Thin-layer and gas–liquid chromatographic identification of neutral steroids in human and rat feces

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Abstract Natural steroids from rat and human feces were fractionated by sequential thin-layer chromatography (TLC) on Florisil, silica gel, and silver nitrate-impregnated silica gel and analyzed by gas–liquid chromatography (GLC). Cholesterol, coprostanol, and coprostanone accounted for more than 95% of the endogenous neutral steroid in human feces, the remainder being predominantly cholestanol. In addition, evidence was obtained for the presence in human feces of trace amounts of epicoprostanol and cholestanone. In rat feces, several cholesterol precursors that probably originated in the skin (and were ingested during fur-licking) were detected in relatively large amounts, accounting for as much as 27% of the total fecal neutral steroids, whereas these steroids were quantitatively trivial in human feces. As with cholesterol, the major dietary plant sterols (sitosterol, campesterol, and stigmasterol) were converted by intestinal bacteria to the corresponding coprostane and ketonic derivatives during intestinal transit in both human beings and rats. This combined use of TLC and GLC provided for the separation of steroids of endogenous and dietary origin that could not be resolved by either system alone. A majority of the fecal steroids could be tentatively identified by their chromatographic behavior in different TCL systems and on GLC, even when reference standards were unavailable.—**McNamara, D. J., A. Proia, and T. A. Miettinen.** Thin-layer and gas–liquid chromatographic identification of neutral steroids in human and rat feces. *J. Lipid Res.* 1981. **22:** 474–484.

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Previous studies (1–3) have shown that human and rat feces contain varying amounts of cholesterol, cholesterol precursors and catabolites, plant sterols, and bacterial byproducts of all the above (**Table 1**). The resolution and identification of individual steroid components of human feces and the differentiation of plant steroids from cholesterol and from the bacterial degradative products of both cholesterol and the plant sterols have been made possible by combined use of thin-layer (TLC) and gas–liquid (GLC) chromatography (3).

In analysis of fecal steroids for cholesterol balance measurements in man, the various steroid classes have been differentiated into three fractions according to TLC mobilities. The three fractions: "cholesterol" (Δ^5 and ring-saturated 5α-sterols), "coprostanol" (ring-saturated 5β-sterols, but also Δ^7, Δ^8, $\Delta^{8,24}$ sterols), and "coprostanone" are further fractionated by GLC to differentiate cholesterol from plant sterol products (3).

Eneroth, Hellström, and Ryhage (1) identified the major steroid components of human feces by GLC and mass spectrometry and reported that the major excretory products consisted of cholesterol, coprostanol, and coprostanone. In the present study, we have attempted to compare and identify the different steroids in human and rat feces using sequential TLC fractionation on Florisil, silica gel G, and silver nitrate-impregnated silica gel G followed by GLC on different liquid phases, thus avoiding the need to utilize the elegant but not always accessible tool of mass spectrometry. The isolation and tentative identification of rat fecal steroids has allowed us to define the origin of these fecal neutral steroids in the rat (4) and has facilitated the precise measurement of sterol balance and whole body cholesterol synthesis in the rat.

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; TMS, trimethyl silyl ether; MOA, methoxyamine; TEPA, triethyl phosphone acetate; RRT, relative retention time.

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MATERIALS AND METHODS

Fecal samples

Human stools were obtained from hospitalized patients at The Rockefeller University Hospital who were fed either solid food diets or liquid formulas with and without fat. Corn sterols were added to fat-containing formulas either as isolates or in the form of refined or distilled corn oil (see below).

Rat stools were collected from male animals of the Wistar strain into which a gelatin capsule containing cholestanol-free [4-^{14}C]cholesterol had been implanted subcutaneously (5) to obtain constant labeling of all miscible cholesterol pools. These animals were maintained on a "cholesterol-free" (ICN Life Sciences Group, Cleveland, OH; composition by weight: 25% casein, 65% sucrose, 6% cellulose, 4% USP XIV salt mixture) to which was added 5% (by weight) of sterol-free synthetic coconut oil (Proctor & Gamble). Stool collections were begun 45 days following implantation of labeled cholesterol: this ensured that all exchangeable body pools were uniformly labeled (6). Stools were also collected from male Wistar rats maintained on Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO).

The collection and homogenization of human feces were carried out as previously described (3). Rat feces were dried at room temperature for 2 days in a vacuum dessiccator followed by grinding in a mortar to a fine powder.

Extraction of neutral steroids was carried out after alcoholic saponification of 1–3 g of human fecal homogenate or 1 g of dried powdered rat feces as described by Miettinen, Ahrens, and Grundy (3).

Reference standards

The following steroids were purchased from Steraloids, Inc. (Wilton, NH): cholesterol, cholestanol, cholestanone, Δ^7-cholestenol, coprostanol, epicoprostanol, epicholestanol, coprostanone, desmosterol, $\Delta^{5,7}$-cholestenol, lanosterol, and 5α-cholestane. Campersterol, stigmasterol, and sitosterol were obtained through the kindness of Dr. Malcolm Thompson (USDA, Beltsville, MD). A mixture of these sterols also was obtained from Dr. Thompson; it contained approximately 6% of ring-saturated 5α-sterols. Dr. William W. Wells (East Lansing, MI) generously provided methostenol, Δ^8-methostenol, Δ^7-coprostenol, and Δ^5-[4-4]-dimethyl-cholesten-3β-ol. Dihydrolanosterol was donated by Dr. R. B. Clayton (Stanford, CA). Sitostanol was isolated from a mixture of corn oil sterols by TLC on silver-nitrate plates.

