (2)	Control diet	14.32±0.46 (12)
12	24	26	16	14.69±0.38 (3)		
19	21	15	11	13.87±0.50 (3)		
24	0	18	14	14.10 (1)		
27	24	21	15	13.91±0.58 (3)		
8	24	24	15	15.21±1.08 (3)	1% sitosterol diet	14.25±0.69 (14)
9	18	11	13	14.63±0.65 (3)		
22	19	18	11	13.86±1.23 (3)		
25	23	21	12	13.61±0.11 (3)		
29	22	23	15	13.77±0.03 (2)		
14	24	24	12	15.56±0.54 (2)	2% sitosterol diet	14.48±0.47 (16)
15	26	24	12	14.33±0.66 (4)		
16	10	23	12	15.68±1.03 (4)		
20	19	19	14	13.43±0.16 (3)		
21	24	23	16	13.41±0.39 (3)		
4	21	19	12	14.02±0.47 (3)	4% sitosterol diet	14.03±0.26 (13)
11	23	24	11	13.30±0.31 (3)		
18	24	24	14	14.33±0.79 (3)		
23	2	1	13	14.35±0.43 (2)		
30	21	20	12	14.40±0.16 (2)		
1 ³	0	0	0		Standard ration	
2	15	10	2	16.60±0.31 (6)		
3	12	0	0	15.29±0.29 (2)		
5	23	2	2	15.76±0.52 (3)		
7	24	14	11	14.79±0.22 (3)		
10	22	10	6	14.36±0.85 (3)		
13	25	0	0	15.08±0.14 (3)		
17 ³	9	0	0	16.74±1.87 (3)		
26	23	17	0	13.90±1.06 (6)		
28	7	11	13	17.67±0.45 (2)		
28 ⁴				25.40±2.95 (2)		

¹Mean ± standard deviation (number of eggs analyzed); these means will be referred to as "control values" for each hen.

²Feeding started on October 20, 1967.

³Internal layers.

⁴Last 2 eggs produced before molting.

of this hen-to-hen variation, careful selection of hens for the subsequent sitosterol feeding experiment was necessary. The hens were divided into groups of which the average egg sterol contents during the control period were similar (table 2)

Molting

When hens start molting, the regularity in egg production ceases; sporadically a few more eggs are laid, but eventually the hen will go through a non-productive period. As exemplified by hen no. 28 (table 2) -- but confirmed in several more cases in the course of the experiment -- the last eggs produced before the "dry" period contained vastly increased amounts of sterols.

Conclusions

1) Since the day-to-day variations in egg sterol contents were insignificant, it is meaningful to compare egg sterol contents observed during the control period with those of the subsequent sitosterol feeding experiment.

2) For the feeding experiment, hens were divided into groups in such a manner that the hen-to-hen variations in egg sterol contents during the control period cancelled out.

3) The consistent differences in sterol levels of eggs produced by different hens would indicate the feasibility of lowering the mean sterol content of eggs by selective breeding.

4) Eggs produced immediately prior to (and during) the period of molting have the potentially harmful property of containing excessive amounts of sterols.

Sitosterol Feeding Experiment

The 4 experimental diets, all containing the same amount of carboxymethylcellulose but various proportions of H³-sitosterol (see above, Purification of Commercial Sitosterol), were fed ad libitum to groups of 5 hens each over a 2-month period, from October 20 until December 17, 1967. Thereafter, the hens were refed their standard ration. The levels of digitonin-precipitable sterols (DgPS) in the eggs were measured and each experimental value obtained was compared with the average control value of the same hen, (figs. 2-5).

Total Sterol Level in Eggs

As shown in figure 2, the feeding of control diet did not effect the egg sterol level. Feeding 1% sitosterol diet, likewise, failed to produce a consistent effect on the total sterol level in eggs (fig. 3). With 2% sitosterol diet (fig. 4), a significant drop in egg sterol level occurred within 7 days of feeding, but to achieve maximal lowering of egg DgPS, feeding had to be continued for another 7-day period. By that time, decreases of about 30% in the original total sterol level were found. It would appear (fig. 2) that the egg sterol levels rebounded between 40 and 60 days of feeding, though the low values obtained a week after refeeding of standard ration cast doubt on the significance of the rebound. After a month of refeeding the standard ration, the egg sterol levels had reverted to normal (fig. 2). When the 4% sitosterol diet was

LEGENDS TO FIGURES 2 - 5

Fig. 2 - Relative egg sterol (DgPS contents of the control group.

Fig. 3 - Relative egg sterol (DgPS contents of the 1% sitosterol group.

Fig. 4 - Relative egg sterol (DgPS) contents of the 2% sitosterol group.

Fig. 5 - Relative egg sterol (DgPS) contents of the 4% sitosterol group.

Differences in egg sterol contents (expressed as mg DgPS per g egg yolk) between experimental eggs and control eggs of the same hens are plotted against the experimental feeding periods after which the eggs were collected. The numbers in the figures correspond to the hen numbers given in table 2. In the control period, ranges of individual egg sterol measurements around the mean control levels, listed in table 2, are plotted for each hen belonging to the particular groups depicted in the figures.

