Reduction and esterification of cholesterol and sitosterol by homogenates of feces

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ABSTRACT Mixtures of cholesterol-1,2-^{3}H and sitosterol-4-^{14}C have been incubated with suspensions of feces in order to compare the behavior of the phytosterol with transformations known to take place with cholesterol under these conditions. Within the limitations of the study, both labeled sterols were esterified to the same extent, and reduction of the Δ^5 double bond to the saturated analogue proceeded equally in both substances. After correcting for procedural losses, the recoveries of ^{3}H and ^{14}C from the incubations were always less than the controls; this strongly indicates destruction of sterol by feces microorganisms.

SUPPLEMENTARY KEY WORDS feces sterols

STEROL BALANCE studies using labeled cholesterol for studying the flux of body cholesterol, first employed by Hellman, Rosenfeld, Insull, and Ahrens (1) and subsequently by Ahrens and coworkers and others (2–7), obviously depend upon a reasonably precise knowledge of the input of sterols and the output of sterols plus their transformation products. It was found that in some, but not all, subjects the determination of sterol output by measurements of fecal sterols and bile acids might not accurately reflect sterol excretion because some of the sterols could be transformed by the action of intestinal microorganisms (8); if the alterations involved destruction of the steroid nucleus, a portion of the sterols would escape measurement. In order to monitor this intraluminal degradation, Grundy, Ahrens, and Salen (9) have used sitosterol, labeled or unlabeled, as a marker. Sitosterol, in contrast to cholesterol, is absorbed to a very limited extent; therefore, any deficit in sitosterol output would be a reflection of loss by microbial action, probably in the large intestine. An identical loss would be suffered by cholesterol, since both molecules possess identical nuclei, differing only by a C-24 ethyl group in the phytosterol. This remarkable identity of behavior prompted us to compare sitosterol and cholesterol on incubation in feces suspension, particularly with regard to double bond saturation and esterification, processes known to occur with cholesterol under these conditions (10–12).

METHODS

Radiochemical Purity of Tracer Sterols

Sitosterol-4-^{14}C (SA, 50 mCi per mmole; Amersham/Searle Corp., Arlington Heights, Ill.) was dissolved in benzene, and 7.45×10^4 cpm was added to 102.3 mg of recrystallized nonradioactive sitosterol containing over 92% β-sitosterol as judged by GLC (728 cpm per mg). The mixture was crystallized once from acetone (728 cpm per mg) and then from benzene–methanol (721 cpm per mg). The labeled compound was therefore over 99% pure by reverse isotope dilution.

Cholesterol-1,2-^{3}H (SA, 30 Ci per mmole; New England Nuclear, Boston, Mass.) was chromatographed on a silica gel plate in ethyl acetate–cyclohexane 3:7. A scan of the plate showed a single peak corresponding to the position of the cholesterol pilot spot. The radioactive area was removed and the silica gel was extracted with benzene. An aliquot (1.34×10^5 cpm) was mixed with

The following trivial names have been used: cholesterol, Δ^5-cholesten-3β-ol; sitosterol, Δ^5-stigmasten-3β-ol; coprostanol, 5β-cholestan-3β-ol; coprositostanol (we prefer this terminology, first used by Rosenheim and Webster [Rosenheim, D., and T. A. Webster. 1941. *Biochem J.* **35**: 928.], because it is related to sitosterol in the same way that coprostanol is to cholesterol), 5β-stigmastan-3β-ol.

Abbreviations: cpm, counts per minute; GLC, gas–liquid chromatography; SA, specific activity; TMS, trimethylsilyl.

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100 mg of nonradioactive cholesterol (1340 cpm per mg) and crystallized from acetone–methanol (1285 cpm per mg) and methanol (1280 cpm per mg). Therefore, cholesterol-1,2-^{3}H was approximately 95% pure.

Saturated Contaminants in Cholesterol-1,2-^{3}H. To 100 mg of nonradioactive 5α-cholestan-3β-ol was added 10 mg of nonradioactive cholesterol and 750,000 cpm of cholesterol-1,2-^{3}H. The mixture was acetylated in acetic anhydride–pyridine and, after isolation, was treated with *m*-chloroperbenzoic acid in chloroform and then chromatographed on an alumina column (11). 5α-Cholestan-3β-ol acetate, eluted with petroleum ether, contained less than 400 cpm. Oxidized material in the polar eluates contained 733,000 cpm; thus the tracer cholesterol was essentially free of saturated contaminants.