TABLE 1. Trivial names of the fecal neutral steroids identified in this report

Name	Structure Based on Changes or Additions to 5α-Cholestan-3β-ol
C_{27} Steroids:	
cholesterol	Δ^5
cholestanol	5α-H
epicholesterol	3α-OH
coprostanol	5β-H
Δ^7-coprostenol	5β-H,Δ^7
Δ^7-cholestenol(lathosterol)	Δ^7
cholestanone	Δ^5,3-one
coprostanone	5β-H,3-one
desmosterol	$\Delta^{5,24}$
dehydrocholesterol	$\Delta^{5,7}$
C_{28} Steroids:	
campesterol	Δ^5-24α-methyl
campestanol	24α-methyl
methylcoprostanol	5β-H,24α-methyl
methostenol	Δ^7-4α-methyl
Δ^8-methostenol	Δ^8-4α-methyl
C_{29} Steroids:	
sitosterol	Δ^5-24α-ethyl
sitostanol	24α-ethyl
ethylcoprostanol	5β-H,24α-ethyl
stigmasterol	$\Delta^{5,trans-22}$-24α-ethyl
stigmastenol	Δ^{22}-24α-ethyl
ethylcoprostenol	5β-H,Δ^{22}-24α-ethyl
C_{30} Steroids:	
lanosterol	$\Delta^{8,24}$-4,4,14α-trimethyl
dihydrolanosterol	Δ^8-4,4,14α-trimethyl

[4-^{14}C]Cholesterol (New England Nuclear Corp., Boston, MA) was purified by TLC as previously described before administration to patients (3). In rat studies, this labeled material was further purified on silver nitrate-impregnated silica gel plates to remove traces of cholestanol.

Radioactivity measurements

Radioactivity measurements were performed with a Packard Tricarb Liquid Scintillation Spectrometer (Model 3003). PPO-POPOP-toluene phosphor solution was prepared from Liquifluor (New England Nuclear, Boston, MA). Quench corrections were performed using [^{3}H]- or [^{14}C]toluene (Packard Instrument Co.) as internal standards. Counting efficiencies were approximately 85% for ^{14}C and 26% for ^{3}H.

Fractionation of steroids by TLC

Thin-layer chromatography plates were prepared with Florisil and silica gel G as previously described (3). Silver nitrate-impregnated plates were prepared as proposed by Morris (7), activated at 120°C for 1 hr, and stored in a light-tight dessiccator. Samples were

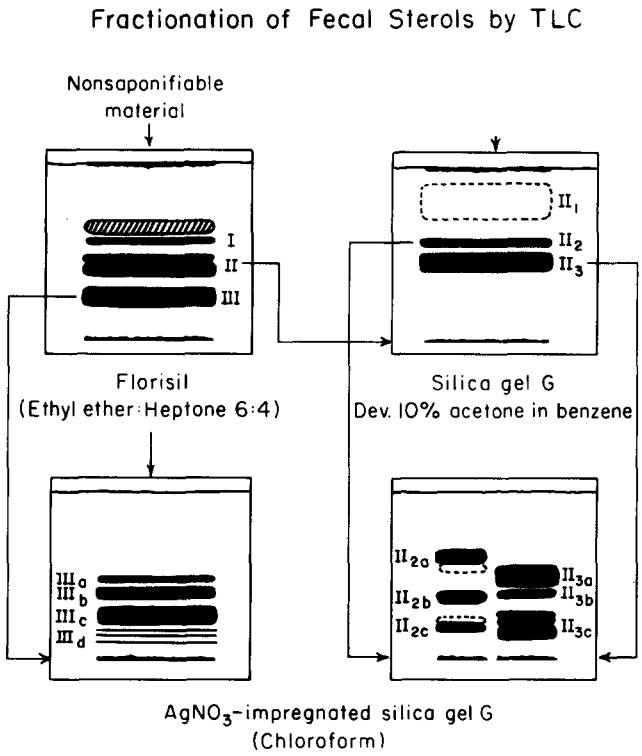

Fig. 1. Flow sheet for fractionation of fecal steroids by successive TLC runs. On initial Florisil TLC, the nonsaponifiable material (including α-tocopherol) obtained from the feces of rats maintained on a sterol-free diet is shown as the shaded area above Fraction I.

applied to the plates as a single band and developed in the indicated solvent in a vapor-saturated chamber. Following separation of steroid mixtures, the plates were sprayed with a half-saturated aqueous solution of Rhodamine G, and steroids were detected under UV light (254 nm).

TLC on Florisil to obtain Fractions I, II and III

Three distinct bands were seen on Florisil plates that were developed in ethyl ether–heptane 60:40 (**Fig. 1**); they were designated Fractions I, II, and III and had the mobilities of coprostanone, coprostanol, and cholesterol, respectively. These three steroid-containing areas of the plate were separately collected (8) and extracted with four 4-ml portions of diethyl ether. An aliquot of each eluate was taken for GLC, another for counting of radioactivity, and the remainder was used for further fractionation on silica gel G and silver nitrate-impregnated TLC plates as illustrated in Fig. 1.

Fraction I containing the ketonic steroids was studied *only* by GLC without further TLC separations. Details of additional TLC separations of Fractions II and III are given below.

TLC of Fraction II

Total Fraction II (silica gel G). TLC of Fraction II on silica gel G plates developed in benzene–acetone 90:10 revealed the presence of three bands (occasionally requiring rechromatography in the same solvent for complete resolution); we have designated them as II_1, II_2, II_3 in order of decreasing mobilities (Fig. 1). These three fractions were eluted individually and studied as described below.

