Dietary β-sitosterol as an internal standard to correct for cholesterol losses in sterol balance studies

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ABSTRACT In the course of carrying out sterol balance studies in 19 patients, we gathered the following evidence that, in some but not all patients, considerable amounts of neutral sterols are "lost" during their passage through the intestinal tract. (*a*) Since plant sterols are largely nonabsorbable in man, they should be totally recovered in the feces; yet in many patients significantly less plant sterol than expected was recovered, the loss amounting to as much as 56% of daily intake. (*b*) In two patients in whom cholesterol-^{14}C and β-sitosterol-^{3}H were instilled into the terminal ileum, from which neither sterol is absorbed, the feces contained 25% less of each isotope than was instilled. (*c*) In four patients fed radioactive cholesterol daily until the isotopic steady state was closely approximated, 28–50% of the isotope could not be accounted for. On the other hand, in five patients fed radioactive bile acids until the isotopic steady state was approximated, input equalled output as predicted.

Since the amount of β-sitosterol absorbed in man is limited (5% or less), this sterol can be used as an internal standard for upward correction of the figure obtained for the amount of neutral steroids excreted. The use of β-sitosterol for this purpose is based on three considerations: (*a*) it passes through the intestine in the same physicochemical state as cholesterol; (*b*) it accompanies cholesterol at every step of its isolation and chromatographic measurement; and (*c*) it is lost to the same extent as cholesterol. Excretion data for fecal neutral steroids can therefore be corrected for irregular fecal flow as well as for the "unexpected loss" referred to.

This loss seems to be due not to errors in stool collection or to technical errors, but to intestinal bacterial degradation of neutral 3β-OH,Δ^{5}-sterols to products not recognized as steroids in the analytical methods used.

KEY WORDS cholesterol · cholesteryl sulfate · β-sitosterol · plant sterols · degradation · internal standards · cholesterol balance · isotopic steady state · chromic oxide

FOR SEVERAL YEARS we have been developing techniques to determine cholesterol balance in man and in laboratory animals. In 1965 (1, 2) we reported methods for the quantitative analysis of dietary sterols and of fecal neutral and acidic steroids; later, we demonstrated the utility of chromic oxide as an internal marker with which to obtain meaningful rates of excretion of fecal steroids despite marked day-to-day variations in fecal flow in formula-fed patients (3).

Although these technical difficulties appeared to be solved, it became increasingly apparent that in many patients cholesterol was being unaccountably lost during its passage through the intestinal tract; thus we were faced with the problem of describing the balance of a disappearing entity. On further investigation we found that in any one patient plant sterols were lost to the same extent as cholesterol. Since the degree of absorption of plant sterols from the human intestine is exceedingly small (4), we reasoned that plant sterols might serve as markers for cholesterol balance studies. This idea was appealing for two reasons: first, the plant sterols partition between oily, aqueous, and particulate phases of intestinal contents in the same manner as cholesterol; and second, during the ultimate quantification by gas–liquid chromatography of neutral steroids derived from cholesterol, the neutral steroids derived from dietary plant sterols are necessarily measured also, and with equal precision (2).

In this paper we report that large amounts of neutral sterols seem to be lost as they pass through the intestine in most of our patients, and state our reasons for con-

Abbreviation: Hyamine, *p*-(diisobutylcresoxy-ethoxyethyl) dimethyl benzylamine.

cluding that these losses are probably due to degradation of the ring structure of neutral steroids by intestinal bacteria. In addition we show that for cholesterol balance studies plant sterols can serve as ideal internal standards: through corrections made possible with these standards, cholesterol balance can be determined with greater accuracy and convenience. These results have been discussed in a preliminary way elsewhere (5–7).

METHODS

Patients

Studies were carried out on 19 patients on the metabolic ward at The Rockefeller University Hospital. The age, sex, and clinical diagnosis of each patient are given in Table 1, along with a description of their lipoprotein pattern according to Fredrickson, Levy, and Lees (9).

Diets

Food intakes consisted exclusively of orally administered liquid formula feedings in which dietary fats contributed 40%, protein 15%, and glucose 45% of total caloric intake, as previously described (10), together with vitamin and mineral supplements. In each case caloric intake was adjusted so as to maintain total body weight at a constant level for the many weeks of the study.

Table 2 lists the dietary fats used in these studies, and their sterol contents. The fats in diets B and C had been subjected to molecular distillation (by Distillation Products Industries, Rochester, N.Y.) and steam deodorization (by E. F. Drew Co., Boonton, N.J.), processes which reduce their sterol contents without impairing their palatability.

Plant sterols were added to diets C, F, G, and H. Corn sterols (Staley), donated by Dr. S. S. Chang of the A. E. Staley Mfg. Co., Decatur, Ill., contained 65% β-sitosterol, 30% campesterol, and 5% stigmasterol. β-Sitosterol (Mann Research Labs, Inc., New York) was purified by Dr. Erold R. Diller, Eli Lilly & Co., Indianapolis, Ind.; the final product contained 90% β-sitosterol and 10% campesterol. Cytellin (also furnished by Dr. Diller) contained 65% β-sitosterol, 30% campesterol, and 5% stigmasterol. Crystalline cholesterol, pure according to gas–liquid chromatographic analysis (Mann Research Labs), was dissolved in corn oil during preparation of diet E, and in diet I egg yolk containing cholesterol was added to cottonseed oil. The cholesterol, total plant sterol, and β-sitosterol contents of the nine formulas fed to these patients were determined by the analytical methods previously described (2).

Isotopes and Their Measurement

Several patients ingested a small amount of radioactive cholesterol as a part of each formula feeding over periods of many weeks. Isotopic cholesterol, dissolved in 10 ml of ethanol, was added to 40 kg batches of formula during homogenization; hence, the daily intake of isotope in each patient was measurable and constant. The following isotopes of cholesterol were used: 4-^{14}C, 1,2-^{3}H, or 7α-^{3}H (New England Nuclear Corp., Boston, Mass.); before use, we established by thin-layer chromatography that all radioactivity was present in cholesterol.

Cholesterol-7α-^{3}H was the first tritiated isotope of cholesterol which was available to us. When this isotope of cholesterol is converted into bile acids, the 7α-^{3}H is lost from the steroid molecule (11); therefore, on this basis alone, measurements of fecal bile acid radioactivity

TABLE 1 CLINICAL DATA

Patient No.	Initials	Age	Sex	Weight (kg) and % of Ideal Wt.*		Diagnosis
		yr		*kg*	(%)	
1	H.Sa.	54	M	78	(117)	Hyperglyceridemia (Type V)†
2	J.C.	14	M	47	(96)	Hypercholesteremia (Type II)
3	E.Y.	39	F	46	(93)	Hypercholesteremia (Type II)
4	J.F.	39	M	78	(105)	IHD‡, hypercholesteremia (Type II)
5	S.C.	18	F	49	(93)	Hypercholesteremia (Type II)
6	R.M.	14	M	48	(98)	Hypercholesteremia (Type II)
7	A.M.	19	M	83	(131)	Hypercholesteremia (Type II)
8	R.G.	58	F	61	(120)	IHD, xanthomatosis, hypercholesteremia (Type II), essential hypertension
9	N.A.	30	M	67	(102)	IHD, hypercholesteremia (Type II)
10	J.H.	39	M	74	(104)	Hypercholesteremia (Type II)
11	J.R.	36	F	53	(98)	IHD, PVD,‡ xanthomatosis, hypercholesteremia (Type II)
12	J.St.	35	M	70	(104)	IHD, hypercholesteremia (Type II)
13	H.Sp.	50	F	78	(137)	IHD, hypercholesteremia (Type II), essential hypertension
14	H.R.	41	F	65	(120)	Hyperglyceridemia (Type IV)
15	J.Sh.	68	F	63	(117)	IHD, normocholesteremia
16	L.Sh.	70	M	64	(109)	IHD, normocholesteremia
17	L.M.	58	F	50	(97)	Hypercholesteremia (Type II), xanthomatosis
18	C.A.	53	M	71	(105)	Hyperglyceridemia (Type V)
19	R.T.	48	M	65	(108)	IHD, hyperglyceridemia (Type V)

* According to life insurance tables (8).