Chromatography of Cholesterol-1,2-^{3}H and Sitosterol-4-^{14}C Mixtures

Labeled sterols were added to the corresponding nonradioactive substances, and 275 mg of a mixture whose composition was 88% cholesterol (65,600 cpm ^{3}H) and 12% sitosterol (74,500 cpm ^{14}C) was prepared; this was chromatographed on 20 g of alumina which had been deactivated so that its water content was 6% (13). The column was developed with benzene–petroleum ether mixtures, benzene, and ether–benzene mixtures. Each fraction as the TMS ether was monitored by GLC on 3% OV-225 on Supelcoport (Supelco, Inc., Bellefonte, Pa.), and a portion was counted. No significant amounts of sterol or radioactivity were eluted before 100% benzene or after ether–benzene 1:9. In the benzene and ether–benzene fractions, 90% of the ^{3}H and 93% of the ^{14}C were obtained; GLC showed that during elution of sterols there was a slight tendency for sitosterol to lead cholesterol.

Incubation Procedure and Isolation of Products

Preparation of the Tracers. A mixture of cholesterol-1,2-^{3}H and sitosterol-4-^{14}C, dissolved in 1 ml of benzene, was pipetted into a glass-stoppered tube and the solvent was removed under a stream of nitrogen. The residue was dissolved in 1 ml of acetone to which 3 drops of Tween 80 was added, followed by 5 ml of 0.9% sodium chloride solution; the emulsion was shaken and immediately introduced into the incubation mixture.

Incubation of Suspensions. Within one-half hour of collection, stool was homogenized with 0.9% sodium chloride (1:1, w/v) for 3 min in a Waring Blendor set at half speed, and the homogenate was divided into two portions ranging from 100 to 250 ml, depending on the amount of stool. The tracers were added to each and the suspensions were homogenized for an additional one-half minute. One portion was immediately poured into 1.5 l of ethanol, swirled, and allowed to stand; this served as the control. The second portion was transferred to a 500-ml Erlenmeyer flask, sealed with paraffin film, and kept in a water bath for 72 hr at 37°C with gentle agitation. The incubation was terminated by mixing the homogenate with 1.5 l of ethanol and swirling for one-half hour. After this, both incubation and control were handled identically.

Extraction of Lipids. The ethanolic suspension was filtered through a Soxhlet cup (6 cm diameter × 17 cm) and the residue was extracted continuously for 24 hr with refluxing ethanol. The filtrate and extract were combined and concentrated in vacuo on a steam bath. The residue was partitioned between 500-ml portions of petroleum ether and 250-ml portions of 70% ethanol in three separatory funnels. The ethanol layers were discarded and the petroleum ether solutions were concentrated to yield the "total lipids."

Column Chromatography of Total Lipids. A portion of the total lipids, depending on the weight of the extract, was chromatographed on deactivated aluminum oxide (13). Usually, about 500 mg of extract on 75 g of alumina was used so that conditions were maintained as nearly constant as possible throughout the studies. Elution was initiated with 200-ml portions of benzene–petroleum ether 1:9, and the columns were developed with the following solvents: benzene–petroleum ether 3:7, benzene–petroleum ether 1:1, ether–benzene 1:19, ether–benzene 1:4, and ethyl acetate. Each fraction was weighed, counted, and analyzed by GLC. *Esterified sterols* were eluted in the first fractions (benzene–petroleum ether 1:9) and *free sterols* were recovered in the ether–benzene mixtures. In all cases, these two fractions contained all of the measurable radioactivity, which amounted to over 95% of the total applied to the column.

Sterol Esters: Saponification. Sterol esters were refluxed for 4 hr in 150 ml of 10% potassium hydroxide in 70% ethanol. The nonsaponifiable portion was extracted with petroleum ether and, after washing three times with 70% ethanol and removing the solvent in vacuo, the fraction designated as *ester sterols* was obtained.