Fig 2— Control diet

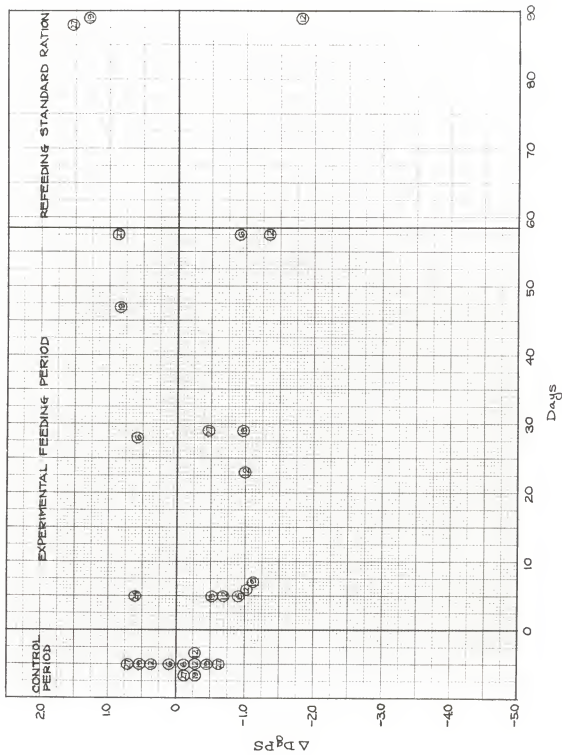


Fig 3 - 1% sitosterol diet

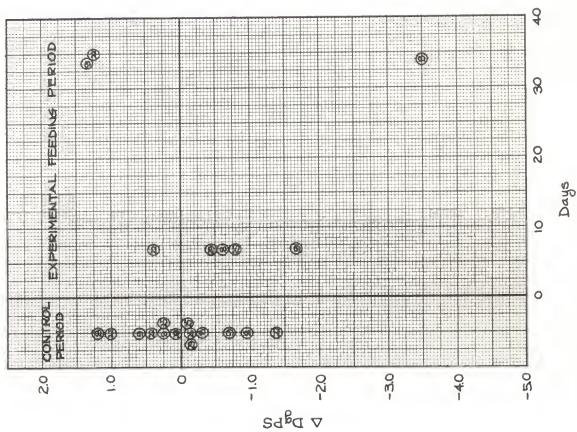


Fig 4 - 2% sitosterol diet

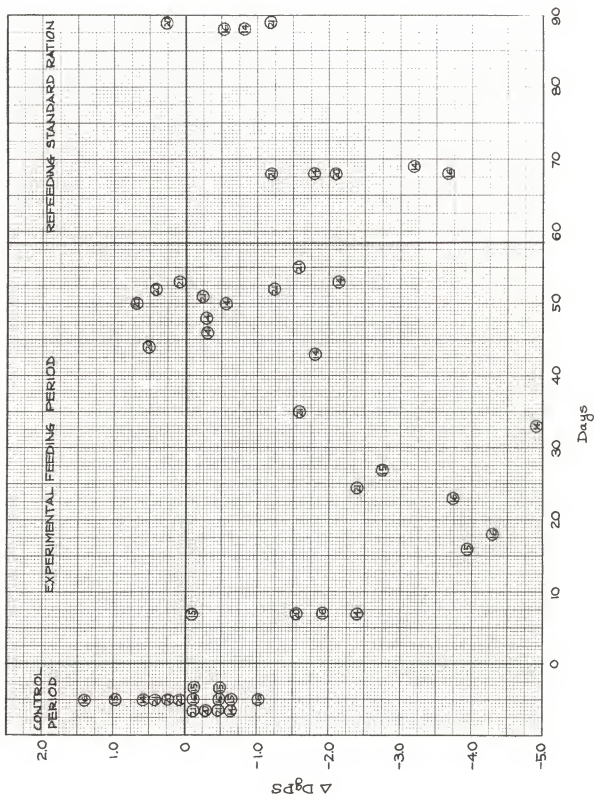
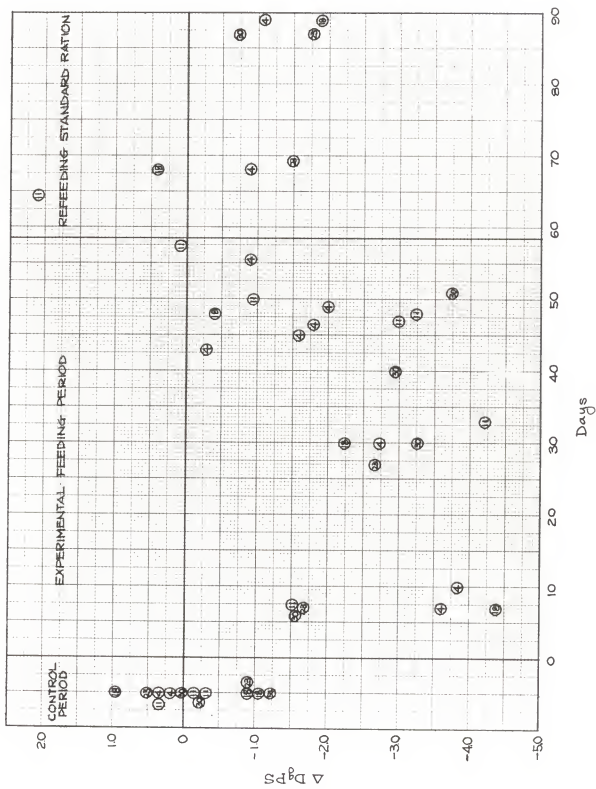


Fig 5 - 4 % sitosterol diet



fed, the maximal depression (about 30%) of egg DgPS was found after only 7 days (fig. 5); egg sterol levels remained below the control average during the entire feeding period and then reverted to normal upon refeeding standard ration. The effects of the various experimental diets on the total sterol content of eggs is most readily evaluated from fig. 6 in which average sterol levels during subsequent experimental feeding periods are plotted. The potent sterol lowering effects of the 2 and 4% sitosterol diets contrast well against the stable control level. The high-sitosterol diets differed in the promptness, but not in the magnitude, of their sterol-lowering effects.