† Type of hyperlipoproteinemia according to Fredrickson et al. (9).

‡ IHD = ischemic heart disease; PVD = peripheral vascular disease.

TABLE 2 DIETARY FATS AND THEIR STEROL CONTENTS

Diet	Dietary Fat	Cholesterol	Total Plant Sterols	β-Sitosterol	Source of Plant Sterols
		mg/21.8 g dietary fat			
A	Corn oil	6.2	200	148	Inherent in dietary fat
B	Distilled corn oil	12.5	63.2	47	Inherent
C	Distilled butter oil	12.5	63.2	41	Corn sterols (Staley)
D	Cottonseed oil	10	80	71	Inherent
E	Corn oil	368	200	148	Inherent
F	Butter	80	40	35	β-Sitosterol (Mann)
G	Butter	80	1820–2800	1183–1820	Cytellin (Lilly)
H	Cottonseed oil	10	2420	1573	Cytellin (Lilly)
I	Cottonseed oil plus egg yolk	206	49	42	Inherent

are meaningless when this isotope is administered to patients. Nevertheless, fecal *neutral* steroid radioactivity is validly measured, and excretion data for fecal acidic steroids can be based on specific activity data for plasma cholesterol (as illustrated in Table 7).

β-Sitosterol-22,23-^{3}H was prepared for us by Dr. M. J. Thompson, Beltsville, Md., and Dr. G. Gupta of The Rockefeller University. The ammonium salt of cholesteryl sulfate, labeled at C-4 with ^{14}C or at C-1 and C-2 with ^{3}H, was prepared from the appropriate isotopes of cholesterol by the sulfation method of Roberts, Bandi, Calvin, Drucker, and Lieberman (12).

Microcombustion of feces, for measurement of total content of ^{3}H or ^{14}C, was performed by the method of Gupta (13); any volatile radioactive compounds would be lost during the drying step that precedes the combustion.

Plasma cholesterol concentration and specific activity were measured twice a week. The mass of plasma cholesterol was measured by the spectrophotometric method of Abell, Levy, Brodie, and Kendall (14); on an aliquot of the same extract, radioactivity was measured in a Packard Tri-Carb scintillation counter (model 3003) as previously described (2).

Expired $^{14}CO_2$ and urinary ^3H_2O were determined for patients who had been fed cholesterol-4-^{14}C and cholesterol-1,2-^{3}H daily until they had reached the isotopic steady state (15). Respired gas was collected in rubber balloons for 1 min every hr and bubbled through 10 ml of Hyamine (Packard Instrument Co., Inc., Downers Grove, Ill.), which trapped the CO_2 for measurement of its radioactivity. Aliquots of 24-hr urine collections were counted directly by adding 1 ml of urine to 18 ml of Bray's solution (16) with appropriate quench correction; other aliquots of urine were distilled and the colorless distillate was counted.

Analysis of Fecal Steroids[1]

Fecal neutral and acidic steroids were isolated separately, and their mass and specific activities were measured by the methods developed recently in this laboratory (1, 2). These procedures permit the essential distinction to be made between cholesterol and plant sterols, the bacterial conversion products (coprostanol and coprostanone) derived from cholesterol, and analogous bacterial products produced from plant sterols during intestinal transit.

Moser, Moser, and Orr (17) recently isolated cholesteryl sulfate (cholest-5-en-3β-yl sulfate) from human feces. The polarity of this compound renders it unextractable in our procedure for isolation of fecal neutral steroids, and the sulfate ester is strongly resistant to hydrolysis by weak alkali at 78°C; therefore, this compound is lost to analysis in the neutral steroid fraction. On the other hand, saponification under pressure (employed to deconjugate tauro- and glyco-bile acids) converts labeled cholesteryl sulfate to products which are not extractable from the acidified saponification mixture with chloroform–methanol; hence cholesteryl sulfate also escapes inclusion in the fecal bile acid fraction.

The concentration of cholesteryl sulfate in feces must therefore be measured independently, as follows. To an aliquot (2 g) of fecal homogenate, sufficient concentrated NH_4OH (about 2 ml) is added to bring the pH to about 11 and to form the ammonium salt of any cholesteryl sulfate present. After the addition of an internal recovery standard, ammonium cholesteryl-1,2-^{3}H sulfate in benzene–ethanol 4:1, the mixture is extracted with 19 volumes of chloroform–methanol 2:1. The extract is filtered and evaporated to dryness; 10 ml of water and 20 ml of ethanol are added; the pH is readjusted to 11 with concentrated NH_4OH, and neutral sterols are removed by extraction twice with 50-ml portions of petroleum ether (bp 60–84°C). The aqueous ethanolic residue containing the sulfate ester is evaporated to about 10 ml under N_2, and the pH of the solution is brought to 7 with 3 N H_2SO_4. Sufficient 3 M pyridinium sulfate is then added to make a 0.3 M solution, and the pyridinium salt of cholesteryl sulfate is extracted into chloroform (2 volumes × 3) (18). The chloroform is evaporated off, and the ammonium salt is re-formed when the residue is dissolved in concentrated NH_4OH–methanol 1:5. After evaporation to dryness, the residue is transferred with chloroform–methanol 2:1 to a 0.5 mm thin-layer plate (20 × 20 cm) of Silica Gel H, pre-

[1] The term fecal *steroid* is used in preference to *sterol* because of the significant amounts of ketonic metabolites of cholesterol that are usually present in neutral and acidic fractions.

viously washed with methanol and activated at 110°C before use. The chromatogram is developed in benzene–methyl ethyl ketone–ethanol–water 3:3:3:1; in this system (18*a*) free C_{27} sterols and sterol esters run at the solvent front whereas the cholesteryl sulfate ammonium salt has an R_f of 0.7; free and conjugated bile acids remain at the origin. The sulfate ester band (detected by means of iodine vapor) is recovered, and the salt quantitatively eluted with methanol. After evaporation of methanol the sulfate ester is solvolyzed according to Palmer (19). The cholesterol released by solvolysis is recovered, converted to its trimethylsilyl ether, and quantified against an internal standard of 5α-cholestane by gas–liquid chromatography, as in our routine procedure for fecal neutral steroids (2). Since this procedure permits only 60–80% of the labeled cholesteryl sulfate added to fecal homogenate to be recovered, the mass of cholesterol derived from cholesteryl sulfate and measured by gas chromatography is corrected upward, using the percentage recovery of radioactivity of labeled cholesteryl sulfate added as internal standard.

RESULTS

Losses of Plant Sterols[2]

Table 3 describes the balance of plant sterols in the first seven patients in this series; studies lasted 15–105 days. Even though the absorption of plant sterols is exceedingly small (4), balance measurements were not begun until the patients had been on their feeding regimen for at least 8 days; this permitted plant steroid excretion in feces to reach a plateau before the first recovery data were collected. The first patient received a formula containing corn oil (diet A) for the entire period. The other six were studied during two periods of approximately the same duration, in the second of which corn oil (diet B) was substituted for butter oil (diet C). The intake of cholesterol and of plant sterols for a given patient was held constant throughout each of the seven studies.

Average daily recoveries of ingested plant sterols in the feces were 90% or more in patients 1–4. However, large losses of plant sterols were noted in patients 6 and 7. Losses of this magnitude could not have been due to systematic laboratory errors, in view of the nearly quantitative recoveries found under the same experimental conditions in patients 1–4.