Fraction II_2 (silver nitrate). Three zones (II_{2a}, II_{2b}, and II_{2c} in order of decreasing mobility) were revealed following TLC of Fraction II_2 on silver nitrate-impregnated plates developed in chloroform (Fig. 1). Fraction II_{2c} was often made up of several distinct bands.

Fraction II_3 (silver nitrate). Three bands (II_{3a}, II_{3b}, and II_{3c} in order of decreasing mobility) were revealed following TLC of Fraction II_3 on silver nitrate-impregnated plates developed in chloroform. If the coprostanol content of total Fraction II was extremely large, as was commonly observed in analyses of human fecal extracts, rechromatography was required to achieve complete separation of these three bands.

TLC of Fraction III (silver nitrate)

In studies of human and rat feces, four zones could be distinguished by TLC on silver nitrate plates developed with chloroform; they were designated Fractions III_a, III_b, III_c, and III_d (Fig. 1). Human neutral steroid extracts usually contained only Fractions III_a and III_c, whereas in rat feces a well defined Fraction III_b was seen, as well as several minor bands of lower mobility designated Fraction III_d.

Gas–liquid chromatography

All GLC analyses were performed on an instrument equipped with a hydrogen flame ionization detector (F and M Biomedical Gas Chromatograph, model 400, Avondale, PA or a Packard GLC, model 873, Downers Grove, IL). Columns were 180 cm glass U-tubes or 360 cm glass coiled tubes, 4 mm i.d., packed with silanized acid-washed Gas Chrom P (100–120 mesh) coated with films of 1% DC-560 (comparable to 1% SE-54), 3% SE-30, or 0.83% HiEff 8B (coatings and supports obtained from Applied Science Laboratories, State College, Pa). Column temperatures ranged from 215 to 265°C, depending on the type of column used, and were monitored with a mercury thermometer (Allihn type; Matheson Scientific Co.,

Philadelphia, Pa). The flash heater and the detecter were kept at 50°C and 40°C, respectively, above the temperature of the column. Nitrogen was used as carrier gas at flow rates of 30–60 ml/min at inlet pressures of 20–30 psi. On average, the GLC columns used had a resolving power of 4,000 theoretical plates for cholesterol.

Preparation of steroid derivatives

Trimethyl silyl ether (TMS) derivatives of neutral sterols were prepared in dimethyl formamide as previously described (3). Ketosteroid derivatives were prepared either as their triethyl phosphone acetates (TEPA) (9) or as methoxyamines (MOA) (10). Under the conditions used for ketosteroid derivatization, the hydroxysteroids could also be silylated if desired.

Steroid numbers

Retention times of steroids relative to 5α-cholestane were converted to steroid numbers as proposed by Vanden Heuvel and Horning (11). By determining the effect of different molecular groups on the steroid numbers of our reference standards, it was possible to calculate relative retention times of many steroids presumed to be present in the fecal samples.

To determine steroid numbers, it is necessary to have only two fixed points: the retentions of 5α-cholestane (=1) and cholesterol (relative to 5α-cholestane) were plotted on a logarithmic scale against their numbers (27 and 29.3, respectively) on a linear scale (12). A line drawn through these two points is then used to calculate all other relative retention times in terms of steroid numbers by the equation: steroid number = 27.00 + 6.53 × (log retention time relative to 5α-cholestane).

Table 2 shows the change in steroid number produced by the presence of different functional groups under the GLC conditions specified in the footnote. This tabulation applies to GLC runs on DC-560 at various temperatures, but steroid number differences are specific to the column packing used. As an example of a typical calculation of relative retention times from steroid numbers, take the case of 24-ethyl coprostanol, for which we had no reference standard. Its retention time can be obtained from the steroid numbers of cholesterol, corprostanol, and 24-ethyl cholesterol (sitosterol), as follows. Under the GLC conditions specified, the steroid number of these three

TABLE 2. Changes in steroid number caused by various structural features, as determined by GLC runs on the nonselective column packing, DC-560[a]

Parent Compound	Comparison Compound	Structural Change	Shift in Steroid Number[b]
Cholesterol	Coprostanol	$\Delta^5 \rightarrow 5\beta$	−0.71
Cholesterol	Cholestanol	$\Delta^5 \rightarrow 5\alpha$	+0.09
Δ^7-Cholestenol	Δ^7-Coprostenol	$5\alpha \rightarrow 5\beta$	−1.04
Cholesterol	Coprostanone	Δ^5,3β-OH → 5β,3-keto	−0.33
Cholesterol	Cholestanone	Δ^5,3β-OH → 5α,3-keto	+0.04
Cholestanol	Cholestanone	3β-OH → 3-keto	−0.05
Coprostanol	Coprostanone	3β-OH → 3-keto	+0.38
Cholesterol	Δ^7-Cholestenol	$\Delta^5 \rightarrow \Delta^7$	+0.41
Coprostanol	Δ^7-Coprostenol	$5\beta \rightarrow \Delta^7$	+0.08
Cholestanol	Δ^7-Cholestenol	$5\alpha \rightarrow \Delta^7$	+0.32
Cholesterol	Desmosterol	$\Delta^5 \rightarrow \Delta^{5,24}$	+0.32
Coprostanol	Δ^{24},Coprostenol	$5\beta \rightarrow \Delta^{24}$	−0.31
Dihydrolanosterol	Lanosterol	$\Delta^8 \rightarrow \Delta^{8,24}$	+0.30
Sitosterol	Stigmasterol	$\Delta^5 \rightarrow \Delta^{5,trans-22}$	−0.43
Methostenol	Δ^8-Methostenol	$\Delta^7 \rightarrow \Delta^8$	−0.20
Δ^7-Cholestenol	Methostenol	4-H → 4α-methyl	+0.56
Cholesterol	Campesterol	24-H → 24α-methyl	+0.85
Cholesterol	Sitosterol	24-H → 24α-ethyl	+1.50
Epicoprostanol	Coprostanol	3α-OH → 3β-OH	−0.11
Epicholestanol	Cholestanol	3α-OH → 3β-OH	−0.04
Coprostanol	Cholestanol	A/B cis → A/B trans	+0.80
Epicoprostanol	Epicholestanol	A/B cis → A/B trans	+0.65

[a] Column 1% DC-560, temperature 244°C, carrier gas (nitrogen) pressure at 30 psi. All sterols were run as their TMS-ethers and ketosteroids as free.

