Effect of cellulose in the diet on the recovery of dietary plant sterols from the feces

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ABSTRACT In one normal subject, J.S., fed several formula diets in a sterol balance study, only 25–58% of the ingested plant sterols were recovered from the stool. The dietary plant sterols were completely recovered from the stools of five other men. Plant sterol recovery was complete in all men when a diet of mixed general foods was consumed.

Since the chief differences in composition of the formula and the diet of mixed general foods were related to the different contents of cellulose and lactose, these components were added to the formula diet of J.S., and plant sterol balance studies were then carried out. The addition of fresh celery or pulverized cellulose to the formula diet partially corrected the usual fecal loss of plant sterols (80% being recovered). Lactose in the formula was only slightly corrective. However, the addition of both cellulose and lactose led to complete recovery of the ingested plant sterols in the feces.

Bacterial cultures of stools were incubated with added cholesterol-4-^{14}C, and a linear relationship between losses of sterol during balance studies and in vitro incubations was observed; that is, a considerable loss of the labeled cholesterol from cultures after the formula diet, but not after the diet of mixed general foods. This in vitro loss was also corrected by the addition of cellulose and lactose to the formula diet.

The loss of the sterol nucleus in the intestinal tract may occur at times because of the lack of certain dietary constituents. It is hypothesized that the metabolism of intestinal tract bacteria is altered when certain constituents are not present in the diet, and that these bacteria may then degrade the sterol nucleus.

SUPPLEMENTARY KEY WORDS cholesterol · β-sitosterol · cholesterol balance · degradation · cholesteryl sulfate · formula diet

Because plant sterols present in the diet are poorly absorbed by the intestinal tract, they should be quantitatively recoverable in the feces. In fact, many years ago, Schoenheimer and others observed that β-sitosterol and other plant sterols fed to rabbits and rats were largely recovered in the feces (1–6), and Gould found that only 5% of the dose of tritiated sitosterol was absorbed in several terminal patients (7). These results were confirmed in later studies (8). In contrast, however, Grundy, Ahrens, and Salen (9), and later Connor, Witiak, Stone, and Armstrong (10) have reported that there was incomplete recovery of ingested plant sterols from the feces of normal human subjects. The reason for these intestinal losses of plant sterols has not yet been precisely identified, but bacterial action in the gut has been suspected.

In sterol balance studies of six healthy volunteer subjects, we found one man who had a consistently negative plant sterol balance while ingesting a formula diet (10). In addition, when his feces were incubated with cholesterol-4-^{14}C, the recovery of the labeled material was incomplete. The ingestion of mixed general foods in a diet similar in composition to the formula diet led to the complete recovery of the plant sterols in the feces, and also the labeled cholesterol in the fecal cultures. The present report is concerned with studies in which a series of diets of differing fiber and carbohydrate content were fed in order to identify the dietary constituents responsible for the plant sterol losses in the feces.

MATERIALS AND METHODS

The first phase of the study was carried out with six normal men who were volunteers from the State Penitentiary, and who were hospitalized at the University of Iowa. The second stage of the study involved additional investigations of the one subject, J.S., who had shown a

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consistently negative plant sterol balance during the initial study (10). Diets were fed either as formulas or as weighed mixed general foods. All dietary periods were 3 weeks in duration. Calories were adjusted in order to maintain a constant body weight. A detailed analysis of all diets is listed in Table 1.

In the first phase of the study, the six men fed cholesterol-free formula diets containing either cocoa butter or redistilled corn oil (diets A or B) as the dietary fat. The plant sterol intake was 296–411 mg/day depending upon caloric intake and type of dietary fat. In the second phase of the study, a single subject (J.S.) was fed diets C and D. Diet C was composed of mixed general foods and contained cellulose and milk. Diet D was a formula diet, similar in composition to diet C but lacking both cellulose and lactose. The cholesterol source in both of these diets was egg yolk. The content of dietary fat and fatty acid compositions were similar. The sequence in which the diets were fed and the various additions to the formula diet are shown in Table 3.

The Determination of Plant Sterol Balance

For the determination of plant sterol balance, two techniques were utilized. The fecal plant sterols for each dietary period were measured and compared with the known dietary intake of plant sterols in order to determine the extent of losses in the intestinal tract. Stools collected over a 7 day period were pooled for analysis. The second procedure was to give a large dose of plant sterols in the diet and then to measure the amount of sterol recovered subsequently in the stool. This test was performed as follows. On the 14th day of each dietary period, 3000 mg of plant sterol was fed, and the stools were analyzed for the next 7 days. Collections were for only 7 days because the plant sterol output in the stool had returned to the base line level after this period of time. Appropriate correction in the calculations was made for the known base line plant sterol output derived from the diet itself. The base line output had been determined for the week before the acute test.