Table 4 presents recovery data for plant sterols at

TABLE 3 BALANCE DATA FOR PLANT STEROLS IN PATIENTS 1–7

Patient	Days:No. of Determinations*	Plant Sterol Intake	Plant Sterol Excretion	Recovery of Plant Sterols	Diet†
		mg/day		%	
1	15:12	975	929 ± 229‡	95	A
2	42:11	280	265 ± 97	95	C:B
3	101:15	225	202 ± 46	90	C:B
4	28:7	317	284 ± 62	90	C:B
5	25:7	249	218 ± 39	87	C:B
6	56:14	300	225 ± 88	75	C:B
7	56:14	402	274 ± 110	69	C:B

* Duration of study (days) and number of successive stool pools analyzed. All stools were collected and analyzed; the ratio of the two figures in this column gives the average stool collection period in days.

† See Table 2 for composition of diets. Patients on these diets ingested less than 80 mg of cholesterol per day.

‡ Mean ± SD (n is the second figure in column 2).

TABLE 4 BALANCE DATA FOR PATIENTS RECEIVING LOW AND HIGH INTAKES OF PLANT STEROLS

	Patient				
	8	9	10	11	12
Low plant sterol intake					
Diet	D	F	F	F	
Days:No. of determinations*	32:8	88:22	72:18	84:21	—
Plant sterol intake, μ (mg/day)	293	166	192	129	—
Plant sterol excretion, $\bar{x}$ (mg/day ± SD)†	172 ± 20	113 ± 44	105 ± 22	90 ± 60	—
Per cent recovery	59	68	55	70	—
High plant sterol intake					
Diet	H	G	G	G	G
Days:No. of determinations*	84:21	120:30	84:21	44:11	56:14
Plant sterol intake, μ (mg/day)	9980	9980	8520	10600	5530
Plant sterol excretion, $\bar{x}$ (mg/day ± SD)†	7850 ± 2390	7310 ± 2440	5650 ± 2440	7830 ± 2840	5220 ± 771
Per cent recovery	79	73	66	74	94
Daily plant sterol loss (mg/day)	2130	2660	2870	2780	308
Total plant sterol loss (g)	178.8	319.4	240.7	122.3	17.24

* See footnote* in Table 3.

† All differences between intake and excretion of plant sterols were significant at P levels < 0.005, as judged by Student's "t" test, where $t = (\bar{x} - \mu)/(\text{SD of } \bar{x})$.

[2] The term *plant sterols* signifies the total of three sterols: β-sitosterol, stigmasterol, and campesterol.

low- and high-intake levels in four other patients. With intakes of plant sterols of 129–293 mg/day, average daily recoveries in feces ranged from 59 to 70%. When the same four patients received the same formula diets supplemented with 8.5–10.6 g of plant sterols (Cytellin) per day, the range of recoveries for total plant sterols was 66–79%, approximately the same as on the low sterol intake. Patient 12, on the other hand, with a daily intake of 5.5 g of plant sterols, excreted 5.2 g per day, an average recovery of 94%; quantitative recovery in this patient indicated again the adequacy of our analytical methods.

Average daily losses of plant sterols are presented in Table 4 along with total accumulated losses through the entire study period in each case. Daily losses ranged from 2.1 to 2.9 g in patients 8–11; in patient 9, 319 g of total plant sterols was lost over a period of 120 days. Since plant sterols are absorbed from the human intestine to only a small extent (4), the magnitude of this loss makes it most unlikely that these large deficits can be explained by intestinal absorption and subsequent storage.

The relative losses of two plant sterols, β-sitosterol and campesterol (the major sterols in Cytellin), were studied in patients 8, 9, and 11 (Table 5). The ratios between the two in feces were essentially identical with that in the diet. Since the two sterols were lost to the same extent, it appeared that the difference in side-chain structure between these two sterols did not affect their rate of loss.

By using the inert marker, chromic oxide, we eliminated the possibility that losses of plant sterols resulted from incomplete stool collections. Each patient received by mouth 300 mg of chromic oxide daily for several days preceding and throughout the study period. As can be seen in Table 6, average daily recoveries of chromic oxide ranged from 91 to 112% during the period in which the balance of plant sterols was measured; quantitative recovery of chromic oxide proved that in all patients the collection of feces was essentially complete. Yet the recoveries of plant sterols, even after correction by means of the chromic oxide data, were only 42–82%. Thus, significant losses of plant sterols were found even though the collections of feces were proved to be complete.

In summary, the above studies led us to the conclusion that incomplete stool collections, systematic analytical errors, and the accumulation of large amounts of plant sterols within the body could all be ruled out as explanations for the losses of plant sterols described. We tentatively concluded that the losses of plant sterols during intestinal transit were due to conversion, presumably by intestinal microorganisms, to products that were no longer recognized as neutral steroids by our methods.

TABLE 5 COMPARISON OF THE β-SITOSTEROL/CAMPESTEROL RATIO IN THE DIET AND IN THE FECAL NEUTRAL STEROIDS OF PATIENTS ON HIGH INTAKES OF PLANT STEROLS

	β-Sitosterol : Campesterol* Ratio		
Patient	8	9	11
Dietary plant sterols	2.13	2.13	2.13
Fecal plant sterols			
1†	2.16	2.13	2.20
2	1.81	2.00	2.16
3	2.24	2.06	2.03
4	2.06	2.03	2.03

* Stigmasterol was also identified in all gas–liquid chromatographic analyses, but, since it comprised only about 5% of the total plant sterols, calculations were confined to the two major plant sterols.

† Analyses were performed on four separate 4-day stool collection periods spaced evenly from beginning to end of each patient's study.

Cholesterol Losses

The extent to which cholesterol might be lost in balance studies cannot be measured in the straightforward manner described above, since significant amounts of cholesterol are absorbed from the intestine each day and mix with newly synthesized cholesterol prior to conversion in the liver to bile acids. The existence of a large, continuously recirculating quantity of cholesterol and bile acids in the body and its distribution into many tissue pools made it necessary to study cholesterol losses during the isotopic steady state (15): this is defined as the equilibrium that develops after prolonged administration of radioactive cholesterol, when theoretically the amount of radioisotopic cholesterol (or its products) excreted is equal to the amount administered.

Cholesterol losses were measured after prolonged daily isotope intake in four patients. After 2–15 wk of oral administration of radioactive cholesterol, the specific activity of plasma cholesterol approached a plateau indicative of the postulated isotopic steady state (Fig. 1). Table 7 presents the results for daily recovery of radioactivity in the two fecal steroid fractions during the steady state in each of the four patients. Recovery of total radioactivity in the two fractions ranged from 50 to 72% of the intake of radioactive isotope.

Fig. 2 presents further evidence that the losses of radioisotopic steroids described above had not occurred during the isolation procedure. Four patients brought to the isotopic steady state by daily ingestion of radiocholesterol provided 35 4-day stool collections in each of which the radioactivity contained was measured in two ways: (*a*) by microcombustion (13) of an aliquot of the unprocessed stool homogenate in order to measure the entire amount of radioactivity excreted per day, and (*b*) by measurement of the radioactivity found in the fecal neutral and acidic steroid fractions excreted daily and isolated from the homogenate by our chemical procedures

TABLE 6 EVIDENCE THAT SIGNIFICANT LOSSES OF PLANT STEROLS OCCUR DESPITE QUANTITATIVE COLLECTION OF FECES

	Patient				
	19	15	15	13	17
Diet	A	A	E	A	D
Days:No. of determinations*	56:14	74:19	96:24	108:27	56:14
Plant sterol intake (mg/day)	882	888	855	995	237
Plant sterol excretion (mg/day ± SD)†	661 ± 140	653 ± 333	372 ± 173	613 ± 313	159 ± 50
Per cent recovery	75	74	44	62	67
Chromic oxide recovery (%)	91	101	105	98	112
Plant sterol excretion (mg/day ± SD, corrected by means of Cr_2O_3 data)†	721 ± 181	645 ± 144	361 ± 75	630 ± 159	142 ± 113
Per cent recovery (after correction with Cr_2O_3 data)	82	73	42	63	60

* See footnote* in Table 3.
† All differences between intake and excretion of plant sterols were significant at P levels < 0.005 (see footnote † in Table 4).

