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Identification and quantification of neutral fecal steroids by gas–liquid chromatography and mass spectrometry: studies of human excretion during two dietary regimens*

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SUMMARY The excretion of fecal neutral steroids was studied in six normocholesterolemic subjects and one hypercholesterolemic subject fed a standardized diet supplemented with butter fat or corn oil.

The neutral steroids in the diets and in feces were identified by gas–liquid chromatography combined with mass spectrometric analyses of the chromatographic effluents. The three main sterols in the diets were cholesterol, β-sitosterol, and a methylcholesterol, probably campesterol.

The fecal neutral steroids were cholesterol and its metabolites, coprostanol and coprostanone, β-sitosterol and methylcholesterol, and their corresponding metabolites. In addition small amounts of cholestanol, 24β-ethylcholestanol, and methylcholestanol were present. Most subjects on both diets excreted coprostanol as the predominant fecal steroid. However, during the corn oil period the proportion of coprostanone and/or cholesterol was increased.

When the normal subjects had been transferred from the butter fat to the corn oil diet the serum cholesterol level fell in all subjects. On the corn oil diet the mean total excretion of neutral steroids in feces increased significantly, primarily as a result of the elevated elimination of plant sterols and plant sterol metabolites. The mean total excretion of cholesterol and cholesterol neutral metabolites was 527 mg/day during the butter and 614 mg/day during the corn oil period. This change, however, was not statistically significant.

The hypercholesterolemic subject responded in the same fashion as the normal subjects.

The possible effect of the two diets on the excretion of "non-dietary" cholesterol as neutral fecal steroids is discussed.

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THE MAIN EXCRETORY products of body cholesterol[1] are bile acids and neutral steroids, which are eliminated with the feces as a complex mixture of metabolites formed by the action of intestinal microorganisms (1). Several investigators have concluded from studies in man that the elimination of body cholesterol as fecal products is greater on diets containing unsaturated fat than on those containing saturated fat. It has been suggested that this increase is more pronounced for the bile acids than for the neutral steroids (2, 3) whereas Hellman et al. (4) found, in the one subject studied, an increase only in the neutral steroid fraction. Spritz et al. (5), in a recent study of five subjects consuming a cholesterol-free formula diet, observed no change in the excretion of bile acids and neutral steroids on substituting saturated by unsaturated fat. The technical difficulties inherent in many methods used for quantitative determination of the fecal bile acids and neutral steroids may explain some of the discrepancies in the results obtained.

The unsaponifiable fraction of feces contains not only cholesterol and its neutral metabolites but also, in

[1] The following systematic names are given to steroids referred to in this report by trivial names:
cholesterol, cholest-5-en-3β-ol; epicholesterol, cholest-5-en-3α-ol; coprostanol, 5β-cholestan-3β-ol; epicoprostanol, 5β-cholestan-3α-ol; coprostanone; 5β-cholestan-3-one; cholestanol, 5α-cholestan-3β-ol; cholestanone, 5α-cholestan-3-one; Δ^4-cholestenone, cholest-4-en-3-one; Δ^7-coprostenol, 5β-cholest-7-en-3β-ol; lathosterol, 5α-cholest-7-en-3β-ol; 7-dehydrocholesterol, cholest-5,7-dien-3β-ol; methostenol, 4α-methyl-cholest-7-en-3β-ol; methostenol, 4α-methyl-cholest-7-en-3β-ol; β-sitosterol, 24β-ethyl-cholest-5-en-3β-ol; campesterol, 24α-methyl-cholest-5-en-3β-ol; stigmasterol, 24β-ethyl-cholest-5,22-dien-3β-ol.