Separation of Saturated and Unsaturated Sterols. This procedure, described earlier (14), was applied to both free and ester sterols. Briefly, the sterols were converted to their formate esters, treated with 30% hydrogen peroxide, and separated by solvent partition between petroleum ether and 90% methanol after hydrolysis. Saturated sterols were recovered unchanged from the petroleum ether. The unsaturated sterols were obtained as 3β,5α,6β-triols from the methanolic phase.

To ensure complete removal of unsaturated material, the saturated sterols were acetylated and treated with *m*-chloroperbenzoic acid prior to chromatography as described previously (11).

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Radioactive Measurements

All samples were simultaneously assayed for ^{3}H and ^{14}C in a Packard Tri-Carb liquid scintillation spectrometer, model 3375, according to the methods described by Bradlow, Fukushima, Zumoff, Hellman, and Gallagher (15). The triol mixtures from the unsaturated sterols were counted in the diotol scintillation mixture of Herberg (16) with use of conversion factors so that all data, expressed as cpm, would be comparable. Where the samples were colored, as in the total lipids, internal standards of cholesterol-^{14}C and cholesterol-^{3}H were counted in identical aliquots of each fraction so that quench corrections could be obtained for each sample independently. Assays were carried out in triplicate. Tritium and ^{14}C values were calculated by the simultaneous equation procedure of Okita, Kabara, Richardson, and LeRoy (17), programmed for a Honeywell 400 computer.

Gas–Liquid Chromatography

GLC measurements were performed on a Barber–Colman instrument, 5000 series, equipped with a flame ionization detector. TMS ethers were prepared by reaction of sterols with Sil-Prep reagent (Applied Science Laboratories Inc., State College, Pa.) overnight at room temperature. The reactants were removed at 60°C under a stream of nitrogen; all injections were made in benzene solution. Quantitation was carried out by comparing the areas under the appropriate peaks with those from standard injections of cholesterol, sitosterol, and coprostanol. No standard coprositostanol was available; therefore, in measuring the substance considered to be coprositostanol, comparisons were made against the area–weight calibration of coprostanol.

RESULTS

Table 1 shows the recoveries of radioactivity in the total lipids from the incubation and control portions of feces homogenates obtained from seven normal men. The marked differences between weights of total sterol in control and incubation are a consequence of using different amounts of the homogenate for each (see Methods); this has no influence on the evaluation of the results. Their sum equals the total sterol in the collection. In all of the studies the recovery of ^{3}H and ^{14}C after incubation was *less* than the recovery from the controls. Losses in the controls are undoubtedly procedural (addition of tracers, transferring the mixture from homogenizer to flask); quenching of radioactivity by the colored solutions, though significant, did not contribute measurably to losses because it was compensated for in each sample by the counting techniques. Since both the incubation and the control were taken from the same homogenate, and since they were processed at the same time under identical conditions, it is reasonable to assume that procedural losses were the same in both. Therefore, the greater loss of radioactivity in the lipids recovered after incubation must have been a consequence of some degradation of cholesterol-^{3}H and sitosterol-^{14}C. If a correction is made for procedural losses (see footnote, Table 1), the adjusted percentage recovery then affords a measure of degradation during incubation. These values range from 72 to 96%, and in six of the seven studies ^{3}H and ^{14}C recoveries differ by less than 2%. These data support the contention of Grundy et al. (9) that sitosterol may be used as a measure of bacterial decomposition of cholesterol since both sterols undergo identical degradative reactions in the intestinal lumen.

A comparison of the behavior of cholesterol-^{3}H and sitosterol-^{14}C with regard to the saturation of the Δ^{5} bond and esterification at C-3 is shown in Table 2. In six experiments, 4 to 53% of the radioactivity in the nonpolar lipids was present in the ester sterols, and in each study the extent of esterification of each tracer was about the same. In incubation VI, no radioactivity was detected in ester sterols; the wide differences in esterification capacity, undoubtedly dependent on the number and types of fecal bacteria, would be expected in such a random selection of subjects. Radioactivity in chromatographic fractions intermediate between sterol esters and free sterols was negligible, so that saturated ketones were not formed to any significant extent from the tracer substances under the incubation conditions.