TABLE 7 RECOVERY OF DIETARY RADIOACTIVE CHOLESTEROL IN THE FORM OF FECAL STEROIDS DURING ISOTOPIC STEADY STATE

	Patient			
	13	14	15	16
Diet	A	A	E	I
Days:No. of determinations*	32:8	28:7	40:9	12:4
Dietary constituents				
Cholesterol (mg/day)	40	36	1587	1040
Plant sterols (mg/day)	995	825	639	216
Radioactive cholesterol (dpm/day × 10^{-6})	2.500	2.868	6.600	6.838
Isotope fed	4-^{14}C	4-^{14}C	7α-^{3}H	7α-^{3}H
Fecal radioactivity				
Neutral steroids (dpm/day × 10^{-6} ± SD)	1.05 ± 0.42	1.91 ± 0.64	3.50 ± 1.33	3.65 ± 1.21
Acidic steroids (dpm/day × 10^{-6} ± SD)	0.189 ± 0.076	0.167 ± 0.054	0.334† ± 0.238	0.464† ± 0.223
Total steroids (dpm/day × 10^{-6})	1.24	2.07	3.84	4.11
Per cent recovery	49.6	72.2	58.2	60.1

* See footnote* in Table 3.
† It is meaningless to measure the radioactivity in fecal acidic steroids when cholesterol-7α-^{3}H is administered, in view of specific loss of tritium at C-7α when this radiocholesterol is converted to bile acids (11). However, a meaningful balance sheet for radioactive sterols can be constructed for patients 15 and 16 by calculating a figure for radioactive fecal acidic steroids as follows: fecal acidic steroids (dpm/day × 10^{-6}) = fecal acidic steroids (mg/day, determined by gas–liquid chromatography) × specific activity of plasma cholesterol (dpm/mg × 10^{-6}). This calculation is based on our consistent finding that in the isotopic steady state the specific activity of fecal acidic steroids equals that of plasma cholesterol.

(1, 2). Results for total radioactivity obtained by analysis of the feces and of the fecal steroids corresponded closely in all patients; nevertheless, the amount of radioactivity recovered during the isotopic steady state was very much less than the intake in all four patients. Since all radioactivity in the dried feces was accounted for by that found in the two fecal steroid fractions, it became clear that incomplete recovery of sterols in these balance studies could not be explained by incomplete isolation of steroids from fecal homogenates.

In patients 10 and 11 (Fig. 2) the tritium not recovered in the stools was sought in urinary water. Assays of $^{3}H_2O$ were made on three successive days in each patient during the isotopic steady state; less than 1% of the tritium lost each day was found there.

The possibility that losses of neutral steroids were due to formation of cholesteryl sulfate (17), with subsequent failure to extract this extremely polar compound and to credit it to the neutral steroids, was tested by determination of cholesteryl sulfate in the feces of 10 patients; these 10 included two of the four patients in whom microcombustion studies were performed during the isotopic steady state. Cholesteryl sulfate was recovered from the stools of all 10 patients. The amount of cholesterol excreted as cholesteryl sulfate ranged from 7.1 to 66.3 mg/day (avg. 26.2 ± 2.5 mg/day), but in no patient did this amount exceed 8.3% of the daily excretion of neutral steroids. Sulfate esters of coprostanol and of the plant sterols were never found. We concluded that cholesterol and β-sitosterol were not being "lost" because of their excretion in the form of unextractable sterol sulfates.

Correction for Losses of Neutral Steroids

Since cholesterol and plant sterols both appeared to be partially lost during intestinal transit, it was reasonable

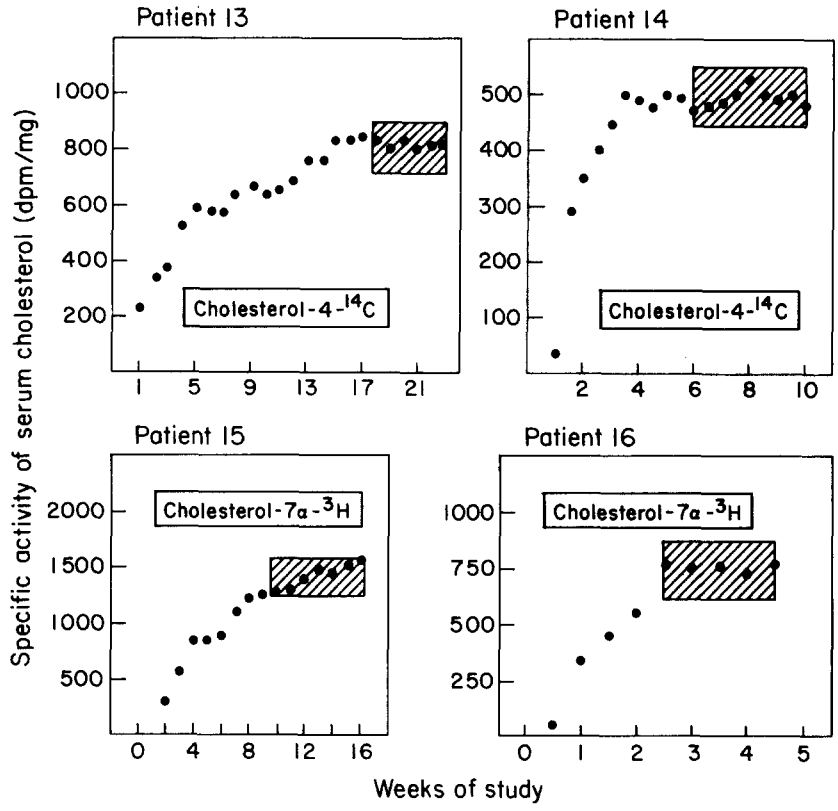

FIG. 1. Approximation to isotopic steady state in four patients. A plateau of specific activity of plasma cholesterol was sought in each patient given isotopic cholesterol daily by mouth. The "steady state" period in each patient is shaded; the data for fecal excretion of isotopic products in that period was used for calculations of cholesterol losses (Table 7).

to ask whether in each patient the two sterols were lost to the same extent. If this were true, then plant sterols could serve as an internal standard to correct for cholesterol losses.

In the four patients in whom the isotopic steady state had been approximated (Table 7 and Fig. 1) the appropriate calculations were made: the percentage recovery of plant sterols was used to correct for cholesterol losses, and the corrected recovery of radioactive fecal neutral steroids was compared to the intake of radioactive cholesterol. The measured amounts of acidic steroids were corrected merely for completeness of collection of feces (with chromic oxide as internal standard).

Fig. 3 presents the results of these calculations: in these four patients the recovery of total fecal radioactivity was raised from 50–72% to 88–99% of the daily intake of labeled cholesterol. (Note that this "correction" raised none of the radiocholesterol recoveries to a figure greater than 100%.) The close approximation of the corrected data to the theoretical values appeared to validate the concept underlying the calculations—namely, that plant sterols and cholesterol were lost to nearly the same degree. In addition, these findings strengthened the basic assumption that the isotopic steady state had been very closely approximated in all four cases, and that losses of radiocholesterol in our balance measurements were not due to continuing disappearance of labeled plasma cholesterol into less highly labeled stores of cholesterol in the tissues.