The fecal neutral sterols and steroids were isolated, and their quantity was determined by gas–liquid chromatography (11, 12). With this technique, plant sterols and their derivatives are readily identifiable. The gas–liquid chromatography was performed on an instrument equipped with a hydrogen flame ionization detector (F & M Biomedical Gas Chromatograph, Model 400; F & M Scientific Corp., Avondale, Pa.). The column was a 4 ft glass U-tube, 4 mm I.D. packed with Diatoport S (80–100 mesh) coated with a 3.8% film of SE-30. Temperatures of the column, detector, and flash heater were 230, 250, and 300°C, respectively. Helium was used as the carrier gas at a flow of 100 ml/min; the inlet pressure was 40 psi.

Aliquots of the formula diets and administered plant sterols were analyzed for plant sterol content and composition according to the same procedure which was utilized for fecal neutral sterols.

The Incubation of Fecal Bacteria with Labeled Sterol

Identical amounts of cholesterol-4-^{14}C were placed in each of two duplicate tubes arranged in four sets. The cholesterol-4-^{14}C (New England Nuclear Corp., Boston, Mass.) had been previously purified by thin-layer chromatography. 0.5 ml of pooled human serum and 10 ml of fresh thioglycolate broth were added to the second, third, and fourth sets of tubes. 0.1 ml of a 1:10 dilution of fresh feces in saline was then added to the third and fourth sets of tubes. The stool was obtained from subject J.S. on the mornings of the days when he was given the acute plant sterol test or 14 days after beginning each specified diet. 10 ml of ethanol was added immediately to the third set of tubes to prevent bacterial growth. All sets of tubes were then incubated anaerobically at 37°C for 72 hr. 10 ml of ethanol was then added to the fourth set of tubes to stop bacterial growth.

TABLE 1 THE COMPOSITION OF THE 3000 CALORIE FORMULA DIET AND GENERAL FOOD DIET

	Plant Sterols	Cholesterol	Fatty Acid Composition		
			Mono-unsaturated	Poly-unsaturated	Saturated
	mg	*mg*		%	
A. Formula, cocoa butter	398	0	34.0	3.0	61.0
B. Formula, corn oil	345	0	31.0	56.0	12.0
C. Mixed general foods	296	500	31.0	49.0	15.0
D. Formula, corn oil	370	500	31.0	56.0	12.0

Vitamins and minerals were added to meet the daily recommended allowances of the National Research Council. In the formula diets, protein from casein was 15% of total calories, the fat was 40%, and the carbohydrate 45% (cornstarch 19.5%, Dextrimaltose No. 1 [Mead Johnson & Co., Evansville, Ind.] 26.2%, and sucrose 44%). Cholesterol was added to diets C and D in the form of egg yolk.

The contents of all tubes were evaporated to 1 ml at 65°C under a gentle stream of nitrogen. The radioactivity of the cholesterol-4-^{14}C in each tube was counted in a Packard Tri-Carb liquid scintillation spectrometer after saponification and hexane extraction by methods previously described (13). Quenching was corrected by an automatic external standard. The aqueous phase of all extracts was evaporated to dryness under nitrogen in counting vials. 0.5 ml of methanol was added to assure complete solubilization of any polar metabolic products of the labeled cholesterol, and the samples were counted. The validity of quench correction was verified by adding known amounts of cholesterol-^{14}C to the hexane and aqueous phases of the extracts, and to the whole cultures which were then dried and suspended in the scintillation fluid with the aid of Cab-O-Sil®. The observed dpm was equal to the dpm added.

Radioactivity in the hexane extract from the second and third sets of tubes was compared with that in the first set in order to verify complete extraction after adding plasma (second set), and plasma, broth, feces, and alcohol (third set). Radioactivity in the hexane extract from the fourth set, which had been incubated 72 hr before stopping growth with alcohol, was then compared with that from the third set.

In three of the formula diet periods, a fifth set of culture tubes was incubated in a sealed glass apparatus attached to a U-tube water trap, and the gas produced from the culture was trapped. At the end of the experiment, the media were acidified to pH 2 with concentrated HCl. Gas was driven from the media by flushing with nitrogen, and any carbon dioxide was trapped in freshly distilled phenethylamine for subsequent liquid scintillation counting (14). The residue of the culture was then suspended in scintillation fluid (PPO–POPOP in toluene with 0.5 ml of methanol added to each 10 ml of the fluid) with the aid of Cab-O-Sil® (Packard Instrument Co., Inc., Downers Grove, Ill.) and counted. Quenching was corrected by an automatic external standard.