Sitosterol in Eggs

Solutions of the digitonin precipitates of egg sterols were assayed for their H^3 contents. The counting results, expressed as digitonin-precipitable sterol CPM per gram egg yolk, are given for individual eggs in table 3. A graphical presentation of averages measured over subsequent experimental feeding periods is given in fig. 7. The greatest differences between the dietary effects on sitosterol incorporation were found in eggs collected in the 7-10-day period: with the 1%, 2%, and 4% sitosterol diets, respectively, 53, 97, and 280 DgPS CPM were found per gram yolk (fig. 7). In most respects, the radioactivity data mirror the effects on total sterol levels: with the 4% diet, almost maximum H^3 -sitosterol levels are already present after one week of experimental feeding. In

Fig 6

average total sterol levels in eggs during various experimental periods. Average sterol levels of eggs collected during the intervals indicated in the Figure and standard errors of the means are given. This Figure is an interpretation of the preceding Figures, 2 - 5, in which individual egg sterol levels are presented.

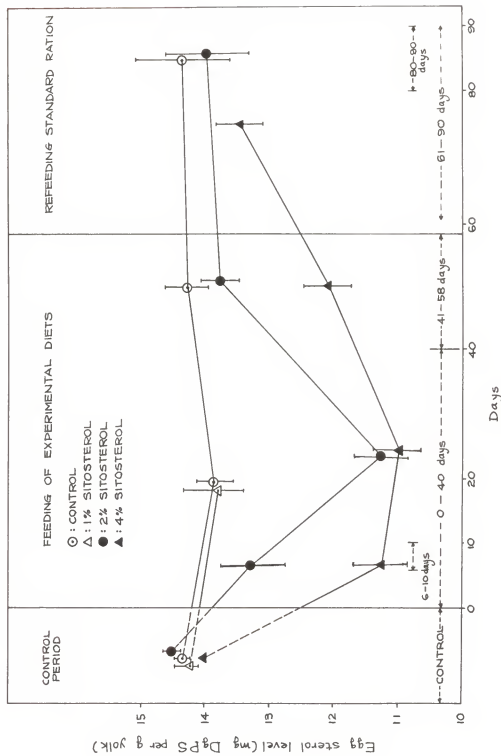


Fig 7

Average H^3 -sitosterol levels in eggs

Averages and standard errors of CPM in digitonin-precipitable sterols per g egg yolk at subsequent periods of feeding the experimental diets are plotted.

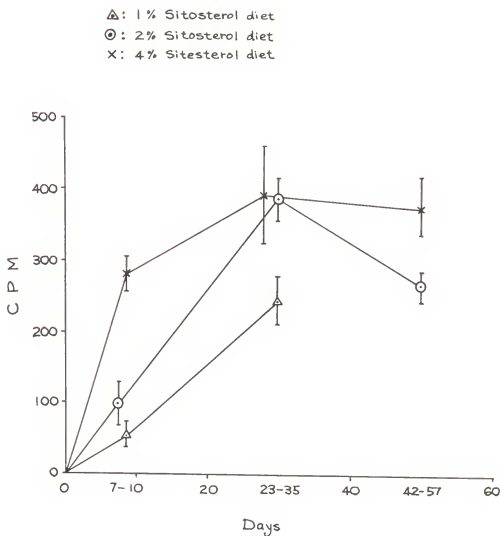


TABLE 3

 H^3 - sitosterol content of eggs

Radioactivity in digitonin-precipitable sterols (DgPS) of individual eggs is expressed as CPM in DgPS per gram egg yolk. Values pooled as indicated in the table are graphically presented in fig. 7

Days	1% sitosterol diet					2% sitosterol diet					4% sitosterol diet				
	Hen number					Hen number					Hen number				
	8	9	22	25	29	14	15	16	20	21	4	11	18	23	30
7	1	14	73	95	83	95	96	22	175						
8											344	327	220	217	336
10											236				
23								351							
27						429									
28														278	
30											455		271		627
33						446						319			
34	173	203							275						
35			372	242	214					408					
42											466				
43						177					389				
44								231							
45											163				
46						181									
47												404			
48						169						557	575		
49										267	272				
50						269			418			411			
51										222					365
52									347	301					
53										293					
54								260			230			574	
55										255					
56											165				
57												264			

contrast, feeding of the 2% diet had to be extended over a longer period to obtain the maximum effect, but the maxima reached with the 2 high-sitosterol diets were equal in magnitude. With the 1% sitosterol diet, only slightly (but significantly) less sitosterol was incorporated into eggs after 35 days of experimental feeding than that found with the other labeled diets. It is conceivable that--had the 1% sitosterol feeding been extended over a longer period--a similar egg sitosterol level would have been reached as that observed with the 2 and 4% sitosterol diets.

Since the specific activity of sitosterol in the experimental diets was 188 CPM per mg, the radioactivity measured in the DgPS of eggs can be converted to mg of H^3 sitosterol present in the eggs. Table 4 shows the results of this calculation. After the first 23 days of experimental feeding, as much as 2 mg of H^3 -sitosterol was found to be present per gram of egg yolk. If, in addition, 1% of the egg sterol fraction consists of unlabeled sitosterol (33), and the eggs contained about 14 mg of DgPS per gram yolk, 0.14 mg sitosterol per gram yolk must be added to the above estimate, which brings the total sitosterol content per gram yolk up to 2.14 mg. The average egg weight in our experiment was 68 grams, and the yolk weights averaged about 21 grams. Thus, an egg collected after the first weeks of experimental feeding contained about 45 mg of sitosterol.