[b] Steroid numbers were calculated from the equation: steroid number = 27.00 + 6.53 × (log retention time relative to 5α-cholestane). For the other column packings used in the present study, the equations for steroid number determinations were 27.00 + 7.48 × (log RRT 5α-cholestane) for 3% SE-30 and 27.00 + 6.84 × (log RRT 5α-cholestane) for 0.83% HiEff.

sterols are 29.30, 28.59, and 30.72, respectively. The difference in steroid number between cholesterol and coprostanol (−0.071, due to loss of the Δ^5 functional group) is subtracted from the steroid number of 24-ethyl cholesterol (30.72) to obtain the steroid number of 24-ethyl coprostanol (30.01). Using the formula: steroid number = 27.00 + 6.53 × (log retention time relative to 5α-cholestane), we obtain a retention time for 24-ethyl coprostanol of 2.89, relative to 5α-cholestane.

However, it may be seen in Table 2 that the introduction or deletion of a specified functional group does not cause identical changes in steroid numbers of all steroids. The introduction of a double bond into coprostanol between C-7 and C-8 changes the steroid number +0.08, whereas the corresponding difference between cholestanol and Δ^7-cholestenol is +0.32. This difference is attributed to the *cis* and *trans* A/B ring configurations of coprostanol and cholestanol, respectively. Note also that the difference in steroid number between 3α-hydroxyl and 3β-hydroxyl isomers is −0.11 in the *cis* A/B ring series and −0.04 in the *trans* A/B series.

Three sets of retention times (relative to 5α-cholestane), on three column packings, of the neutral steroids we have identified in our studies of human and rat feces are shown in **Table 3.** The relative retention times were either determined directly or cal-

TABLE 3. Retention times (relative to 5α-cholestane) of rat fecal neutral steroids isolated by successive TLC runs shown in Fig. 1

TLC Fraction	Steroid Compound	1% DC-560[a]		3% SE-30[b]		0.83% HiEff 8B[c]	
		Ref/Calc.[d]	Found	Ref/Calc.[d]	Found	Ref/Calc.[d]	Found
I	Coprostanone	2.00	1.99	1.72	1.72	5.38	5.40
	Methyl coprostanone	2.59	2.57			7.22	7.24
	Ethyl coprostenone	2.84	2.83			7.63	7.60
	Ethyl coprostanone	3.27	3.27	2.81	2.81	9.00	8.92
II_1	Cholestanone	2.28	2.29				
	Δ^7-cholestenone	2.57	2.56				
	Campestanone	2.95	2.93				
	Stigmastenone	3.29	3.30				
	β-sitostanone	3.78	3.77				
II_{2a}	Epicoprostanol	1.82	1.83	1.69	1.74		
	Dihydrolanosterol	3.19	3.20	2.81	2.74	2.57	2.55
	Δ^7-lanosterol	3.47	3.41			3.08	2.80
II_{2b}	Lanosterol	3.54	3.53	3.06	3.02	3.32	3.30
II_{3a}	Coprostanol	1.75	1.74	1.65	1.65	1.38	1.38
	Methylcoprostanol	2.27	2.27	2.06	2.07	1.85	1.85
	Ethylcoprostenol	2.48	2.49	2.25	2.25	1.95	1.96
	Ethylcoprostanol	2.88	2.87	2.55	2.54	2.31	2.31
II_{3b}	Δ^7-coprostenol	1.80	1.80	1.71	1.75	1.41	1.40
	Methostenol	3.16	3.18	2.79	2.71	3.16	3.18
	Δ^8-methostenol	2.95	2.95	2.56	2.59	2.66	2.66
II_{3c}	*$\Delta^{7,24}$-methostenol*	3.53	3.55				
	$\Delta^{8,24}$-methostenol	3.25	3.20				
III_a	Cholestanol	2.32	2.35	2.07	2.07		
	Campestanol	3.03	3.02	2.60	2.61		
	Stigmastenol	3.27	3.29	2.79	2.81		
	β-sitostanol	3.81	3.80	3.23	3.23		
III_b	Δ^7-cholestenol	2.60	2.60	2.29	2.29	2.62	2.60
III_c	Cholesterol	2.25	2.24	2.03	2.03	2.17	2.16
	Campesterol	2.94	2.93	2.55	2.55	2.90	2.89
	Stigmasterol	3.18	3.20	2.74	2.74	3.05	3.08
	β-sitosterol	3.70	3.71	3.17	3.17	3.60	3.60
III_d	Desmosterol	2.52	2.53	2.18	2.23		
	$\Delta^{5,7}$-cholesterol	2.58	2.57				

[a] Column temp. 245°C.
[b] Column temp. 265°C.
[c] Column temp. 224°C.
[d] RRT's of reference compounds are shown in Roman letters; those in italics were calculated from steroid number data, as exemplified in Table 1 for GLC on DC-560 columns.

culated from steroid number values derived for each individual column packing.