Independent evidence was obtained that plant sterols were lost to the same extent as cholesterol. A mixed solution of cholesterol-4-^{14}C and β-sitosterol-22,23-^{3}H, containing 2 mg of each sterol in 5 ml of ethanol, was injected into the terminal ileum through a small polyvinyl tube in two patients; the tube was immediately flushed with 10 ml of physiological saline twice. This instillation site was chosen because cholesterol absorption from the colon is thought to be negligible. Since Hirsch, Ahrens, and Blankenhorn (20) had shown that the ileocecal valve is situated, on the average, 261 cm from the nose, the tube was passed through the nose to a distance of 275–300 cm; opaque material was injected and X-ray photographs were taken which showed that the end of the tube was located in ileum in the right lower quadrant of the abdomen. Stools collected daily for several days after instillation of the mixture of isotopic sterols were analyzed for both isotopes in total neutral steroids and in each of three neutral steroid subfractions

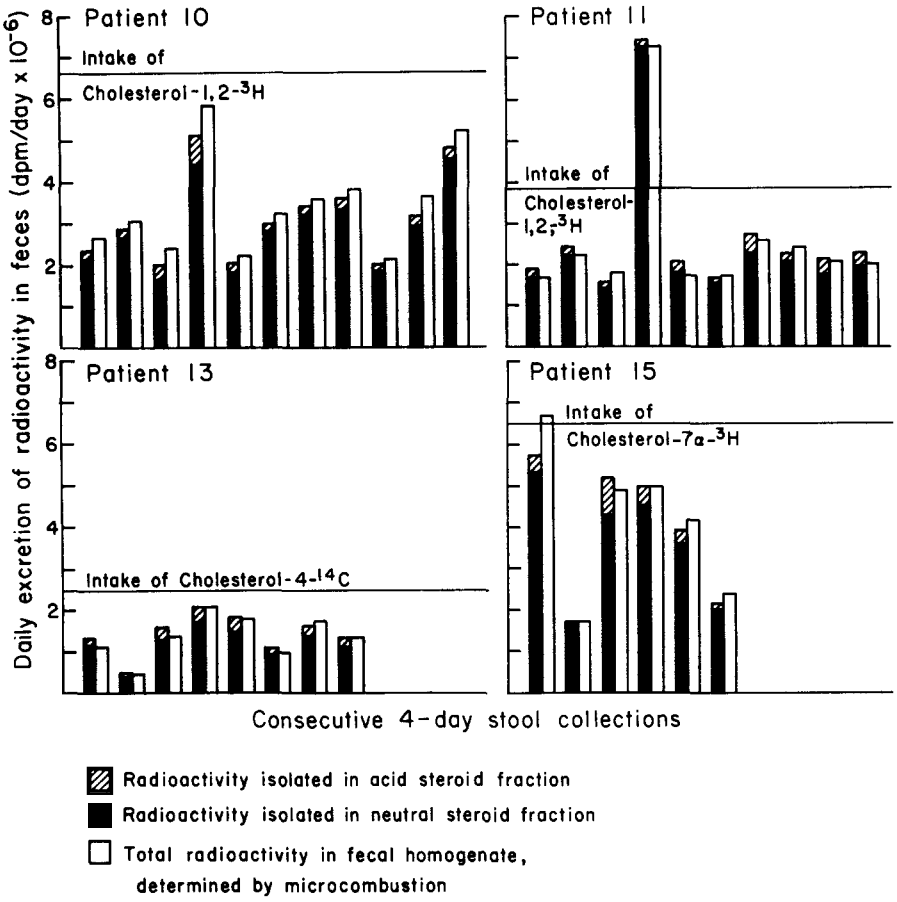

FIG. 2. Losses of radioactivity during isotopic steady state. Fecal radioactivity determined by extraction of steroids (1, 2) and by microcombustion of fecal homogenate (13) are compared to the daily intake of isotopic cholesterol. Similarity of results with the two methods indicated that radioactivity was present only in fecal steroids and was not lost at any step in the isolation procedures. Finding radioactive bile acids in patient 15, albeit in small amounts relative to neutral steroids, was undoubtedly due to the radioimpurity of the isotope used (tritium at 7β-, 3-, 4-, and 6-positions).

(cholesterol, coprostanol, and coprostanone). In patient 18, 78% of the labeled β-sitosterol and 74% of the labeled cholesterol were recovered (Table 8); excretion of both isotopes was essentially complete by the end of the 5th day. In patient 15, 76% of β-sitosterol and 73% of cholesterol were recovered, but excretion was much slower and probably not complete even by the 13th day. Only traces of cholesteryl sulfate were found in the stool collections of patient 15, and no $^{14}CO_2$ could be detected in the respiratory gases of either patient on 10 examinations over a 4 day period after instillation of the two isotopes.

The plasmas of both patients were counted for radioactivity 18 hr after isotope instillation into the ileum, and daily thereafter, as a crude index of absorption from the colon of the labeled sterols or labeled products derived from them. The highest radioactivity was noted in both patients in the first samples, drawn at 18 hr. At that time the percentages of total administered radioactivity found in the plasma of the two patients were exceedingly small—in patients 15 and 18, respectively, β-sitosterol-22,23-^{3}H: 0.12% and 0.05%, and cholesterol-4-^{14}C: 0.34% and 0.63%, if the plasma volume is taken to be 4.5% of body weight (21). Thus, in both studies the two isotopic sterols were excreted together throughout the recovery period, no significant amount of either was detected in plasma, and both were lost to almost the same degree.

These losses, we believe, are due to bacterial conversion to products as yet unidentified; in contrast, we easily identified and measured the known bacterial conversion products: coprostanol (from cholesterol) and 24-ethyl-coprostanol (from β-sitosterol) as well as the minor components, coprostanone and 24-ethyl-coprostanone. Fig. 4 shows that in both patients similar proportions of conversion products were formed from cholesterol and from β-sitosterol.