TABLE 4

A. Radioactivity in egg DgPS

Average H^3 content of DgPS per gram egg yolk, standard errors of the means, and (in parentheses) the numbers of eggs analyzed are presented

Group ¹	Period		
	7 - 10 days	23 - 35 days	42 - 57 days
1%	53 \pm 19 (5)	241 \pm 35 (5)	
2%	97 \pm 31 (4)	382 \pm 31 (5)	261 \pm 0 (13)
4%	280 \pm 25 (6)	390 \pm 68 (5)	372 \pm 41 (13)

B. H^3 -sitosterol content of eggs

The specific activity of dietary sitosterol was 188 CPM per mg. Using this value, the average values of CPM in DgPS per g yolk presented above were converted to average values for mg sitosterol present per gram egg yolk

Group ¹	Period		
	7 - 10 days	23 - 35 days	42 - 57 days
1%	0.28 \pm 0.10	1.28 \pm 0.19	
2%	0.52 \pm 0.16	2.03 \pm 0.16	1.39 \pm 0.11
4%	1.49 \pm 0.13	2.07 \pm 0.36	1.98 \pm 0.22

¹Groups are listed by the sitosterol contents of the diets they received.

Cholesterol in Eggs

Apart from sitosterol and a negligible amount of other plant sterols, the total DgPS in eggs (fig. 2-6) consist of cholesterol. The cholesterol levels of the experimental eggs can thus be found if the calculated H³-sitosterol contents (table 4) and 1% of the control DgPS level (unlabeled sitosterol) are subtracted from the total sterol levels (fig. 6). This calculation is presented in table 5. The results show that even the 1% sitosterol feeding resulted in a significant drop of the egg cholesterol level. With the 2 and 4% sitosterol diets it was possible to lower the egg cholesterol levels by an average of as much as 37%.

Miscellaneous Observations

Health of the Hens. Throughout the experiment, the hens maintained a healthy appearance. Their food intake was normal, and they did not lose body weight.

Blood Sterols. Blood samples were taken from each hen after 53 days of experimental feeding and, again, after 84 days when the hens had been refed their standard ration for a period of 27 days. The hens fed the sitosterol-containing diets showed, in general, a decreased total blood sterol level after 53 days as compared with 84 days; the effects were larger with the 2 and 4% sitosterol diets than they were in the 1% group, but the magnitudes of the blood cholesterol lowerings were quite variable, ranging from highly significant decreases of up to 70% to insignificant increases of about 10%. Significant

TABLE 5

A. Total sterol (DgPS) contents of eggs

Averages, standard errors, and (in parentheses) number of eggs analyzed are given (cf. fig. 6). Sterol contents are expressed as mg DgPS per g yolk

Group ¹	Period			
	Control	6 - 10 days	15 - 40 days	41 - 60 days
Control	14.32 _± 0.13 (12)		13.85 _± 0.28 (10)	14.26 _± 0.34 (4)
1%	14.25 _± 0.18 (14)	13.58 _± 0.35 (5)	14.23 _± 1.30 (5)	
2%	14.48 _± 0.12 (16)	13.28 _± 0.52 (4)	11.24 _± 0.23 (7)	13.70 _± 0.31 (12)
4%	14.03 _± 0.07 (13)	11.28 _± 0.47 (6)	11.05 _± 0.46 (6)	12.10 _± 0.38 (11)

B. Cholesterol content of eggs

From above total sterol contents, the H³-sitosterol levels listed in table 4B and, in addition, 1% of the control sterol level (unlabeled sitosterol) have been subtracted. The results are expressed in mg cholesterol per g yolk

Group ¹	Cholesterol content of eggs			Percent reduction in egg cholesterol content			
	Period - days			Period - days			
	Control	6 - 10	15 - 40	41 - 60	6 - 10	15 - 40	41 - 60
Control	14.18	13.71	14.12		3.3	0	
1%	14.11	13.16	12.81		6.7	9.2	
2%	14.34	12.62	9.07	12.22	12.0	36.8	14.8
4%	13.89	9.65	8.84	9.98	30.5	36.4	28.2

¹Groups are listed by the sitosterol contents of the diets they received.

changes in blood sterol levels were not found in hens fed the experimental control diet, nor in hens maintained on standard ration (the "double control" group).

Due to the low specific activity of the dietary sitosterol and, furthermore, because of the fact that small aliquots of the DgPS solution had to be taken to avoid excessive quenching due to methanol, the counting rates of blood DgPS samples were very low (albeit significantly higher than background). The large probable errors associated with such low counting rates do not warrant a precise quantitative evaluation of the H^3 -sitosterol content of blood, and hence, the total DgPS levels in blood could not be corrected for the presence of sitosterol. However, since a sample counting rate of as little as 1 CPM above background would have meant the presence of 29 mg of sitosterol per 100 ml plasma, and the samples were found to contain from 0.5 to 3 CPM, the presence of quite significant amounts of H^3 -sitosterol in blood was indicated.

Production and Appearance of Eggs. Eggs were produced with the same regularity during the experimental feeding period as they were during the preceding control and the subsequent re-feeding periods. The eggs had a normal appearance, and neither their total weights nor their yolk weights were affected.

DISCUSSION

Sitosterol

The results of the experiment in which intestinal absorption of sitosterol was measured in laying and nonlaying hens fed a 2% sitosterol diet confirm the observation by Schön (17) on humans, namely that sitosterol dispersed on carboxymethylcellulose is well absorbed. The finding that the laying hens absorbed sitosterol with about 60% efficiency and the non-layers with about 85% may indicate that a step involved in intestinal sitosterol absorption in the laying hen had become rate-limiting. Hence, the greatly larger amounts of sitosterol ingested by the laying hens were absorbed with an efficiency that was slightly lower than that in the non-layers, who consumed less of the sitosterol-supplemented diet. That this rate-limiting step may be "pushed" in the direction of sitosterol absorption by loading the system with the plant sterol is suggested by various observations: 1) In absolute amounts, the laying hens absorbed more sitosterol than did the non-layers; 2) When the H^3 -sitosterol diets were fed over an extended time period, maximal effects (lowering of the egg cholesterol levels as well as incorporation of sitosterol in eggs) were found during the shorter feeding periods when higher levels of sitosterol were administered in the diet.