RESULTS

Composition of Fraction III

A. Fraction III from Florisil TLC

Human feces (plant sterol-free diet). Fecal steroids obtained from Fraction III on Florisil were isolated from the feces of patients who had been maintained on plant sterol-free formulas that were either fat-free or contained 40% of total calories as butter oil. GLC analysis of this fraction showed only one component with the retention time of cholesterol. However, cholestanol, were it present, could not have been reliably separated from cholesterol by either the TLC or GLC systems employed up to this stage of fractionation (but see below).

Human feces (plant sterol-containing diets). Corn oil-rich diets contain a mixture of sterols that are made up of campesterol, stigmasterol, and sitosterol (99% of total sterols). After passage through the human intestine these plant sterols had the same TLC mobility on Florisil (Fraction III) and the same GLC retention times as the dietary sterols; they were totally absent from the feces of patients on plant sterol-free diets.

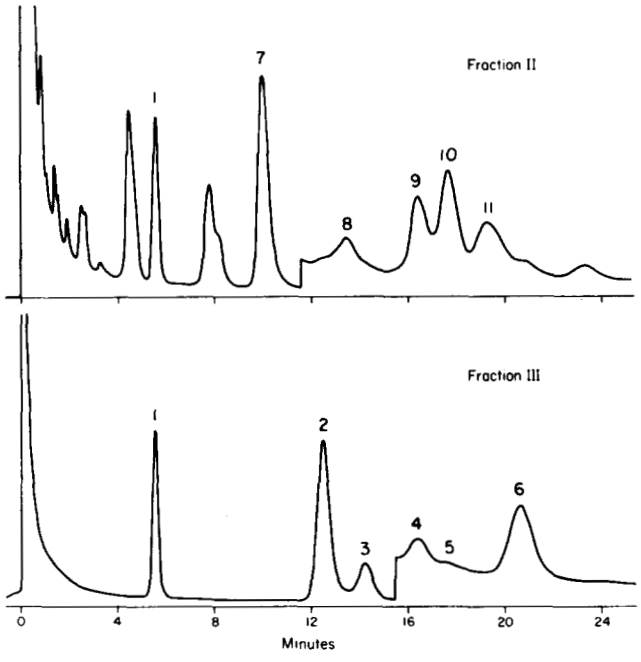

Fig. 2. GLC patterns on 1% DC-560 of Fractions II and III obtained from rats on a sterol-free diet. Diet analysis demonstrated the presence of cholesterol (0.7 mg/day/rat) and plant sterols (0.4 mg/day/rat). Peak 1 = 5α-cholestane (internal standard); 2 = cholesterol; 3 = Δ^7-cholestenol; 4 = campesterol; 5 = stigmasterol; 6 = sitosterol; 7 = coprostanol; 8 and 9 = 5β-saturated plant sterols, plus unidentified cholesterol precursors; 10 = methostenol and dihydrolanosterol; and 11 = lanosterol plus undefined compounds.

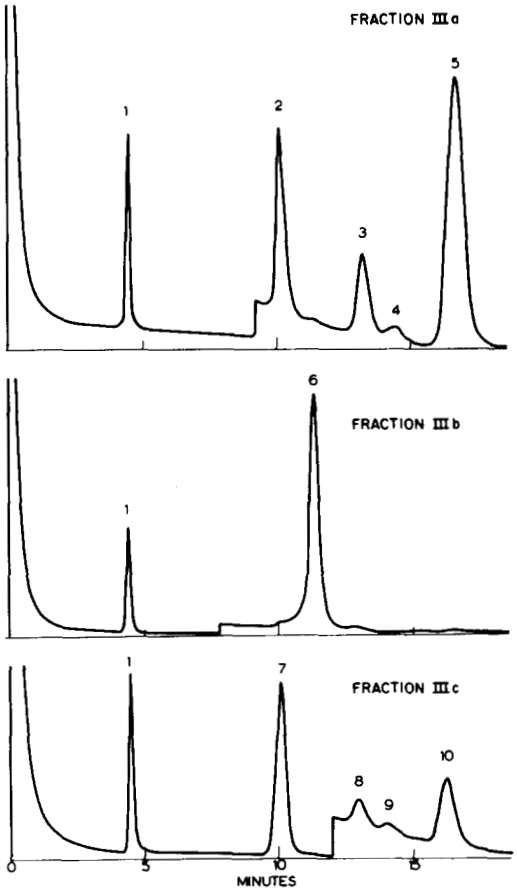

Fig. 3. GLC patterns on 1% SE-30 of Fraction III separated by TLC on silver nitrate-impregnated silica gel G plates. The following identifications were made: 1 = 5α-cholestane (internal standard); 2 = cholestanol; 3 = campestanol; 4 = stigmastanol; 5 = sitostanol; 6 = Δ^7-cholestenol; 7 = cholesterol; 8 = campesterol; 9 = stigmasterol; and 10 = sitosterol.

Rat feces. GLC patterns and relative retention times from Fraction III of rat feces from animals fed a standard chow diet were almost identical on all GLC columns to Fraction III steroids obtained from human feces with the exception that an additional peak with the retention time of Δ^7-cholestenol was consistently found in rat feces (**Fig. 2**). Derivatization of Fraction III with TEPA or MOA caused no change in GLC patterns of the TMS-ethers, indicating the absence of ketosteroids.