TABLE 1 BASIC DATA OF THE SUBJECTS EXAMINED

Subject	Sex	Age	Caloric Intake (per Day)	Dietary Fat as Per Cent of Total Calories Consumed	Body Weight*	
					Butter Period	Corn Oil Period
		yrs			*kg*	*kg*
1 (G − l)	F	33	1900	41	58.1	57.7
2 (L − d)	F	21	1900	41	54.6	54.4
3 (S − n)	F	21	1900	41	67.5	66.2
4 (W − n)	M	32	2060	40	64.4	65.0
5 (T − l)	M	22	2270	40	73.2	73.2
6 (F − d)	F	23	2066	40	55.5	56.1
7 (A − r)	F	45	1450	40	66.9	63.2

* Mean of five to seven determinations during each dietary period.

subjects on ordinary diets, plant sterols closely related chemically to cholesterol. Whereas a good deal of information is available on the composition of fecal neutral steroids of endogenous origin (for a recent review see reference 1), little is known about the nature of the fecal steroids derived from various dietary plant sterols. The only reports on this subject are those of Rosenheim and Webster (6) and Coleman et al. (7), who demonstrated the conversion of β-sitosterol into 24β-ethylcoprostanol in vivo and in vitro. It seems reasonable to expect that intestinal microorganisms effect transformations of plant sterol molecules similar to those brought about in the cholesterol molecule. The pronounced interference of such compounds with the quantitative determination of cholesterol and neutral cholesterol metabolites must be taken into account when nonisotopic methods are being used. Even when plant sterols have been excluded from the diet many conventional methods are inadequate. Using colorimetry alone or in combination with digitonide precipitation several investigators have determined fecal neutral steroids quantitatively without separating them individually. Because of the unequal response of different steroids to these reagents (8–10) erroneous results could be obtained. The errors might be enlarged when the effects of different diets are compared, since these diets could alter the composition of the neutral cholesterol metabolites (3). To overcome difficulties of this kind Goldsmith et al. (3) separated the steroid mixture into three fractions by glass paper chromatography prior to quantification. This procedure did not, however, allow of a selective determination of cholesterol and its neutral metabolites in the presence of plant sterols. To avoid errors involved in most chemical determinations of fecal neutral steroids, Hellman et al. (4) used isotope methods, whereas Spritz et al. (5) isolated the bile acids and sterols quantitatively and then weighed them.[2]

[2] E. H. Ahrens, Jr., personal communication.

Recently gas–liquid chromatography (GLC) has been applied to the analysis of some fecal neutral steroids (11–15). This technique has given superior separations of neutral steroids and can be used for quantitative work. We therefore considered it of interest to apply GLC to the reinvestigation of the effect of different dietary fats on the excretion of neutral fecal steroids in man. Identification of the various compounds, detected when neutral fecal lipid fractions are analyzed by GLC, was achieved with an instrument combining GLC and mass spectrometry recently developed in this laboratory (16).

MATERIALS AND METHODS

Subjects

Of the seven subjects studied (Table 1) six were cadets from the Salvation Army School in Stockholm. The cadets lived at the school and were occupied with their ordinary work during the whole experiment. Subject 7 was hypercholesterolemic and was hospitalized in a metabolic ward.

Diets

Throughout the experiment all subjects were given a standardized diet. Breakfast consisted of coffee or tea, toast, and marmalade. For lunch and dinner the subjects consumed one of four prepared dishes which were served in rotation. The dishes (kindly supplied by A. B. Findus, Hälsingborg, Sweden) were composed of either meat (chicken or veal) or fish (cod and flounder) together with potatoes, vegetables, and fat (corn oil or butter). The amount of fat corresponded to 51% of the calories in the meat portions and to 62% in the fish portions. During the first part of the experiment the dietary fat was made up of butter; after 4 weeks this was replaced by corn oil. For lunch and dinner each subject was served in addition white bread and fruit. The daily intake of bread, marmalade, and fruit was approximately equal for each subject during the two dietary periods. The subjects were instructed to consume the specially prepared dishes completely.

During the first 10 days the intake for each subject was adjusted in order to maintain constant weight. The only significant deviation was observed with Subject 7, whose weight decreased by 3.7 kg during the experiment.

Collection of feces started on the 10th day after the beginning of the experiment. Feces from Subjects 1–3 were collected throughout the remainder of the dietary experiment. It was not possible to collect feces from Subjects 4–7 for 14–16 days immediately following the change of diet, but after that time feces were again collected for a period of 9–19 days.