A rough estimate of the sitosterol balance in hens may be attempted on the basis of these findings and those reported in the literature. If the laying hen absorbed 60% per day of the sitosterol contained in 50 g of 1.83% sitosterol-containing

diet, its daily intake would amount to about 550 mg. In rats, 30% of the sitosterol absorbed is oxidized by the intestinal mucosa and excreted into feces (10). If the hen resembles the rat in this respect, about 180 mg of sitosterol might have been eliminated along this pathway, leaving 370 mg of absorbed sitosterol in the hen to be accounted for. Assuming that 4.5% of the 2025 g hen was plasma containing about 30-90 mg % sitosterol (see above, Blood Sterols), then there was a plasma pool of about 60 mg sitosterol. With the liver containing, in the steady state, about the same amount of the plant sterol, and an egg about 45 mg, there remains about 200 mg of ingested sitosterol unaccounted for. Since little, if any, sitosterol is incorporated in animal tissues (18), about 200 mg of sitosterol may have been converted per day to bile acids under the conditions of the experiment.

Suitability of H^3 -Sitosterol for Biological Experimentation

After H^3 -sitosterol, prepared by exchange with tritiated water in the presence of platinum dioxide catalyst (37), has been treated with KOH in order to remove labile H^3 from the compound, the resulting preparation has its H^3 firmly attached to the sterol. The work of Gould, Werbin, and others (15, 27, 34, 42) on rats, humans, and guinea pigs has demonstrated the adequacy of tritium as a label for sitosterol in metabolism studies. These findings confirm this in hens: the feces in the absorption experiment contained the same percentage of administered sterol and administered radioactivity, indicating that

in the feces the label was still attached to the sterol. Also, rechromatography of formerly chromatographed H^3 -sitosterol resulted in complete recovery of material and label in the sterol-containing fractions. The constant specific activity of dietary H^3 -sitosterol was relied upon to convert measurements of radioactivity into quantities of sitosterol present in the materials counted.

Lowering of the Cholesterol Levels in Eggs

One of the most significant observations is that sitosterol supplemented in finely dispersed form to a standard ration is capable of lowering the cholesterol level of eggs by as much as 37%. The results obtained with the 2% sitosterol diet (fig. 4) might suggest that the egg cholesterol levels rebound after an initially large drop. Such rebounding could then point to adaptational changes in the activities of cholesterol producing enzymes. However, the significance of this rebounding is disavowed for various reasons: 1) When present, adaptations of cholesterologenic enzyme activities occur within a day after imposing a stimulating condition upon animals (42); 2) No effect of sitosterol upon the rate of cholesterol synthesis by liver has been found (24); 3) Low egg cholesterol levels were found in the 2% sitosterol group after 7 days of refeeding standard ration (fig. 4); 4) Three out of the 4 high values (fig. 4) during the "rebounding" period were obtained from eggs produced by hen number 20, which had just recovered from a lapse in egg

production that had continued over most of the experimental feeding period and was attributed to molting. It is conceivable that the first eggs produced after molting contain elevated levels of cholesterol, just as the last eggs produced before molting did (e.g., table 2, hen number 28); 5) No significant rebounding was noticed when the 4% sitosterol diet was fed (figs. 5 and 6).

During the control period, when standard ration was fed, it was found that the cholesterol level in eggs produced by the same hen was remarkably constant (table 2). Because of this small day-to-day variation in egg sterol levels, the differences between hens were very significant. Considering that there were hens producing eggs with over 17 mg of sterols per g yolk and other hens laying eggs with only about 13 mg of sterols per g yolk, selective breeding of hens appears a promising means of producing low-cholesterol eggs.

The question may be asked: Can cholesterol be completely eliminated from eggs, or is there a limit below which the egg cholesterol level by sitosterol feeding may not be decreased? This question could probably be answered if all functions of cholesterol in an egg were known, but they are not. In their chemical nature, cholesterol and sitosterol are so much alike that the plant sterol could substitute for cholesterol. But the structural difference, the extra ethyl group on sitosterol, would seem to exclude the possibility of sitosterol taking the place of cholesterol in structural components of the egg and

of a developing embryo (such as cell membranes and myelin). In a fertilized egg, therefore, a limitation to which the cholesterol level could be lowered is clearly indicated. In other eggs, too, cholesterol is likely to remain the preponderant sterol as it is in plasma. An indication that an egg cholesterol-lowering limit may have been approached under the experimental conditions is that the magnitudes of the effects of the 2% and the 4% sitosterol diets were similar (table 5).

Incorporation of Sitosterol into Eggs

Pharmaceutical use of sitosterol has been recommended because of its potency in lowering blood and tissue cholesterol levels and since no harmful side-effects of administering even vast doses of sitosterol have ever become apparent. Therefore, the incorporation of about 45 mg of sitosterol in eggs is important. Compared to an estimated average daily consumption of about 500 mg of plant sterols by humans (15), the 45 mg contained in an egg may seem a small amount. But the 45 mg of sitosterol was concentrated in a 20 g yolk containing about 180 mg of cholesterol in the experimental eggs. On the basis of evidence obtained when sitosterol was administered simultaneously with cholesterol (1-4) and, further more, since the presence of even small amounts of sitosterol in the intestines has been shown to cause severe impairment of cholesterol absorption, it may be expected

that absorption of the 180 mg of egg cholesterol is prevented by the simultaneous presence of 45 mg of sitosterol in the intestine.