B. Fraction III from silver nitrate TLC

Fraction III obtained by TLC on Florisil was rechromatographed on silver nitrate plates to separate compounds according to the degree of unsaturation (Fig. 1). GLC patterns of Fractions III_a, III_b, and III_c are presented in **Fig. 3.**

Fraction III_a, moving on TLC with the mobility of cholestanol, showed a single GLC peak with retention time of reference cholestanol, provided the sample was obtained from patients on plant sterol-free diets. Three additional components were observed when the diet contained corn sterols: these compounds had the same GLC relationship to campesterol, stigmasterol, and sitosterol as cholesterol had to cholestanol (Table. 3). Accordingly, these sterols were considered to be ring-saturated 5α-plant sterols produced by bacterial action. Radioactivity was present in Fraction III_a in rats endogenously labeled with [4-^{14}C]cholesterol (cholestanol-free): the specific activity of fecal cholestanol (Fraction III_a) was the same as that of fecal cholesterol (Fraction III_c) indicating that the former was derived from the latter (4).

Fraction III_b behaved both on TLC and GLC in the same manner as the reference standard Δ^7-cholestenol. This component was found in greater amounts in rat than in human feces; it was estimated that human beings on a fat-free formula diet excreted 1–3 mg/day of this sterol, and that it must have been of endogenous origin, since it was not present in the diet. Its production from cholesterol or cholesterol products was ruled out by the absence of radioactivity in this fraction both in human and in rat feces after pre-labeling the host with [4-^{14}C]cholesterol.

Fraction III_c contained cholesterol and the major plant sterols, judging by TLC mobility and GLC retention times. In fecal extracts obtained from patients pre-labeled with [4-^{14}C]cholesterol, almost all of the radioactivity of Fraction III was found in III_c. Furthermore, the specific activities of cholesterol obtained from Florisil and from silver nitrate plates were the same, indicating that the cholesterol peak obtained from Florisil was virtually free of non-cholesterol materials.

Fraction III_d from human feces was practically free of sterols; however, in studies of rat feces the pattern was variable. Components with the retention times on silver nitrate plates of desmosterol and of $\Delta^{5,7}$-cholestenol were often seen; however, radioactivity was never encountered in this subfraction after pre-labeling the rat with [4-^{14}C]cholesterol, indicating that these components were not of cholesterol origin.

Composition of Fraction II

A. Fraction II from Florisil TLC

Human feces (plant sterol-free diet). Fraction II obtained from feces of patients on plant sterol-free diets by Florisil TLC showed one major peak on GLC corresponding in retention time to coprostanol, and several smaller peaks (seen only at high sensitivity) with retention times greater than coprostanol. The largest of these minor components corresponded in retention time to methostenol.

Rat feces (plant sterol-free diet). The GLC pattern of Fraction II from feces of rats on a plant sterol-free diet was more complicated than that found in the human being. GLC peaks emerging before coprostanol (Fig. 2) are believed to be long-chain alcohols similar to those found in rat skin lipids (13), whereas those following coprostanol represent the cholesterol precursors described below.

Human and rat feces (plant sterol-containing diets). When the diet of human beings or rats was rich in corn sterols, three additional components appeared in the GLC pattern. These components were present in the same relative amounts as the three plant sterols fed. They also had the same relative separation factors on GLC, but their retention times were less than those of the dietary plant sterols and differed from them to the same degree that the retention time of coprostanol differed from that of cholesterol. Thus, it is likely that these compounds were the 5β-saturated analogues of campesterol, stigmasterol, and sitosterol produced by reduction by intestinal bacteria, analogous to the reduction of cholesterol to coprostanol. In rat and human stools these plant products obscured the GLC pattern of the cholesterol precursors illustrated in Fig. 2 in feces from rats fed sterol-free diets.

TLC of standards revealed that cholestanone, if present, would be found in Fraction II. Treatment of this fraction with TEPA and MOA did not produce a change in the GLC pattern, however, indicating that ketosteroids were not present even when sought at high GLC sensitivity.

B. Fraction II from silica gel G TLC

Fraction II from Florisil TLC was rechromatographed on silica gel G plates to obtain a separation of coprostanol and related plant sterols from cholesterol precursors and to obtain a better identification of the latter compounds. In studies of rats pre-labeled with [4-^{14}C]cholesterol, neutral steroids of endogenous origin were identified as before by their radioactivity.

Fraction II_1. This fraction contained no radioactivity. Cholestanone, if present, would have moved in this fraction, but it was not found.

Fraction II_2. GLC of this zone showed many components including those with GLC retention times of lanosterol and dihydrolanosterol. No radioactivity was present in this fraction, indicating the absence of endogenous cholesterol products.

Fraction II_3 revealed the presence of coprostanol, the three 5β-saturated plant sterols, methostenol, and other sterols discussed below.

C. Fraction II_2 from silver nitrate plates

Fraction II_{2a} showed four major peaks on GLC (**Fig. 4**), one of which (number 2) had the retention time of dihydrolanosterol (8). The other components were not identified: their steroid numbers on 1% DC-560 were 28.27, 30.48, 31.78, 32.28, and 32.69. The retention time of the peak immediately following peak 2 (dihydrolanosterol) did not correspond to that calculated for Δ^7-dihydrolanosterol.

Fraction II_{2b} showed one major component corresponding in GLC characteristics to lanosterol (peak 3).