Evidence That No Losses of Acidic Steroids Occur

To determine whether bile acids also were lost within the

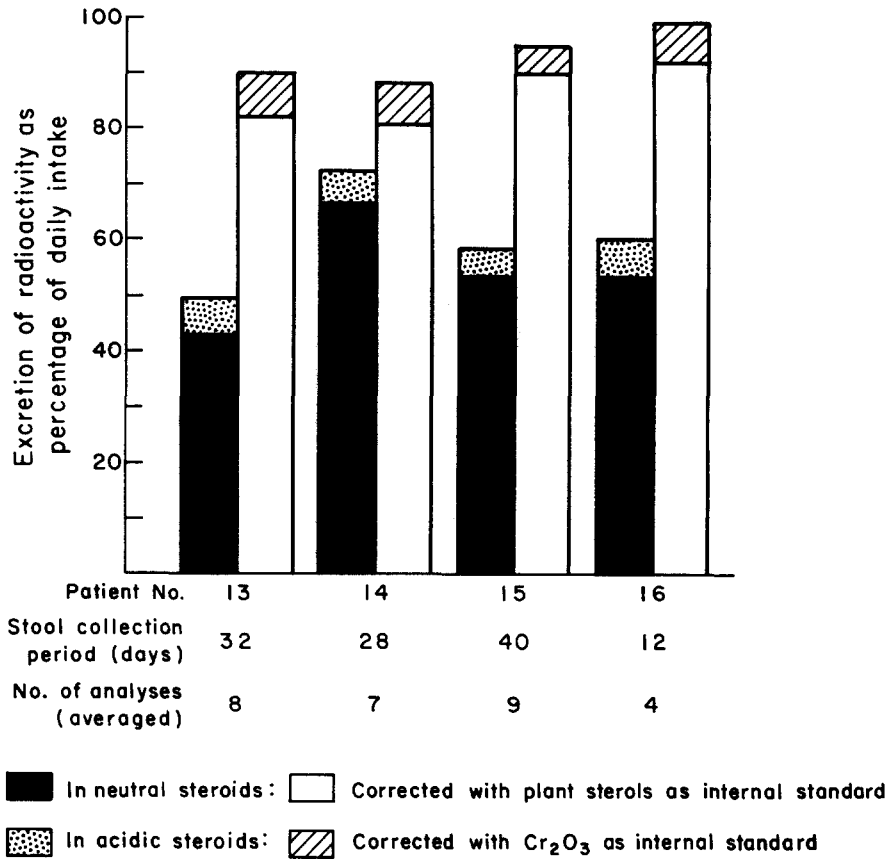

FIG. 3. Use of dietary plant sterols to correct for losses of fecal neutral steroids. The excretion of radioactivity during the isotopic steady state in four patients is plotted, with uncorrected values on the left, and after correction with internal standards on the right. After correction, the mean excretion of radioactivity in fecal steroids (see Table 7) closely approached the daily intake of isotopic cholesterol.

intestinal tract, we fed small daily doses of cholic acid-24-^{14}C in formula diets to five patients for several weeks. We considered that the isotopic steady state had been reached when the specific activities of fecal bile acids reached a plateau; at that time, by definition, one unit of radioactive bile acids should be excreted for each unit administered by mouth.

Table 9 records data on bile acid excretion during the steady state in those five patients. Uncorrected mass values are shown, as well as mass values corrected for completeness of stool collections, with chromic oxide as internal standard. Finally, the raw data were corrected with the recovery of radioactive cholic acid serving as internal standard for total bile acid losses. Calculations were made as follows: (*a*) daily excretion of acidic steroids (corrected for fecal flow) = (daily intake of Cr_2O_3 [mg]/mg of Cr_2O_3 per g of fecal homogenate) × (mg of fecal acidic steroids/g of fecal homogenate); (*b*) daily excretion of acidic steroids (corrected for cholic acid losses) = (daily intake of cholic acid-24-^{14}C [dpm]/-dpm of cholic acid-24-^{14}C per g of fecal homogenate) × (mg of fecal acidic steroids/g of fecal homogenate).

The findings in Table 9 indicated that in each patient the daily excretion of bile acids corrected with chromic oxide was essentially the same as that corrected with cholic acid-24-^{14}C. Thus, bile acids were completely recovered, even though the recovery of β-sitosterol was only 55–62% of the amount ingested daily during the same study period.

Corrections for Variations in Fecal Flow

For many years chromic oxide has been used as an internal standard to correct for variations in fecal flow. Stanley and Cheng (22) and Whitby and Lang (23) have published the most extensive data in support of the use of this marker in patients fed solid foods. Its usefulness in formula-fed patients has recently been validated in this laboratory (3), and other applications for such an internal standard were pointed out. However, chromic oxide is not always an ideal standard, and, since it cannot be incorporated reliably into materials fed at each feeding, it must be administered as a supplemental medication. This introduces the possibility of error through lack of patient cooperation or through mistakes by medication nurses. These errors can be eliminated completely if plant sterols are used as in-

ternal standards to correct variations in fecal flow, since plant sterols can easily be incorporated in every formula feeding.

Table 10 demonstrates this application in eight studies of seven patients in each of whom large numbers (8–19) of successive stool collections were made (these were usually 4-day pools). The raw data for fecal neutral steroids are given, together with standard deviations; coefficients of variation ranged from 15 to 67%, with a mean of 39%. When the raw data were corrected according to variations in recovery of β-sitosterol, the coefficients of variations in successive stool collection periods were greatly reduced, with a range of 5–11% and a mean of 8%.

TABLE 8 Recovery in Feces of Isotopic Cholesterol and β-Sitosterol Instilled by Tube into the Terminal Ileum

		Cholesterol-4-^{14}C		β-Sitosterol-22,23-^{3}H	
		dpm × 10^{-6}	%	*dpm* × 10^{-6}	%
Patient 18					
Administered dose		84.8	100	563.9	100
Fecal excretion					
days	*stool wt*				
0–2	165	0		0	
3–4	208	61.0	71.9	427.8	75.9
5	167	1.7	2.10	10.9	1.9
6–7	128	0.07	0.10	0.22	0.03
Total		62.8	74.1	438.9	77.8
Patient 15					
Administered dose		88.8	100	602.6	100
Fecal excretion					
days	*stool wt*				
0–1	70	2.20	2.5	15.2	2.5
2–3	110	9.67	10.9	67.9	11.3
4	60	5.51	6.2	38.0	6.3
5	136	11.1	12.5	81.5	13.5
6	207	12.8	14.4	90.1	15.0
7	120	8.08	9.1	56.5	9.4
8	143	5.37	6.0	37.9	6.3
9	97	3.70	4.2	25.5	4.2
10	116	2.70	3.0	18.3	3.0
11	138	1.75	2.0	11.9	2.0
12	61	0.64	0.7	4.30	0.7
13	262	1.21	1.4	8.17	1.3
Total		64.7	72.9	455.2	75.5

DISCUSSION

During our investigations of the numerous technical aspects involved in carrying out studies of cholesterol balance in man, we came to realize that in many patients we could not account for a large percentage of the cholesterol and plant sterols fed each day. This observation threatened to eliminate the possibility of carrying out balance studies for cholesterol until we found that it is possible to correct for cholesterol losses by using dietary plant sterols as an internal standard.

Three lines of evidence are presented in this report which suggest that during intestinal transit cholesterol is converted to products no longer recognized as neutral steroids and that recoveries of plant sterols can be used to correct for these losses. (*a*) Since man absorbs hardly any plant sterols and since only trivial amounts of absorbed plant sterols appear to be converted to bile acids (see below), these sterols should be almost quantitatively recoverable from feces in the fraction designated "neutral steroids"; yet in many patients we cannot account for sizable portions of the plant sterols fed, even when the collection of feces is shown to be complete and when the isolation of neutral steroids from feces is found to be quantitative. (*b*) When radioactive cholesterol is adninistered daily until the isotopic steady state is approximated, radioactivity found in fecal steroids is significantly less than that administered. However, the percentage of radioactivity from dietary cholesterol found in the feces is almost exactly the same as the percentage of dietary plant sterols recovered there. (*c*) When cholesterol and β-sitosterol labeled with different

TABLE 9 Evidence That Losses of Fecal Bile Acids Are Insignificant When Collections of Feces Are Quantitative and When Losses of Dietary Plant Sterols Are Large

			Fecal Acidic Steroids				
				Corrected with Internal Standards		Percentage Recovery (Corrected Cr_2O_3)	
Patient	Diet	Days:No. of Determinations*	Uncorrected†	Cr_2O_3	Cholic Acid-24-^{14}C	^{14}C-Bile Acids	β-Sitosterol
			mg/day			%	
8	D	36:9	401 ± 48‡	376 ± 85	362 ± 64	104	55
9	F	48:12	283 ± 79	256 ± 62	228 ± 40	112	62
10	F	32:16	110 ± 31	111 ± 13	118 ± 19	94	56
11	F	40:10	124 ± 115	92 ± 17	86 + 18	107	62
17	D	40:10	214 ± 75	208 ± 43	184 ± 38	113	60

* See footnote* in Table 3.

† The term "uncorrected" refers to raw data for daily excretion of fecal bile acid·, based on calendar collection periods.

‡ ±SD.