Thoughts for Continued Research

The low-cholesterol, sitosterol-containing eggs will have to be fed to experimental animals in order to evaluate their effect on the blood and tissue cholesterol levels of the consumer. Furthermore, since lowering of the levels of other atherogenic lipids under influence of sitosterol feeding has been reported (8, 9) in a number of animal tissues, it is worth measuring the levels of these lipid classes in the experimental eggs and in their eventual consumers. There is no reason to doubt that the administration of plant sterols to lactating animals may lead to production of low-cholesterol, sitosterol-containing milk. The nutritional qualities of the experimental products remain to be measured. Also, the hatchability of eggs produced by sitosterol-fed hens must be investigated.

Even though much work remains to be done, an important step forward may have been made towards removing an atherogenic factor from the human diet,

SUMMARY

In recent years, eggs have obtained notorious status among atherogenic nutrients because of their abundance in

suspect lipid classes, notably cholesterol. Based on the well-documented observation that plant sterols, predominantly sitosterol, have a potent effectiveness in lowering cholesterol levels in blood and tissues, it was expected that a decrease in egg cholesterol content would result by feeding laying hens diets supplemented with sitosterol.

Sitosterol has to be administered in a finely dispersed form, otherwise it is not absorbed from the intestines. A 20% (w/w) emulsion of sitosterol in 3% carboxymethylcellulose was prepared and portions of this emulsion were blended into standard chicken ration. The diets prepared each contained the same amount of carboxymethylcellulose (0.5%, w/w) but different proportions of sitosterol, namely 0% (control diet), 1%, 2%, or 4%. Sitosterol added to the diets was labeled with 188 CPM of well-purified, tritiated sitosterol per mg sterol. This specific radioactivity value was used to convert CPM measured in a sample to mg sitosterol contained in the sample.

The 2% sitosterol diet was fed to 2 laying and 2 non-laying hens for one day, after which standard ration was refed. Food intake was measured and feces, collected daily for 6 days, were analyzed for sterols and radioactivity. Since sitosterol was found to be absorbed with 60% efficiency by the layers (and with 85% efficiency by the non layers), the experimental diet was deemed an adequate means of administering sitosterol to hens.

Over a 2.5-month period, standard diet was fed to hens housed in individual cages. A record of egg production was kept for each hen, and the eggs were analyzed for their sterol content. Differences in the sterol level of eggs obtained from the same hen were remarkably small, but differences between hens of as much as 30% were observed. Hence, selective breeding of hens appears to be a promising method of producing eggs with a low cholesterol content. The last eggs produced before the hen enters a non-productive period while molting were found to contain up to twice the cholesterol level found normally in eggs of the same hen. On the basis of productivity and average egg sterol contents, 4 groups of 5 hens each were selected for the subsequent sitosterol-feeding experiment, while the remaining hens were maintained on standard ration.

The 4 groups of hens each received one of the four experimental diets for a period of about 2 months, after which the standard ration was refed. Eggs, collected daily, were analyzed for their sterol and radioactivity contents. The carboxymethylcellulose alone (control diet) did not influence the egg sterol level. The 1% sitosterol diet had no consistent effect on the level of total sterols in eggs, but after the total sterol levels had been corrected for their H^3 -sitosterol contents, a significant decrease (9%) in egg cholesterol level was found after about 1 month of experimental feeding. The 2 and 4% sitosterol diets both

decreased egg cholesterol levels by about 36%, and these low egg cholesterol levels reverted back to normal upon refeeding standard ration. Also, the maximal sitosterol incorporations in these 2 groups of hens were found equal in magnitude, namely about 45 mg sitosterol per egg. The only difference between the 2 high-sitosterol diets was that maximal effects were found after 1 week with 4% sitosterol diet, and after about 2 weeks with the 2% diet.

Based on evidence reported in the literature, it was felt that the amounts of sitosterol included in the experimental eggs are sufficient to block intestinal absorption of the decreased quantities of cholesterol contained therein, so that an important step towards removal of an atherogenic factor from the diet has been made.

LITERATURE CITED

1. Pollak, O. J. 1953 Successful prevention of experimental hypercholesteremia and cholesterol atherosclerosis in the rabbit. *Circulation* 7: 696.
2. Pollak, O. J. 1953 Reduction of blood cholesterol in man. *Circulation* 7: 702.
3. Betzien, G., H. Brachars, P. B. Diezel, H. Franke, R. Kuhn, and T. Seidl 1961 Zur Wirkung des Sitosterins auf den Cholesterin-Stoffwechsel beim Warmblütter. *Arzneimittel-Forsch.*, 11: 751.
4. Ryzhova, A. F. 1965 Effect of the combined injection of p-aminobenzoic acid and beta-sitosterol on the course of experimental atherosclerosis in rabbits. *Byul. Eksp. Biol. Med.*, 60: 83.
5. Nutrition Reviews 1965 Experimental atherosclerosis in rabbits. 23: 329.
6. Peterson, D. W. 1951 Effect of soybean sterols in the diet on plasma and liver cholesterol in chicks. *Proc. Soc. Exptl. Biol. Med.*, 78: 143.
7. Gerson, T., F. B. Shorland, and G. G. Dunckley 1963 The effect of B-sitosterol on cholesterol and lipid metabolism in the rat. *Nature* 200: 579.
8. Gerson, T., F. B. Shorland and G. G. Dunckley 1964 The effect of B-sitosterol on the metabolism of cholesterol and lipids in rats on a diet low in fat. *Biochem. J.*, 92: 385.
9. Gerson, T., F. B. Shorland, and G. G. Dunckley 1965 The effect of B-sitosterol on the metabolism of cholesterol and lipids in rats on a diet containing coconut oil. *Biochem. J.*, 96: 399.
10. Swell, L., E. C. Trout, Jr., H. Field, Jr., and C. R. Treadwell 1959 Intestinal metabolism of C¹⁴-phytosterols. *J. Biol. Chem.*, 234: 2286.
11. Gerson, T., F. B. Shorland, and Y. Adams 1961 The effects of corn oil on the amounts of cholesterol and the excretion of sterol in the rat. *Biochem. J.*, 81: 584.
12. Swell, L., T. A. Boiter, H. Field, Jr., and C. R. Treadwell 1956 The absorption of plant sterols and their effect on serum and liver sterol levels. *J. Nutrition* 58: 385.