RESULTS

Plant Sterol Balance in Man

The dietary intake and fecal excretion of plant sterols by the six healthy human subjects during the last 2 weeks of each dietary period are summarized in Table 2. Dietary plant sterols consisted of sitosterol, stigmasterol, and campesterol. The sterols found in the stool were the original plant sterols plus their respective stanol and stanone derivatives. These derivatives result from the reduction of the Δ^5 double bond, and, in the case of the stanone, from the oxidation of the 3-β-hydroxy group to the 3-ketone derivative by the intestinal bacteria. While receiving formula diets which differed only in the source of fat, subjects 1–4 showed a consistent recovery of 91% or more of the ingested plant sterols. There was 90% recovery of ingested plant sterol from subject 5 in the third period, while only 74% and 79% were recovered in the first two periods. We consistently failed to recover 25–48% of ingested plant sterol from the feces of subject 6 (J.S.). The patients varied considerably with regard to the percentage of the ingested plant sterol recovered in feces as the parent sterol and as the bacterially-altered derivatives. However, in subject 6 the incomplete recovery of plant sterols involved both the parent sterol and its derivatives in a nearly constant ratio for all dietary periods.

Plant Sterol Balance Tests During Diets of Different Cellulose Content

The results of the plant sterol balance tests carried out in subject 6 (J.S.) during the final week of 3-week periods of varying diets are presented in Table 3. With the standard formula diet D, during periods 2 and 8, 39 and 47% of the 3000 mg of ingested plant sterol were recovered in feces. When the subject was fed a diet of similar composition but provided as mixed general foods, 107–122% of the plant sterol was recovered in the stool during test periods 1, 6, and 7.

TABLE 2 THE DIETARY INTAKE AND FECAL EXCRETION OF PLANT STEROLS AND THEIR BACTERIAL DEGRADATION DERIVATIVES DURING DIFFERENT FORMULA DIET PERIODS

	I. Cocoa Butter					II. Corn Oil					III. Cocoa Butter					
		Output					Output					Output				
Subject	Intake	Sterol	Stanol and Stanone	Total	Recovery	Intake	Sterol	Stanol and Stanone	Total	Recovery	Intake	Sterol	Stanol and Stanone	Total	Recovery	Mean % Recovery All Periods
	mg		*mg*		%	*mg*		*mg*		%	*mg*		*mg*		%	
1	374	348	0	348	93	300	323	27	350	117	374	378	2	380	102	104
2	398	123	252	375	94	324	110	314	424	131	398	32	290	322	81	102
3	374	72	270	342	91	300	57	316	373	124	374	59	337	396	106	107
4	398	77	289	366	92	324	366	41	407	126	398	411	17	428	108	109
5	411	53	251	304	74	357	53	228	281	79	411	81	287	368	90	81
6	398	22	198	220	55	324	90	153	243	75	398	56	111	167	42	57

TABLE 3 THE EFFECT OF DIETARY CONSTITUENTS ON RECOVERY OF PLANT STEROL IN THE FECES AND CHOLESTEROL-4-^{14}C IN FECAL CULTURES

Dietary Period	Diet	Additions to Diet	Lactose	Cellulose	Plant Sterol Balance			Cholesterol-4-^{14}C in Fecal Cultures		
					Intake*	Recovery	Recovery	Preincubation	After 72 hr Incubation	Recovery
			g	g	mg		%	dpm†		%
1	C (mixed general foods)	None	46	7.3	3000	3199	107	1.628×10^5	1.608×10^5	98.7
2	D (formula)	None	0	0	3000	1159	39	1.337×10^5	1.080×10^5	80.4
3	D	Celery (400 g)	0	2.4	3000	2072	69	1.551×10^5	1.448×10^5	93.3
4	D	Ethyl cellulose‡	0	3.5‡	3000	2335	78	1.559×10^5	1.410×10^5	90.4
5	D	Ethyl cellulose	0	7.5‡	3000	2395	80	8.105×10^4	7.367×10^4	92.7
6	C	None	46	7.3	3000	3525	117	1.216×10^4	1.202×10^5	98.8
7	C	None	46	7.3	3000	3666	122	—	—	—
8	D	None	0	0	3000	1415	47	1.050×10^5	7.526×10^4	71.6
9	D	Lactose	46	0	3000	1779	59	7.278×10^4	5.031×10^4	68.3
10	D	Lactose, ethyl cellulose	46	7.5	3000	3206	107	1.428×10^5	1.398×10^5	97.8

* Cytellin(R), furnished by Dr. Erold R. Diller (Eli Lilly & Co., Indianapolis, Ind.) containing β-sitosterol 65%, campesterol 30% and stigmasterol 5%.