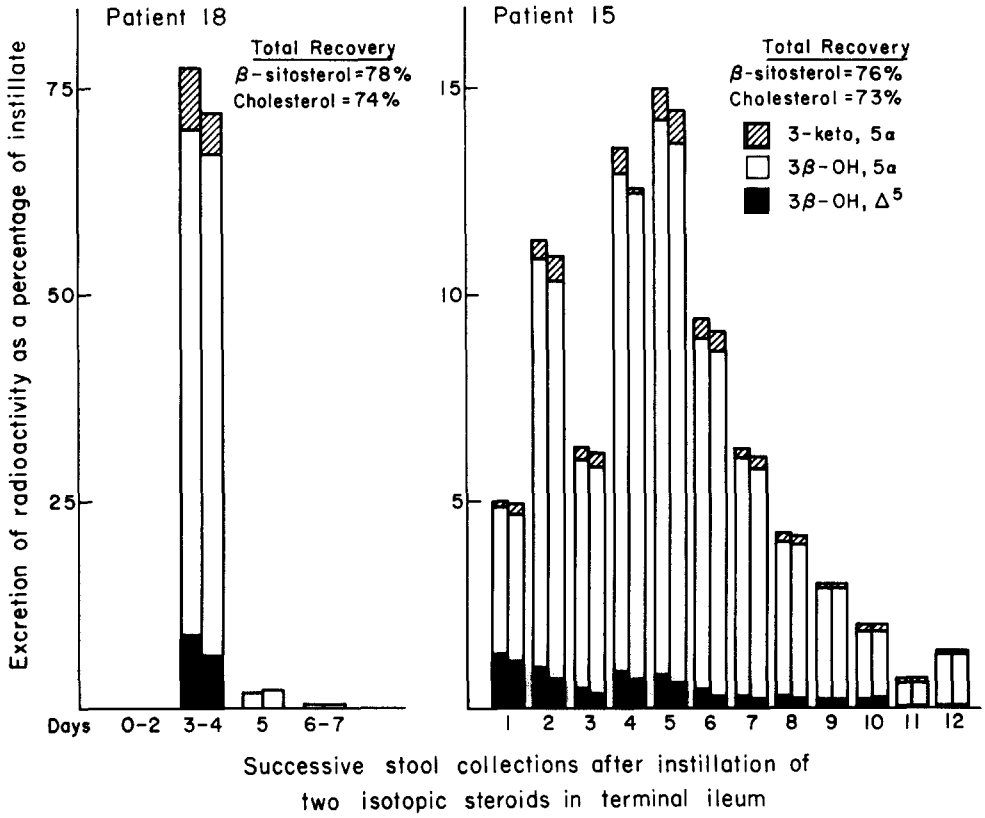

FIG. 4. Similar losses of cholesterol and β-sitosterol instilled into the terminal ileum in two patients. Pairs of bars compare fecal excretion of neutral steroid products of β-sitosterol-22,23-^{3}H (on left) and cholesterol-4-^{14}C (on right) in successive stool collection periods. The two labeled sterols were converted to secondary products similarly, and the total recoveries of steroids derived from the two sterols were incomplete to almost the same extent.

isotopes are instilled together into the terminal ileum so that they are not exposed to the absorbing surfaces of the jejunum and upper ileum but are exposed to colonic bacteria prior to their excretion, losses of the two labeled sterols are the same.

Taking all three findings into consideration, it is reasonable to deduce that cholesterol and the three commonly ingested plant sterols, during their passage through the intestine, are degraded to products which are no longer recognizable as neutral steroids by our methods. This line of reasoning is based on the assumption that the plant sterols are absorbed in man to very slight degree; indeed, Schoenheimer (24) concluded that they are totally nonabsorbable. However, in 1956 Gould and associates (4) demonstrated that in man a small amount of dietary β-sitosterol can be absorbed, and more recently Gould, Jones, LeRoy, Wissler, and Taylor (personal communication) have concluded that some—not more than 5%—of the β-sitosterol in the diet may be absorbed. Their calculations are based on measurements of radioactivity in plasma after oral administration of labeled β-sitosterol, and on analysis of tissue stores in patients fed this radioisotope shortly before death. They showed also that β-sitosterol is cleared from the plasma by the liver into the neutral sterol fraction of the bile, indeed more rapidly than cholesterol itself is cleared. In this connection it is relevant to recall the study of Hanahan and Wakil (25) in 1953 which claimed that ergosterol can be converted by the rat into bile acids. The question of conversion of β-sitosterol to bile acids was not directly addressed by Gould et al., but Fujimoto and Lupo diPrisco (personal communication) have not been able to demonstrate significant amounts of labeled acidic steroids in the bile in two patients with T-tubes in the common bile duct who were given β-sitosterol-4-^{14}C orally, nor in another such patient given the isotope intravenously.

In feeding experiments here[3] we have confirmed Gould's findings regarding the appearance of tritiated β-sitosterol in plasma. At no time after the daily oral administration of 21.3 μc to a patient for 83 days could more than 0.41% of the radioactivity administered daily be detected in plasma. Furthermore, in two other pa-

[3] Salen, G., S. M. Grundy, and E. H. Ahrens, Jr. Unpublished data.

TABLE 10 CORRECTION OF EXCRETION DATA OF FECAL NEUTRAL STEROIDS FOR DEGRADATION AND FECAL FLOW, USING DIETARY PLANT STEROIDS AS INTERNAL STANDARD

	Patients							
	8	9	11	13	14	15	17a	17b
Days:No. of determinations*	44:11	72:18	76:19	32:8	28:7	68:17	56:14	14:7
Diet	D	F	F	A	A	A	D	D
Fecal neutral steroids (uncorrected)								
mg/day	426	433	442	367	446	428	318	458
±SD	±150	±65	±305	±160	±210	±234	±105	±84
Coeff. of variation (%)	35	15	67	44	47	55	33	18
Average daily recovery of plant sterols	63.1	66.9	68.5	60.4	54.8	81.9	67.5	56.3
Fecal neutral steroids (corrected)								
mg/day	675	647	645	608	814	522	471	813
±SD	±59	±45	±72	±37	±38	±36	±46	±74
Coeff. of variation (%)	9	7	11	6	5	7	10	9

* See footnote* in Table 3.

tients given tritiated β-sitosterol intravenously the label appeared to be cleared into the bile more rapidly than simultaneously administered cholesterol-4-^{14}C; the feces contained more than 95% of its tritiated steroids in the neutral steroid fraction, with less than 5% in acidic steroids.

Thus, the available evidence in man supports the premise that very little dietary β-sitosterol is absorbed by the human intestine and that what is absorbed is rapidly returned to the intestinal lumen. The use of β-sitosterol as an internal standard to correct for losses of cholesterol therefore appears to be valid in man. Whether this applies equally well to rats remains to be determined, since several investigators have concluded that plant sterols are absorbed in the rat to a significant degree. Ivy, Lin, and Karvinen (26) were unable to account in the feces for about 22% of fed plant sterols; Roth and Favarger (27) noted losses of 28% of plant sterols; and Swell, Boiter, Field, and Treadwell (28) were unable to recover up to 23% of dietary plant sterols in the feces. More recently, Swell et al., (29) found that approximately 53% of orally administered β-sitosterol-^{3}H could not be recovered in the feces. In none of these four studies was the "absorbed" β-sitosterol sought within the whole rat, and the possibility of sterol degradation in the intestinal lumen was not considered. In 1967 Borgström measured the amount of β-sitosterol appearing in the thoracic duct lymph of rats and reported a figure of 5% for absorption, as compared to 50% for cholesterol (30); these estimates agree remarkably well with those made by indirect methods in man by Gould and coworkers (personal communication).