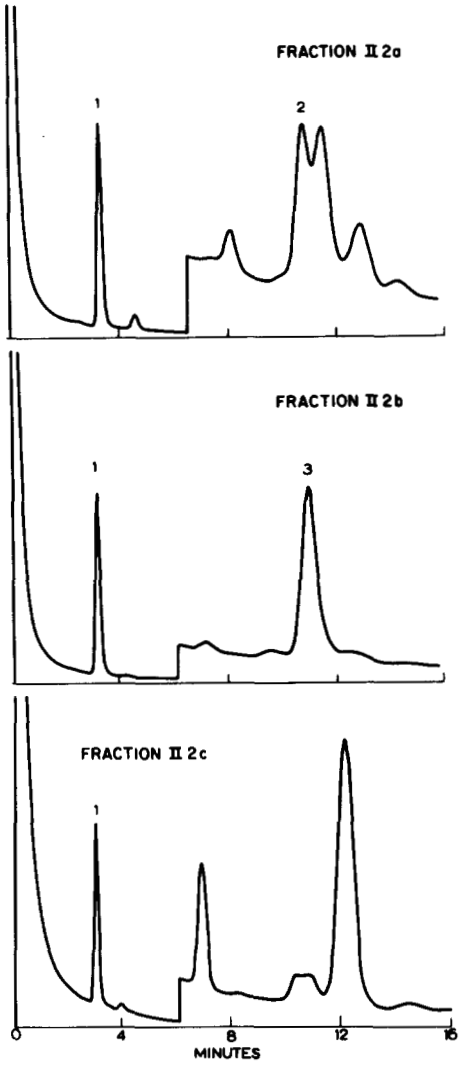

Fig. 4. GLC patterns on 1% DC-560 of Fraction II_2 following sequential separations by TLC on silica gel G and silver nitrate-impregnated silica gel G. The following identifications were made: 1 = 5α-cholestane (internal standard); 2 = dihydrolanosterol; and 3 = lanosterol.

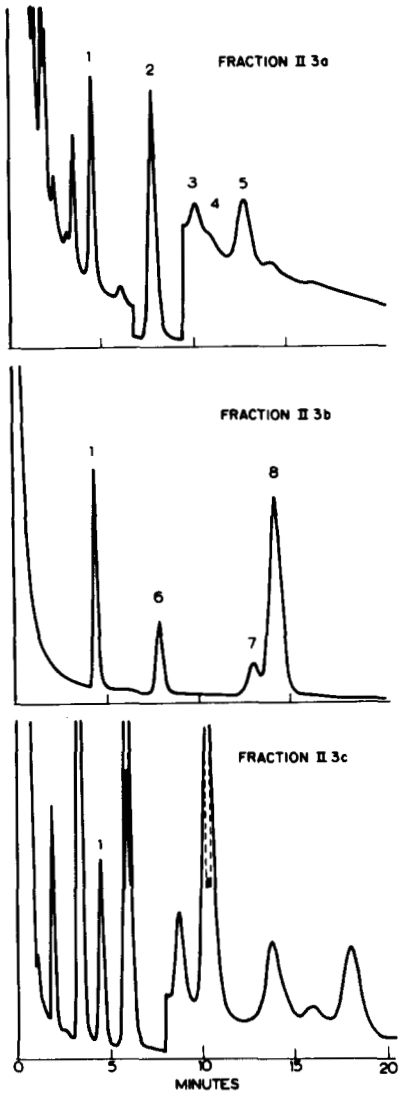

Fig. 5. GLC patterns on 1% DC-560 of Fraction II_3 following sequential separations by TLC on silica gel G and silver nitrate-impregnated silica gel G. The following identifications were made: 1 = 5α-cholestane (internal standard); 2 = coprostanol; 3 = methyl coprostanol; 4 = ethyl coprostenol; 5 = ethyl coprostanol; 6 = Δ^7-coprostenol; 7 = Δ^8-methostenol; 8 = methostenol.

Traces of other unidentified components were also seen (Fig. 4).

Fraction II_{2c} contained a series of components in small concentrations; none of these were identified by comparison to reference standards, nor could we deduce their structures from their numbers.

D. Fraction II_3 from silver nitrate plates

Fig. 5 shows GLC patterns of the three zones isolated by TLC on Fraction II_3 on silver nitrate plates. *Fraction II_{3a}* contained coprostanol and three compounds with retention times similar to those calculated for the 5β-ring saturated plant sterol analogues of

campesterol, stigmasterol, and sitosterol: only these components of Fraction II_3 contained radioactivity. GLC of *Fraction* II_{3b} showed three peaks that were identified on the basis of TLC retention times and GLC characteristics as Δ^7-coprostenol, Δ^8-methostenol, and methostenol. The GLC patterns of *Fraction* II_{3c} were extremely complex: no reliable identifications could be made.

Fig. 5 shows that Δ^7-coprostenol (II_{3b}, peak 6) and an unidentified compound in Fraction II_{3c} could be separated from coprostanol (II_{3a}, peak 2). Thus, measurements of coprostonal made solely by GLC of total Fraction II are overestimates due to contamination with these other products. This was clearly shown when the specific activity of coprostanol determined by GLC of total Fraction II was found to be 5% lower than that determined by GLC of Fraction II_{3a} in analyses of stools of rats on plant sterol-free diets (4).

Likewise, measurements of plant sterols in this fraction are overestimated somewhat by the presence of cholesterol precursors co-chromatographing on GLC, but in the case of both coprostanol and the plant sterols this contamination is usually less than 3% in analysis of fecal extracts derived from rats on plant sterol-free diets. When rat diets contained plant sterols, the contamination of the plant sterol peaks by cholesterol precursors was negligible in relationship to the total. In extracts of human feces these cholesterol precursors were never detected.

E. Detection of trace steroids in Fraction II total

Cholestanone was detected in the stools of a patient who had been maintained on a cholesterol intake of 2.8 g/day for several weeks. A large quantity of coprostanol was excreted with small amounts of cholestanol, Δ^7-cholestenol, and cholestanone. Cholesterol itself was almost totally absent.

GLC of Fraction II_1 obtained from silica gel G plates revealed the presence of three minor components: one of these had the retention time of cholestanone, and another the retention time calculated for Δ^7-cholestenone.