13. Ivy, A. G., T. M. Lin, and E. Karvinen 1955 Absorption of dihydrocholesterol and soya sterols by the rat's intestine. *Am. J. Physiol.*, 183: 79.
14. Haust, H. L., and J. M. R. Beveridge 1963 Type and quantity of 3B-hydroxysterols excreted by subjects subsisting on formula rations high in corn oil. *J. Nutrition*, 81: 13.
15. Werbin, H., I. L. Chaikoff, and E. E. Jones 1960 The metabolism of H³-B-sitosterol in the quinea pig: Its conversion to urinary cortisol. *J. Biol. Chem.*, 235: 1629.
16. Herrmann, G. R., and A. Samawi 1962 The effects of various serum cholesterol lowering procedures and agents in patients with coronary artery disease. *Texas Reports Biol. Med.*, 20: 599.
17. Schön, H. 1959 Sterol-balance experiments in humans. *Nature* 184: 1872.
18. Swell, L., and C. R. Treadwell 1961 Metabolic fate of injected C¹⁴-phytosterols. *Proc. Soc. Exptl. Biol. Med.*, 108: 810.
19. Dunham, L. W., R. E. Fortner, R. D. Moore, H. W. Culp, and C. N. Rice 1959 Comparative lymphatic absorption of B-sitosterol and cholesterol by the rat. *Arch. Biochem. Biophys.*, 82: 50.
20. Beveridge, J. M. R., W. F. Convell, G. A. Mayer, and H. L. Austin 1958 The hypocholesterolemic effect of corn oil attributed to plant sterols. *Canad. J. Biochem.*, 36: 895.
21. Hernandez, H. H., D. W. Peterson, I. L. Chaikoff and W. G. Dauben 1953 Absorption of cholesterol-4-C¹⁴ in rats fed mixed soybean sterols and B-sitosterol. *Proc. Soc. Exptl. Biol. Med.*, 83: 498.
22. Hernandez, H. H., and I. L. Chaikoff 1954 Do soy sterols interfere with absorption of cholesterol? *Proc. Soc. Exptl. Biol. Med.*, 87: 541.
23. Swell, L., T. A. Boiter, H. Field, Jr., and C. R. Treadwell 1954 The esterification of soybean sterols in vitro and their influence on blood cholesterol. *Proc. Soc. Exptl. Biol. Med.*, 86: 295.
24. *Nutrition Reviews* 1962 Mechanism for lowering serum cholesterol concentrations. 20: 72.

25. Swell, L., E. C. Trout, Jr., G. V. Vahouny, H. Field, Jr., S. von Schuching, and C. R. Treadwell 1958 Influence of H^3 -B-sitosterol on sterol excretion. Proc. Soc. Exptl. Biol. Med., 97: 337.
26. Gould, R. G., L. V. Lotz, and E. M. Lilly 1957 In G. Popják and E. LeBreton (Editors) International conference on the biochemical problems of lipids, Interscience Publishers, Inc., New York, p. 353; and J. Glover and C. Green, Ibid., 1955: p. 359.
27. Gould, R. G. 1955 Absorbability of beta-sitosterol. Trans. N. Y. Acad. Sci., 18: 129.
28. Swell, L., T. A. Boiter, H. Field, Jr., and C. R. Treadwell 1959 Absorption of H^3 -sitosterol in the lymph fistula rat. Proc. Soc. Exptl. Biol. Med., 100: 140.
29. Glover, J., and C. Green 1957 The distribution and transport of sterols across the intestinal mucosa of the guinea pig. Biochem. J., 67: 308.
30. Glover, J., and G. A. Morten 1958 Absorption and metabolism of sterols. Brit. Med. Bull., 14: 226.
31. Schön, H., and P. Engelhardt 1957 Zur Frage der Resorption des B-sitosterols. Naturwissenschaften 44: 116.
32. Nutrition Reviews 1964 The effect of corn oil on fecal sitosterol and cholesterol in man. 22: 35.
33. Weiss, J. P., R. M. Johnson and E. C. Naber 1967 Effect of some dietary factors and drugs on cholesterol concentration in the egg and plasma of the hen. J. Nutrition, 91: 119.
34. Werbin, H., I. L. Chaikoff and M. R. Imada 1960 Radiochemical purity of beta-sitosterols tritiated by catalytic exchange and by the Wilzbach procedure. Arch. Biochem. Biophys., 89: 213.
35. Marker, R. E., and E. J. Lawson 1937 Sterols. XXVI. Sitosteryl chloride, stigmasteryl chloride and related compounds. J. Am. Chem. Soc., 59: 2711.
36. Shoppee, C. W. 1946 Steroids and the walden inversion. Part II Derivatives of Δ^5 -cholestene and Δ^5 -androstene. J. Chem. Soc., 1147.