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Serum Cholesterol

Blood samples were taken every week and the serum cholesterol was determined using the Tschugaeff reaction. The extraction procedure of Folch et al. (17) was used.

Analysis of Diet Portions

The different diet portions were homogenized in chloroform–methanol 1:1 (v/v) with an Ultra-Turrax homogenizer (Janke and Kunkel, KG, Staufen i.Bra., West Germany) for 5 min. The homogenate was filtered and the residue washed twice with the same solvent mixture. The combined filtrates were transferred to a separatory funnel, water was added, and the organic layer collected. The aqueous phase was reextracted three times with chloroform. An aliquot (1/100) of the combined chloroform extracts was evaporated under reduced pressure until it started to foam, when 0.5 ml of 2 N KOH and 0.5 ml of methanol per mg dry weight of the extract (determined on a separate sample) were added. After saponification for 1 hr at 60° the hydrolysate was extracted three times with petroleum ether. The combined organic phases were washed with 50% aqueous ethanol until neutral and then taken to dryness under reduced pressure. The whole fraction was chromatographed on a silicic acid column (Mallinckrodt 100 mesh, activated for 5 days at 120°). For 200 mg of extract 5 g of silicic acid was used. The column was washed with benzene (20 ml/g silicic acid) and the sterols were eluted with 3% acetone in benzene. As a check on the quantitative recoveries of the sterols in the chromatographic procedures a tracer amount of labeled cholesterol was added. All the radioactivity was recovered in the 3% acetone in benzene fraction. Sterols in this fraction were subjected to GLC.

Another aliquot of the combined chloroform–methanol extracts from the diet portions was taken for analysis of fatty acids. It was evaporated to dryness and then saponified at room temperature for 12 hr using the same amounts of alkali and methanol as used for the sterols. The unsaponifiable material was extracted with petroleum ether and the aqueous phase acidified to pH 3 with 6 N HCl and again extracted with petroleum ether. The organic phase was washed until neutral with small volumes of water and dried over Na_2SO_4. An aliquot was taken to dryness under a stream of nitrogen, redissolved in diethyl ether–methanol 9:1, and methylated by treatment with diazomethane.

Analysis of Feces

Feces were collected in plastic bags and immediately stored at −20°. The contents of the bags were then transferred into 1000 ml plastic bottles, thawed in about 0.5 liter chloroform–methanol 1:1 at room temperature, and homogenized for 5 min. The bottles were sealed with a screw-cap through which the shaft of the homogenizer was introduced. The plastic bags were cut into pieces and washed with chloroform–methanol. This solution, together with the fecal homogenate, was transferred to a Soxhlet extractor of the siphon-type and continuously extracted for 48 hr at reflux temperature in a final volume of 1–1.5 liters of chloroform–methanol. The extract was stored at room temperature in dark, tightly stoppered bottles until analyzed (within 1–14 days).

Extracts from 3–5 days of feces were pooled and an aliquot (1–2% of the pooled mixture) was concentrated under reduced pressure to 5–10 ml (until foaming); 0.5 ml of 2 N KOH and 0.5 ml of methanol per mg dry weight (determined on a separate sample) were added and the final solution was refluxed for 1 hr. After extracting the hydrolysate three times with petroleum ether (bp 40–60°) (occasional emulsion formation being overcome by the addition of NaCl), the extracts were combined, washed with 50% aqueous ethanol in water until neutral, dried over Na_2SO_4, and concentrated under reduced pressure. The remaining solution was transferred to a test tube and taken to dryness under a stream of nitrogen.

Reference Compounds

The reference compounds used were prepared in this laboratory or obtained commercially (Southeastern Biochemicals, Morristown, Tenn., and Fluka AG, Buchs, Switzerland).

The compounds used in the quantitative work were purified to constant melting point and contained a maximum of 3% of impurities as judged by GLC and thin-layer chromatography. They were dried at 10^{-2} mm Hg for 48 hr at room temperature. Analysis of carbon and hydrogen content: cholesterol ($C_{27}H_{46}O$) calculated C = 83.93%, H = 11.91%, found C = 84.26%, H = 12.06%, coprostanol ($C_{27}H_{48}O$) calculated C = 83.51%, H = 12.37%, found C = 83.55%, H = 12.51%.