Epicoprostanol was found with some difficulty in stools from the above-mentioned patient by GLC of the area lying just ahead of Fraction II_{2a} on silver nitrate plates; it was necessary to remove coprostanol contaminants of Fraction II_3 by preliminary re-chromatography on silica gel G. These data indicate that the isomerization of 3β-hydroxyl to 3α-hydroxyl can occur to a small extent in the human intestinal lumen: epicoprostanol was found in only one out of three patients who were fed 500–700 mg/day of cholesterol. No similar conversions of plant sterols were noted.

Cholesterol precursors were sought in patients on fat-free, low-cholesterol, plant sterol-free, and plant-sterol-containing formulas. Multiple re-chromatography was carried out to remove the relatively large amounts of coprostanol and 5β-saturated corn sterol congeners. Results of these studies showed that many of the components found in rat fecal steroids (Figs. 4 and 5) could also be identified in human stools. The amounts of cholesterol precursors in human stools were small: they ranged from 1 to 7 mg/day for methostenols plus lanosterols in the 15 subjects studied. On a per kilogram basis, a comparable excretion of the same precursors would be approximately 50- to 100-fold greater in rat feces.

Composition of Fraction I

By comparison with reference standards, only coprostanone moved on Florisil TLC with the mobility of Fraction I. On GLC of fecal extracts from rats on plant sterol-free diets, a single peak was found with retention time of coprostanone, whereas three additional peaks appeared when the diet was rich in plant sterols. Furthermore, it was observed that these compounds did not form TMS-ethers; their retention remained unchanged after carrying out TMS-derivatization for one hour; and this also was observed with TMS-treatment of coprostanone itself. Due to the presence of the latter compound in Fraction I and due to non-reactivity of all Fraction I components in the TMS-reaction, we propose that the most likely structure assignment is a series of ring-saturated 5β-3-keto plant sterol derivatives, i.e., methyl coprostanone, ethyl coprostenone, and ethyl coprostanone. Accordingly, relative retention times were calculated from their steroid numbers: they corresponded closely to the GLC retention times noted on both non-polar and polar liquid phases. The same compounds were also found in rat stools, where the amount of ketosteroids was usually about 1.2–2.5% of total sterols even on ordinary rat chow.

DISCUSSION

Any attempt to perform meaningful sterol balance measurements in man or in rats (and presumably any other animal) requires that the large number of fecal steroids excreted daily are identified as to structure and origin (endogenous and exogenous). The present study has applied sequential TLC on Florisil, silica gel and silver nitrate-impregnated silica gel, coupled with GLC analysis on polar and non-polar phases, to identify the major and minor steroid components of human and rat feces. In the accompanying study (4),

the origin of these sterols in the rat has been investigated and their respective sources identified. Eneroth et al., (1) using column chromatography, GLC, and mass spectrometry identified a number of cholesterol and plant sterols and their respective metabolites in fecal samples from patients on solid food diets; the present study extends these findings to the identification of a number of minor sterol constituents of human feces and to the multiple sterols present in the feces of the rat.

The fact that a number of these sterols arise by bacterial degradation of endogenous and exogenous sterols is well established. Studies by Kellogg and Wostmann (14) have demonstrated that germfree rats excrete neither coprostanol, nor coprostanol analogues of the plant sterols, nor coprostanone; in the rat these conversions occur primary in the cecum (15). In addition, the excretion of bacterial metabolites of endogenous and exogenous sterols increases with age from birth to adulthood (16) as the number and variety of intestinal microflora increase; these conversions can be completely obliterated in patients treated with neomycin (17). Other studies have addressed the rate of bacterial biohydrogenation of the Δ^5-double bond (18).

In man, approximately 95% of the fecal steroids of endogenous origin consist of cholesterol, coprostanol, and coprostanone. Epicoprostanol (1), cholestanol (1, 19), and Δ^4-cholestenone (20) have all been previously identified in human feces; yet cholestanol, which constitutes the most abundant of the minor sterols, only contributes 2% of the fecal neutral steroid output in man (3).

Previous studies have documented the fact that in man (21) and the rat (22) the plant sterols and cholesterol are metabolized by the intestinal bacteria to the same extent; in addition, there is bacterial degradation of neutral 3β-OH, Δ^5-sterols to products not recognized as steroids by the analytical methods used. The extent of this degradation has been shown to vary depending on species (23), intestinal transit time (24); and diet (25).

Cholesterol precursors. Both methostenol and Δ^7-cholestenol have previously been reported to exist in rat stools (26–29) but not in human feces (1). In the present study, Δ^7-cholestenol and methostenol have been identified in human feces as minor components. In the rat, numerous sterol precursors have been identified: the origins of these sterols, whether endogenous, dietary, or due to fur-licking, is the subject of the accompanying study (4) which demonstrates that when animals are restrained from fur-licking, the concentration of these cholesterol precursors in feces is markedly reduced. The present data (Figs. 4 and 5) demonstrate that there is still a large number of unidentified peaks in some of the fractions that may represent still other cholesterol precursors and possibly secondary conversion products produced by the action of intestinal bacteria (30, 31). Unless these precursors originating in the rat skin are properly isolated, the estimation of truly endogenous cholesterol synthesis by the rat can be seriously overestimated.

In human feces from patients prelabeled with [^{14}C]cholesterol and fed a plant sterol-free diet, the major steroids produced endogenously from cholesterol were shown to be cholesterol, coprostanol, and coprostanone; Δ^7-cholestenol and methostenol, while present in minor quantities, were not labeled and thus were not products of endogenous cholesterol metabolism. Upon feeding a diet containing plant sterols, fecal steroids were found to include all steroids listed above plus unabsorbed plant sterols, and also their ring-saturated 5α and 5β analogues and their respective ketones.

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