37. Gould, R. G. 1958 H^3 -cholesterol. In Organic Synthesis With Isotopes, part II (A. Murry, III and D. L. William), Interscience Publ., New York, p. 1694.
38. Creech, B. G., and B. W. Sewell 1962 A rapid micro procedure for determination of free and esterified cholesterol in blood serum using silicic acid separation. *Analyt. Biochem.*, 3: 119.
39. Zlatkis, A., B. Zak and A. Boyle 1953 A new method for the direct determination of serum cholesterol. *J. Lab. Clin. Med.*, 41: 486.
40. Sperry, W. M., and M. Webb 1950 A revision of the Schönheimer-Sperry method for cholesterol determination. *J. Biol. Chem.*, 187: 97.
41. Dixon, W. J., and F. J. Massey, Jr. 1957 Introduction to Statistical Analysis, 2nd edition, McGraw-Hill Book Company, Inc., New York.
42. Werbin, H., D. M. Bergenstal, R. G. Gould, and G. V. LeRoy 1957 Evaluation of tritium cholesterol as a tracer in man. *J. Clin. Endocrinol. and Metabolism* 17: 337.
43. Hill, R., W. W. Webster, J. M. Linazasoro, and I. L. Chaikoff 1960 Time of occurrence of changes in the liver's capacity to utilize acetate for fatty acid and cholesterol synthesis after fat feeding. *J. Lipid Res.*, 1: 150.

ACKNOWLEDGMENTS

Gratitude is expressed to Justus Liebig Universitat, Giessen, W. Germany, who sent me as an exchange student to Kansas State University. I wish to express my deepest gratitude to Dr. Rudolf Clarenburg, Research Advisor, Professor of Department of Physiology, College of Veterinary Medicine for his assistance in setting up the project, his personal guidance and constructive criticism in the final preparation of the manuscript, and finally for presiding at the final examination.

Appreciation is expressed to Dr. Lucille M. Wakefield, Head of the Department of Foods and Nutrition, who arranged for my financial assistance and reviewing the manuscript. Without her continuous assistance, it would not have been possible for me to have completed the studies.

I wish to thank Dr. Beth Fryer, Major Professor and Professor of Foods and Nutrition for guidance throughout the course of my studies, and reviewing the manuscript.

I also want to express my appreciation to the Department of Physiology and various members providing me laboratory facilities which made my research work possible.

The writer also wishes to thank Mrs. Alice L. Christman, Audyne E. Self for their kindness, and Mrs. Vesta Kerr for typing the manuscript.

My personal appreciation is specially expressed to my husband, Dr. Young Sup Chung, mother-in-law, my son, and family for their moral support, understanding and patience to see my studies through.

REMOVAL OF AN ATHEROGENIC FACTOR FROM THE HUMAN DIET

by

INHI A. KIM CHUNG

Diplom, Justus Liebig Universität, Giessen, W. Germany, 1966

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Foods and Nutrition

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1968

In recent years, eggs have obtained notorious status among atherogenic nutrients because of their abundance in suspect lipid classes, notably cholesterol. Based on the well-documented observation that plant sterols, predominantly sitosterol, have a potent effectiveness in lowering cholesterol levels in blood and tissues, it was expected that a decrease in egg cholesterol content would result by feeding laying hens diets supplemented with sitosterol.

Sitosterol has to be administered in a finely dispersed form, otherwise it is not absorbed from the intestines. A 20% (w/w) emulsion of sitosterol in 3% carboxymethylcellulose was prepared and portions of this emulsion were blended into standard chicken ration. The diets prepared each contained the same amount of carboxymethylcellulose (0.5%, w/w) but different proportions of sitosterol, namely 0% (control diet), 1%, 2%, or 4%. Sitosterol added to the diets was labeled with 188 CPM of well-purified, tritiated sitosterol per mg sterol. This specific radioactivity value was used to convert CPM measured in a sample to mg sitosterol contained in the sample.

The 2% sitosterol diet was fed to 2 laying and 2 non-laying hens for one day, after which standard ration was refed. Food intake was measured and feces, collected daily for 6 days, were analyzed for sterols and radioactivity. Since sitosterol was found to be absorbed with 60% efficiency by the layers (and with 85% efficiency by the non layers), the experimental diet was deemed an adequate means of administering sitosterol to hens.

Over a 2.5-month period, standard diet was fed to hens housed in individual cages. A record of egg production was kept for each hen, and the eggs were analyzed for their sterol content. Differences in the sterol level of eggs obtained from the same hen were remarkably small, but differences between hens of as much as 30% were observed. Hence, selective breeding of hens appears to be a promising method of producing eggs with a low cholesterol content. The last eggs produced before the hen enters a non-productive period while molting were found to contain up to twice the cholesterol level found normally in eggs of the same hen. On the basis of productivity and average egg sterol contents, 4 groups of 5 hens each were selected for the subsequent sitosterol-feeding experiment, while the remaining hens were maintained on standard ration.

The 4 groups of hens each received one of the four experimental diets for a period of about 2 months, after which the standard ration was refed. Eggs, collected daily, were analyzed for their sterol and radioactivity contents. The carboxymethylcellulose alone (control diet) did not influence the egg sterol level. The 1% sitosterol diet had no consistent effect on the level of total sterols in eggs, but after the total sterol levels had been corrected for their H^3 -sitosterol contents, a significant decrease (9%) in egg cholesterol level was found after about 1 month of experimental feeding. The 2 and 4% sitosterol diets both decreased egg cholesterol levels by about 36%, and these low egg cholesterol levels reverted back

to normal upon refeeding standard ration. Also, the maximal sitosterol incorporations in these 2 groups of hens were found equal in magnitude, namely about 45 mg sitosterol per egg. The only difference between the 2 high-sitosterol diets was that maximal effects were found after 1 week with 4% sitosterol diet, and after about 2 weeks with the 2% diet.

Based on evidence reported in the literature, it was felt that the amounts of sitosterol included in the experimental eggs are sufficient to block intestinal absorption of the decreased quantities of cholesterol contained therein, so that an important step towards removal of an atherogenic factor from the diet has been made.