† Mean values of duplicate determination whose maximum difference in no instance exceeded 1.27% of the mean.

‡ Ethyl cellulose, Cellosize(R) (W. P.-440 H; Union Carbide Corp., Chemicals Div., New York).

In order to determine which substances in the general food diet, but absent in the formula diets, might have prevented the loss of fecal plant sterol, we added several constituents of the general food diet to the formula diet. The ingestion of 400 g of fresh celery with the standard formula increased the recovery of the 3000 mg of plant sterol to 2072 mg or 69% (period 3). Because the effect of celery might have resulted from its cellulose content, the subject was then given formula diets with increasing amounts of cellulose. With the ingestion of 3.5 g of cellulose per day, the recovery of plant sterol was 78%; with 7.5 g of cellulose (periods 4 and 5) the recovery was 80%. The carbohydrate composition of the formula diet was then altered to include 46 g of lactose because the general food diet had contained lactose from milk. The plant sterol recovery with lactose was somewhat greater (period 9). When both cellulose and lactose were added to the formula, there was complete recovery of the ingested test meal of plant sterols (period 10).

The Recovery of Cholesterol-4-^{14}C from 72-hr Fecal Cultures

The recovery of labeled cholesterol from the first three sets of duplicate tubes was virtually complete in all instances, with differences of less than 2%. The recovery of the labeled cholesterol from the third set of tubes served as a control with which the radioactivity found in the fourth set of tubes incubated with live bacteria, could be compared. With a background diet of mixed general foods (periods 1 and 6), the recovery of the labeled cholesterol from the 72-hr cultures was 98.7 and 98.8% (Table 3). When formula diet D was fed, only 80.4 and 71.6% of the added labeled cholesterol could be found after incubation (periods 2 and 9). The addition of celery or cellulose to the formula diet increased the recoveries of the cholesterol to 93.3, 90.4, and 92.7% in periods 3, 4, and 5, respectively. The lactose in the diet did not improve the recovery of the labeled cholesterol (period 9). In the final dietary period when the subject received both 7.5 g of ethyl cellulose and lactose in the formula, the recovery was 97.8%.

In an effort to find the labeled cholesterol not recovered from the bacterial cultures to which it had been added, we also measured the radioactivity of the aqueous phase after saponification and hexane extraction of the cultures. No radioactivity in the aqueous phase was found. When the culture apparatus was designed to trap all carbon dioxide produced during incubation, no radioactive carbon dioxide was obtained. In a final effort to identify the fraction containing the missing ^{14}C, the whole culture after incubation was evaporated to dryness under a stream of nitrogen, suspended with Cab-O-Sil®, and counted. The counts observed in the whole culture suspensions were the same as those observed in the hexane extracts of duplicate cultures. No additional radioactivity was observed in the unextracted whole culture.

DISCUSSION

The evidence seems clear that severe losses of plant sterols can occur at times in the intestinal tracts of some human beings. In the absence of methodological errors or appreciable intestinal absorption of plant sterols as both of these possibilities have largely been ruled out (7–10), it is reasonable to suppose that the bacteria of the lower gut may be stimulated to degrade the sterol nucleus.

The hypothesis that the incomplete fecal recovery of ingested plant sterol in one subject resulted from feeding

a formula diet is supported by the following three lines of evidence: (*a*) as the diet was changed from formula to solid foods and back to formula again, in successive dietary periods, plant sterol recovery was always complete on a solid food diet and incomplete with a formula diet; (*b*) the addition of the same quantity of constituents present in the solid food diet (cellulose and lactose) to the formula diet led to the complete recovery of plant sterols in the stool; and (*c*) the recovery of ring-labeled cholesterol from fecal cultures was incomplete during formula diet feeding, complete with mixed food diets, and also complete after feeding a formula diet to which cellulose and lactose were added.

Söhngen's experiments more than 50 years ago showed that certain soil microorganisms could grow in a culture media containing cholesterol as the sole source of carbon (15). Other workers have confirmed this observation with both soil organisms and bacteria from the intestinal tracts of patients (16–20). In these studies not all of the end products of the cholesterol "breakdown" have been identified.