Gas–Liquid Chromatography

A Pye argon chromatograph modified for a 2 m x 5 mm U-tube column and connected with a standard ionization detector placed in a separately heated oven was used. For details see Sjövall (18). The stationary phase QF-1, 0.5–4%, was applied on acid-washed, acetone-washed, and silanized Gas Chrom P, 100–140 mesh (Applied Science Laboratories, Inc., State College, Pa.). as described by Sjövall et al. (19). Similarly treated support coated with 1% SE-30 was kindly given to us by Dr. E. Horning. The QF-1 and SE-30 columns had respectively 2500–4000 and 4000 theoretical plates, calculated for cholesterol.

Standard conditions: flash heater temperature, 280°; column temperatures 200° (QF-1) and 195° (SE-30);

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detector voltage, 1500 v; detector temperature, 240°; argon pressure, 1 kg/cm² (14 psi). For identification purposes samples were run both on QF-1 and on SE-30 before and after preparation of trimethylsilyl (TMS) ethers or dimethylhydrazones. All *quantitative* analyses were performed using the unsubstituted steroids on 0.5% QF-1 as stationary phase.

The TMS ethers were prepared as follows: 1 mg of the saponified extract was dissolved in 0.2 ml hexamethyldisilazane (Fluka, Buchs, Switzerland) and 0.2 ml dimethylformamide (treated with calcium carbide, distilled, and stored over neutral aluminum oxide, Woelm, grade I). After standing at room temperature overnight the reaction was complete for all 3-hydroxysterols tested. The ethers were either injected directly from the reaction mixture or from a heptane solution (prepared by adding 1.0 ml redistilled heptane to the dimethylformamide solution, mixing the solvents by slight tapping, and separating the phases by centrifugation). Dimethylhydrazones were prepared according to the procedure of Horning et al.[3] (20) using 0.1–0.2 ml 1,1-dimethylhydrazine (Fluka AG, Buchs, Switzerland) for 0.1–0.5 mg of steroids. After standing at room temperature overnight the solution was taken to dryness under a stream of nitrogen. The reaction was complete for the saturated 3-ketosteroids tested. Addition of 0.05 ml glacial acetic acid to the reaction mixture converted Δ^4-cholestenone into its dimethylhydrazone. Both types of derivatives had a tendency to hydrolyze on standing at room temperature and the GLC analysis was therefore performed shortly after the completion of the appropriate reaction. All retention times given were measured relative to cholesterol and represent the mean of three determinations.

In quantitative studies, samples were dissolved in redistilled acetone prior to GLC and analyses were made in duplicate for most samples. One 4 µl portion corresponding to 10–20 µg of the extract and another corresponding to 20–40 µg were injected with a 10 µl Hamilton syringe. Peak areas were measured by triangulation and compared with those obtained for known amounts of cholesterol, coprostanol, and coprostanone.

Mass Spectrometry of GLC-Column Effluents

The outlet of a GLC column was connected to a modified Atlas CH4 mass spectrometer. The compound instrument thus formed is referred to in the following sections as the GC-MS instrument (since it can be used with gas–solid as well as gas–liquid chromatography). The flash heater, column oven, and type of glass column used were essentially as described by Haahti et al. (21). The column (4 m x 2 mm) was packed with 1% SE-30. Operating conditions: flash heater temperature, 290°;

[3] E. C. Horning and W. J. A. VandenHeuvel, personal communication.