In each incubation, cholesterol-^{3}H and sitosterol-^{14}C were transformed to their saturated analogues in almost identical yields (Table 2). 4 to 91% of the radioactivity in the free fraction was associated with saturated sterols, and in the esters, virtually all of the ^{3}H and ^{14}C (>93%) was present in the saturated sterol fraction. This is in accord with earlier studies which demonstrated preferential esterification of 3β-hydroxy-5β-sterols by feces bacteria and suggested that during incubation cholesterol is converted to coprostanol prior to esterification (12, 14). It should be pointed out that all of the controls were carried through identical steps and no radioactivity was detected in the ester sterols nor in the saturated sterols; this served as a check on the validity of the separation procedures.

Tables 3 and 4 present the analyses of sterols in milligrams per gram of nonpolar lipids (petroleum ether-soluble lipids) in the homogenates. In Table 3, the amounts represent the sum of the quantities of four sterols (cholesterol, coprostanol, sitosterol, and coprositostanol) as measured by GLC in the free and ester sterol fractions. Additional peaks in the chromatograms, if present, contributed little to the total sterol values and were ignored. It can be seen that the quantity of ester sterol before incubation ranged from barely measurable

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TABLE 1 RECOVERIES OF RADIOACTIVITY AFTER INCUBATION OF CHOLESTEROL-1,2-^{3}H AND SITOSTEROL-4-^{14}C WITH FECES HOMOGENATES*

Study	Recovery in Total Lipids: Control ^{3}H	Control ^{14}C	Incubation ^{3}H	Incubation ^{14}C	Incubation Corrected† ^{3}H	Incubation Corrected† ^{14}C	Total Sterols: Control	Total Sterols: Incubation
	%						*mg*	
I	86	95	83	85	96	89	290	696
II	85	91	61	66	72	73	450	1090
III	93	97	82	84	88	87	446	736
IV	94	96	79	83	84	86	376	784
V	87	90	81	84	93	93	615	695
VI	91	85	77	74	85	87	1005	920
VII	87	80	72	68	83	85	400	320

* I–IV contained 124,200 cpm cholesterol-^{3}H and 68,400 cpm sitosterol-^{14}C in incubations, and 62,100 cpm ^{3}H and 34,200 cpm ^{14}C in controls. V–VII contained 497,000 cpm cholesterol-^{3}H and 274,000 cpm sitosterol-^{14}C in incubations and controls.

† The ratio of % recovery in incubation:% recovery in control × 100 for each isotope (see text).

amounts up to about 16% of the sterols and in all cases, except in VI, significant esterification occurred. The percentage esterification during incubation, 5–44%, agreed reasonably well with the radioactive data in Table 2. Table 4 shows the actual sterol distribution in the free and ester fractions in two representative studies. The data clearly demonstrate that phytosterols undergo esterification during incubation.

Increases in quantities of saturated sterols were seen in both the free and ester fractions after incubation in six of the seven experiments (Table 3). In study VII, there was no increase of saturated sterols in the free fraction, which is in accord with the low conversion of tracer material.

TABLE 2 DISTRIBUTION OF RADIOACTIVITY IN ESTER STEROLS AND SATURATED STEROLS AFTER INCUBATION OF CHOLESTEROL-1,2-^{3}H AND SITOSTEROL-4-^{14}C WITH FECES HOMOGENATES

Study	Radioactivity in Ester Sterols ^{3}H	Radioactivity in Ester Sterols ^{14}C	Radioactivity in Saturated Sterols*: Ester ^{3}H	Ester ^{14}C	Free ^{3}H	Free ^{14}C
	%		%			
I	4	4	†	†	47	55
II	53	53	98	98	90	91
III	30	29	97	98	88	84
IV	33	27	97	98	72	72
V	17	17	93	93	75	79
VI	0	0	0	0	91	91
VII	11	13	†	†	4	13

* The percentages refer to the proportion of the total radioactivity in that particular fraction which was present in saturated sterols.

† Not measured.

DISCUSSION

The danger of identifying a material, i.e., coprositostanol, based solely on its chromatographic behavior is fully recognized. Nevertheless, the presence of a peak bearing a similar relationship to sitosterol as coprostanol does to cholesterol in seven GLC systems,[1] as well as its persistence throughout the isolation and separation steps, both chemical and physical, offers convincing evidence that the peak represents the reduction product of sitosterol (coprositostanol) or possibly a mixture of reduced C_{29} phytosterols. Several groups of investigators have identified reduced phytosterols in feces (7, 18, 19), and Miettinen, Ahrens, and Grundy have shown that, after coprostanol, coprositostanol makes up the greatest proportion of reduced sterols (18).