The question of degradation of sterols by intestinal bacteria has been examined by a number of previous investigators, all of whom have concluded that the ring structure is not broken in mammalian systems. Schoenheimer's belief (31) that the ring structure of cholesterol is not destroyed in the lumen of the intestine was supported by studies in rats by Chaikoff and his colleagues (32) and by Siperstein and Chaikoff (33). These workers were unable to find $^{14}CO_2$ in the respired gases when cholesterol-4-^{14}C was given either intravenously or orally. Likewise, Hellman and his colleagues (34) failed to detect respiratory $^{14}CO_2$ in patients fed cholesterol and steroid hormones labeled at C-4 with ^{14}C.

Thus, even though destruction of cholesterol and related sterols by soil bacteria is well known and has been studied in detail (35–37), it has been generally accepted that cholesterol is not degraded in the mammalian intestine; indeed, all previous cholesterol balance studies have been carried out on that assumption. However, Cook and associates (38) were unable to account for all the cholesterol that they had fed to rats. And in 1952, Curran and Brewster (39) isolated a coliform organism from man which utilized cholesterol-4-^{14}C as its sole carbon source, degrading it to $^{14}CO_2$; this was confirmed by Wainfan, Henkin, Rittenberg, and Marx (40), who showed in vitro that cholesterol can be digested by mixed organisms isolated from rat feces.

In five of the 19 patients of the present study the recovery of plant sterols was found to be essentially complete over long periods; in the remainder, large and persistent losses were noted over long periods in which numerous sequential collections of feces were analyzed. Finding theoretical recoveries in some patients and low recoveries in other suggests to us that losses were of biological rather than methodological origin. With respect to methodologic losses, it may be relevant to note that Moser et al. (17) recently isolated cholesteryl sulfate from feces in several patients. Nevertheless, in those four patients in whom we noted incomplete recoveries of radioactive cholesterol and its products during the isotopic steady state, we found the same total radioactivity in feces by microcombustion analysis as could be accounted for in our chromatographic analyses of neutral and acidic steroids (Fig. 2); this equivalence of recovery

of radioactivity by two different procedures makes most unlikely any significant losses of radioactivity in the form of unextracted sterol sulfates. However, in a more direct attack on this possibility we found only small amounts of cholesteryl sulfate in the stools of two of these four patients and of eight others. Our evidence that the excretion of cholesteryl sulfate amounts to less than 5% of that of fecal neutral steroids is in excellent agreement with the recent report of Eneroth and Nyström (41), who used a more complex procedure for its isolation and measurement.

Thus, the weight of all our evidence leads us to believe that neutral sterols can be degraded to nonsteroidal products during intestinal transit. We recognize that direct proof of this conclusion rests on identification of the split products of this degradation, in which we have not yet had success. Since we have not found $^{14}CO_2$ in expired air nor $^{3}H_2O$ in urine in our isotopic steady state experiments, we postulate two possible pathways by which tritium and ^{14}C from ring A of cholesterol have eluded us: (*a*) by formation of small fragments which are absorbed from the gut to enter pools which are turning over very slowly, or (*b*) by formation of small fragments which remain in the intestinal lumen and which volatilize and are lost prior to analysis of dried feces by microcombustion (short-chain fatty acids or aldehydes, or methane, could exemplify such fragments).

We are currently seeking to identify the products of degradation of neutral steroids after anaerobic incubation of freshly collected feces with labeled sterols in a closed system.[3] In cultures of some but not all patients' feces we find losses of 15–40% of cholesterol-4-^{14}C during incubation, judging by the recovery of neutral steroids after mild saponification of the incubation mixture and extraction with petroleum ether, steps which are part of our routine method (2). The remaining radioactivity is found in the alkaline aqueous residue; a significant portion of this radioactivity is converted into cholesterol on solvolysis (12), as if it were cholesteryl sulfate. Yet, in studies of one such patient, where sizable amounts of cholesteryl sulfate appeared to be produced in vitro, we were unable to detect any sulfate ester in freshly excreted feces, nor, as noted above, did we find significant amounts of cholesteryl sulfate in the stools of 10 other patients. It remains for future experiments to clarify these findings and to determine whether, perhaps, sulfation is the first step in degradation of the ring structure of cholesterol and of plant sterols. Failure to find β-sitosteryl sulfate in the feces that contained cholesterol sulfate weakens this possibility.

It is relevant to recall that cholesterol, campesterol, stigmasterol, and β-sitosterol all share the 3β-OH,Δ^5-structure and differ only in their side chains. These facts suggest that an initial enzymatic attack on the sterol molecule may take place upon the steroid ring structure rather than upon the side chain. We have no evidence at this time as to whether neutral sterols with a different configuration at C-5 (e.g., coprostanol [3β-OH,5β-H] and cholestanol [3β-OH,5α-H]) are lost during intestinal transit, but the acidic steroids, which have a 5β-H configuration, are evidently degraded less than cholesterol and possibly not at all. Thus, whether the presence of a Δ^5-double bond or the configuration (*cis* or *trans*) of the A/B ring system is of greater importance in determining the extent of degradation remains to be examined.

β-Sitosterol as an Ideal Internal Standard for Cholesterol Balance Studies

The ideal internal standard for sterol balance studies should have the following characteristics: (*a*) it should have chemical and physical properties similar to those of cholesterol but be different enough not to be absorbed from the intestine; (*b*) it should be a substance found in the same physicochemical phase of the intestinal contents as cholesterol, at all levels of the intestinal tract; and (*c*) it should accompany cholesterol at every step in the isolation of neutral steroids from feces, yet be reliably measurable in the presence of cholesterol and its metabolic products. The plant sterols, especially β-sitosterol, satisfy these criteria; β-sitosterol is plentifully distributed in most plant fats and is readily available commercially.

The analytical methods available (1, 2) for isolation and quantification of fecal neutral steroids assure the reliable measurement of β-sitosterol in the same gas–liquid chromatogram used for measurement of cholesterol. Thus, the quantitative relationship between the contents of cholesterol and of β-sitosterol in any sample of feces analyzed can be determined from a single gas–liquid chromatogram. *Any losses of β-sitosterol will be associated with equal losses of cholesterol, whether these losses be mechanical (incomplete stool collections), analytical (losses during isolation procedures), or bacterial.* If the daily intake of β-sitosterol is constant, excretion data for neutral steroids derived from cholesterol can be obtained in corrected per diem terms by multiplying those data by the percentage recovery of β-sitosterol in the same samples. In this calculation the results will simultaneously be corrected for variation in fecal flow rates as well. Corrected results for neutral steroid excretions will always be higher than uncorrected data, but they will show less day-to-day variation; this makes statistical comparison between test periods more reliable.

Since bile acids are apparently not lost during intestinal transit, some internal standard other than β-sitosterol must be used for quantification of bile acid excretion on a reliable per diem basis. Chromic oxide

serves this purpose well, even though it is an inert inorganic compound of high density (sp gr 5.2), which presumably travels through the intestine in the particulate phase of intestinal contents. As pointed out by Davignon, Simmonds, and Ahrens (3), patients who fail to excrete as much chromic oxide as they ingest are not reliable for balance studies: use of this marker serves to identify these patients so that they may be eliminated from such studies. However, in the patients who excrete chromic oxide ideally (and they are the majority), fecal bile acid excretion can be expressed in valid per diem terms by relating the fecal bile acid content of a given stool collection to the chromic oxide content of another aliquot of that collection, as a percentage of daily intake of that marker.

Once the fact has been established that a given patient excretes chromic oxide ideally (3), it is no longer necessary to make total *stool collections; it is sufficient to analyze casual specimens collected at the convenience of patient, ward nurse, and investigator. A true value for daily fecal neutral steroid excretion can be obtained through use of β-sitosterol as internal standard, and for daily fecal bile acid excretion, through use of chromic oxide as standard.*

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