Unlike Curran and Brewster who recovered $^{14}CO_2$ from cultures of *Escherichia coli* incubated with randomly ^{14}C-labeled cholesterol (17), we did not recover any labeled CO_2 from the stool cultures incubated with cholesterol-4-^{14}C. We searched for polar derivatives such as cholesteryl sulfate (9, 19) or polar products of cholesterol catabolism in the aqueous phase of the saponified culture material and in the complete culture material, but the missing label was not able to be found.

Our inability to identify the missing label led us to concur with Grundy et al. that the metabolic breakdown products may be substances which escape into the environment (9). Another possibility to account for the losses might be the absorption of the labeled breakdown products in the body and their subsequent dilution in large body pools. No evidence is yet available to support either of these views.

We suggest, on the basis of these experimental results, that perhaps the intestinal bacteria normally utilize the cellulose of the diet as a carbon source to meet their growth requirements. When this carbon source is not provided, as in the formula diet, the intestinal bacteria find alternate sources of carbon in the plant sterol nucleus, thereby causing its loss from the intestine. Why this phenomenon is not universal in all subjects given a formula diet, or if it even occurs when a mixed general food diet is consumed is not known. This hypothesis is strengthened by the correlation of the in vitro bacterial incubation studies with what occurred in vivo. The stool cultures (even though a carbon source was provided in the incubation medium) "destroyed" or lost the sterol nucleus whenever the straight formula diet had been fed. These losses were minimal or less when cellulose was added or when solid foods were fed.

The action of the intestinal bacteria on the sterol nucleus to form stanol and stanone derivatives is apparently independent of their "degrading" capacity. Subjects 3 and 5 (Table 2) converted most of the plant sterols to stanol derivatives but exhibited no plant sterol losses. Subject 6 (J.S.) had consistent plant sterol losses, but converted plant sterols to stanols to a degree similar to subjects 3 and 5.

This study was supported by U.S. Public Health Service Research Grants HE-11,485, and AI07587, by the American and Iowa Heart Associations, and by the Clinical Research Center Grant M01-FR-59.

Manuscript received 8 January 1970; accepted 13 April 1970.

References

1. Schoenheimer, R. 1929. *Hoppe-Seyler's Z. Physiol. Chem.* **180:** 16.
2. Schoenheimer, R. 1929. *Hoppe-Seyler's Z. Physiol. Chem.* **180:** 24.
3. Schoenheimer, R. 1929. *Hoppe-Seyler's Z. Physiol. Chem.* **180:** 36.
4. Behring, V., and R. Schoenheimer. 1930. *Hoppe-Seyler's Z. Physiol. Chem.* **192:** 97.
5. Schoenheimer, R. 1931. *Science (Washington).* **74:** 579.
6. Swell, L., T. A. Boiter, H. Field, Jr., and C. R. Treadwell. 1956. *J. Nutr.* **58:** 385.
7. Gould, R. G. 1955. *Trans. N. Y. Acad. Sci.* **18:** 129.
8. Gould, G., R. J. Jones, G. V. Leroy, R. W. Wissler, and C. B. Taylor. 1969. *Metab. Clin. Exp.* **18:** 652.
9. Grundy, S. M., E. H. Ahrens, Jr., and G. Salen. 1968. *J. Lipid Res.* **9:** 374.
10. Connor, W. E., D. T. Witiak, D. B. Stone, and M. L. Armstrong. 1969. *J. Clin. Invest.* **48:** 1363.
11. Grundy, S. M., E. H. Ahrens, Jr., and T. A. Miettinen. 1965. *J. Lipid Res.* **6:** 397.
12. Miettinen, T. A., E. H. Ahrens, Jr., and S. M. Grundy. 1965. *J. Lipid Res.* **6:** 411.
13. Connor, W. E., R. Johnston, and D. S. Lin. 1969. *J. Lipid Res.* **10:** 388.
14. Yeh, S. Y., J. H. Cavanaugh, and L. A. Woods. 1966. *J. Pharm. Sci.* **55:** 1212.
15. Söhngen, N. L. 1913. *Zentrabl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. Orig.* II Abt. **37:** 595.
16. Turfitt, G. E. 1948. *Biochem. J.* **42:** 376.
17. Curran, G. L., and K. C. Brewster. 1952. *Bull. Johns Hopkins Hosp.* **91:** 68.
18. Stadtman, T. C., A. Cherkes, and C. B. Anfinsen. 1954. *J. Biol. Chem.* **206:** 511.
19. Wainfan, E., G. Henkin, S. C. Rittenberg, and W. Marx. 1954. *J. Biol. Chem.* **207:** 843.
20. Cook, R. P., D. C. Edwards, C. Riddell, and R. O. Thomson. 1955. *Biochem. J.* **61:** 676.