Throughout the isolation and fractionation steps, the procedures were deliberately designed to be generic; that is, to separate free from ester sterols and to remove unsaturated from saturated sterols without effecting separation of the C_{27} sterols from phytosterols. In this way the amount of each isotope within a group permitted a comparison between the behavior of tracer cholesterol and sitosterol. Precise agreement between the data from radioactive assay and from GLC measurements would occur only if the tracer were completely mixed with the sterols in the homogenate. The brief homogenization of the labeled suspension and the gentle agitation during the incubation were probably insufficient to achieve uniform distribution. It would be expected, however, in a sterol balance study, that tracer sterol administered in the diet would be thoroughly mixed before excretion so that distribution of radioactivity would afford a more accurate measurement of distribution of mass.

Although not strictly comparable to the conditions in the large intestine, the results of this investigation

[1] Unpublished data from this laboratory.

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TABLE 3 STEROL CONTENT OF LIPIDS FROM FECES HOMOGENATES*

Study	Free Sterol				Ester Sterol				
			Saturated					Saturated	
	C†	I†	C	I	C	I	Esterified	C	I
	mg/g PE†		*%*		*mg/g PE*		*%*	*%*	
I	288	233	86	89	5.7	20.0	5	55	87
II	284	127	86	90	54.6	179	44	95	97
III	306	190	93	94	34.7	103	22	96	98
IV	319	189	85	90	18.6	128	34	88	96
V	290	212	85	93	4.6	22.3	6	59	89
VI	178	153	95	96	0.9	0.5	0	—	—
VII	125	86	85	84	2.8	28.3	20	91	98

* Measured by GLC.
† C, control; I, incubation; PE, petroleum ether-soluble lipids (see Methods).

TABLE 4 DISTRIBUTION OF STEROLS IN FECES BEFORE AND AFTER INCUBATION

Sterol	Study III				Study IV			
	Free Sterol		Ester Sterol		Free Sterol		Ester Sterol	
	C*	I	C	I	C	I	C	I
	mg/g PE							
Coprostanol	256	160	30.4	91.2	245	151	15.0	109
Cholesterol	16.2	7.7	0.7	1.6	39.3	14.1	1.1	3.6
Coprositostanol	27.9	18.4	2.9	9.4	26.9	18.1	1.4	14.4
Sitosterol	5.4	4.1	0.7	0.4	8.2	5.1	1.1	1.4

* See Table 3 for abbreviations.

support the finding that bacterial degradation of the steroid nucleus can occur intraluminally (9); nonprocedural losses can only be ascribed to microbiological action since the incubation represents a closed system with regard to absorption or secretion of sterol. In addition, during incubations of feces suspensions, sterols of plant and animal origin appear to be degraded to approximately the same extent. It should be stressed that bacterial degradation of the steroid nucleus which may be observed during sterol balance studies (7, 9, 18) is *not* an in vivo transformation in the sense of occurring within the tissues of the individual. There is convincing evidence that, *in man*, with the exception of aromatization during the biosynthesis of estrogens, no alteration of the ring structure takes place (20, 21).

The similarity in behavior of cholesterol and sitosterol toward feces microorganisms extends to double bond saturation and esterification, processes where no losses of sterol are involved. Thus the large amounts of saturated C_{29} sterol in the ester fraction were present as a consequence of esterification of coprositostanol which was first formed by reduction of the 5-6 double bond in sitosterol; this is analogous to the steps in the formation of coprostanol esters (22). In a later study it was shown that of the four cholestan-3-ols epimeric at C-3 and C-5, only coprostanol (3β,OH,5β-H) was significantly esterified in feces homogenates (12). Therefore, it appears likely that the structural difference between cholesterol and sitosterol is sufficiently remote from ring A so that it has no effect on either reduction or esterification. That this is true under in vivo conditions has been conclusively shown by Salen, Ahrens, and Grundy (23), who demonstrated the esterification of sitosterol-22,23-^{3}H as well as its conversion to bile acids in man.

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