TABLE 2 GLC and Mass Spectrometric Data of Neutral Steroids

Compound Designation	Tentative Structure*	Retention Times Relative to Cholesterol: 0.5% QF = 1 Calc.	0.5% QF = 1 Found	0.5% QF = 1 Derivatives† Calc.	0.5% QF = 1 Derivatives† Found	1% SE = 30 Calc.	1% SE = 30 Found	1% SE = 30 Derivatives† Calc.	1% SE = 30 Derivatives† Found	Interpretation of Mass Spectrometric Data: Mol. Wt.	Side Chain	Double Bonds in the Nucleus	Double Bonds in the Side Chain	Hydroxyl Groups in the Nucleus
F	5β-3-one (coprostanone)	1.89‡	1.89	1.00‡	0.99	0.99‡	0.99	1.39‡	1.39	388	C_8H_{17}	0	0	0
G	24α-methyl-5β-3-one	2.54§	2.55	1.34§	1.35	1.32§	1.31	1.85§	1.83	402	C_9H_{19}	0	0	0
H	24β-ethyl-5β-3-one	3.20§	3.20	1.69§	1.69	1.70§	1.69	2.39§	2.32	416	$C_{10}H_{21}$	0	0	0
A	5β-3β-ol (coprostanol)	0.86‡	0.86	0.53‡	0.53	0.89‡	0.89	0.97‡	0.97	388	C_8H_{17}	0	0	1
C	24α-methyl-5β-3β-ol	1.15§	1.14	0.71§	0.71	1.19§	1.23	1.29§	1.31	402	C_9H_{19}	0	0	1
D	24β-ethyl 5β-3β-ol	1.45§	1.44	0.91§	0.90	1.53§	1.55	1.67§	1.65	416	$C_{10}H_{21}$	0	0	1
F1 = B	Δ^5-3β-ol (cholesterol)	1.00‡	1.00	0.70‡	0.71	1.00‡	1.00	1.26‡	1.26	386	C_8H_{17}	1	0	1
F2 = J	24α-methyl-Δ^5-3β-ol	1.34‡	1.34	0.94‡	0.93	1.33‡	1.37	1.73‡	1.67	414	C_9H_{19}	1	0	1
F3	24β-ethyl-$\Delta^{5,22}$-3β-ol	1.40†	1.40	0.97†	0.97	1.44†	1.45	1.88†	1.88	412	$C_{10}H_{19}$	1	1	1
F4 = E	24β-ethylΔ^5-3β-ol	1.69‡	1.67	1.20‡	1.20	1.72‡	1.73	2.17‡	2.15	400	$C_{10}H_{21}$	1	0	1
I	5α-3β-ol (cholestanol)	1.08‡	1.08	0.77‡	0.76	1.00‡	1.00	1.26‡	1.24	388	C_8H_{17}	0	0	1
L	24α-methyl-5α-3β-ol	1.45§	1.44	1.03§	0.99	1.33§	1.37	1.73§	1.67	402	C_9H_{19}	0	0	1
K	24β-ethyl-5α-3β-ol	1.83§	1.82	1.30§	1.28	1.72§	1.73	2.18§	2.15	416	$C_{10}H_{22}$	0	0	1

* All notations refer to specific structural features of cholestane.
† Trimethylsilyl ethers of 3-hydroxysterols and 1,1′-dimethylhydrazones of 3-ketosteroids.
‡ Values obtained for reference compounds.
§ Calculated on the basis of retention time relationships of analogous reference compounds.

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column temperature, 250°; carrier gas, helium. The gas flow into the column was about 8 ml/min. The carrier gas was partially eliminated from the effluent by means of molecular filters, giving a flow rate of 0.1–0.2 ml/min at the entrance to the ion source. The accelerating voltage was kept at 3000 v and the electrons for molecule bombarding have an energy of 20 ev. The rapid scanning procedure used was slightly modified from that previously described by Ryhage (22). For further details on the construction see Ryhage (16).

Biological extracts were dissolved in acetone and 1–3 μl of the solution, corresponding to 20–30 μg, were injected into the GC–MS instrument. Detailed mass spectra were obtained even when only 1–2 μg of the individual steroids were analyzed.

RESULTS

Preliminary studies showed that the gas–liquid chromatograms of neutral lipid fractions from both food and feces had many peaks, the retention times of which were different from those of reference compounds available to us. These unknown substances could not be isolated in a classical manner, but by extensive gas chromatography, alone and in combination with mass spectrometry, it has been possible to identify the main steroids observed.

Qualitative Analyses of Food Sterols

According to GLC analyses only one sterol (F1) was present in butter fat diets, whereas four (F1–F4) were found in the corn oil-containing diets. The retention times of these compounds are given in Table 2. The gas chromatogram reproduced in Fig. 1 was obtained when a purified extract of a portion of the diet containing corn oil was analyzed with the GC–MS instrument. The chromatographic separation was not as effective with this compound instrument but this did not interfere with the interpretation of the mass spectrometric data.

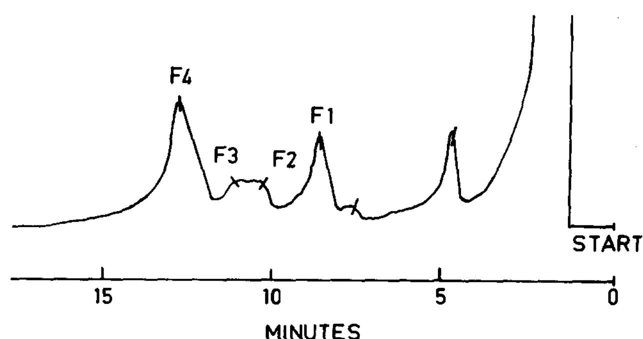

Fig. 1. Gas–liquid chromatogram of trimethylsilyl (TMS) ethers of sterols from a diet containing corn oil, analyzed with a gas chromatograph–mass spectrometer instrument. Subsequent identifications were made as follows: F1, cholesteryl TMS ether; F2, methylcholesteryl TMS ether; F3, stigmasteryl TMS ether, and F4, β-sitosteryl TMS ether. The compound at 5 min was not a steroid. Conditions, glass column; 1% SE-30 on 100–120 mesh Gas Chrom P; 250°; inlet helium flow 8 ml/min.

Compound F1

Gas chromatographic and mass spectrometric data for F1 were identical with those found for cholesterol. The mass spectrum recorded for the trimethylsilyl ether of compound F1 is shown in Fig. 2. Peaks are found at m/e 458 [molecular ion, (M)], 368 [M − 90 (trimethylsilanol)], 353 [M − (15 + 90)], 329 (M − 129), 255 [M − (side chain +90)], and 129. The last mentioned peak is the base peak in this and in all other mass spectra obtained from 3-trimethylsilyl ethers of Δ^5-sterols. The fragment at m/e 129 most probably contains the trimethylsilyl group, the carbon atoms from positions 2, 3, and 4, and four hydrogen atoms. Essentially the same cracking pattern was obtained for cholesteryl TMS ether when analyzed in a 180° instrument (Fig. 3) with a temperature in the heated gas inlet of 210°. The slight differences observed were probably due to the different experimental conditions used. Thus, it was noticed that variations in the column temperature of the GC–MS instrument did not alter the specific fragmentation but in some instances changed the relative intensity of many peaks.

Compound F2

The GLC behavior of F2 was not identical with any of those from available reference compounds. It is obvious from the chromatogram (Fig. 1) that the TMS ethers of F1 and F2 were not completely separated with the GC–MS instrument. This explains why fragments of cholesteryl TMS ether are found in the mass spectrum recorded for the TMS ether of compound F2 (Fig. 4). In addition, peaks of high intensities are seen at m/e 382 (M − 90), 367, 343, and that of the parent ion at m/e 472. These peaks indicate a structure which differs from that of cholesteryl TMS ether by addition of 14 mass units. This addition could be caused either by an elongation or a branching of the side chain; the mass spectra do not distinguish between the possibilities. The hydroxyl group in compound F2 was most probably attached to carbon 2 or 3, since the base peak was found at m/e 129. Digitonide precipitation according to the method of Sperry (23) with subsequent splitting of the precipitate as recommended by Issidorides et al. (24) strengthened the arguments for the 3β configuration of the hydroxyl group. To our knowledge the only sterol described that has a structure compatible with these data is campesterol (24α-methylcholesterol) (25, 26).

Compound F3

This compound was present in smaller amounts than the other sterols. Its retention times as well as the mass

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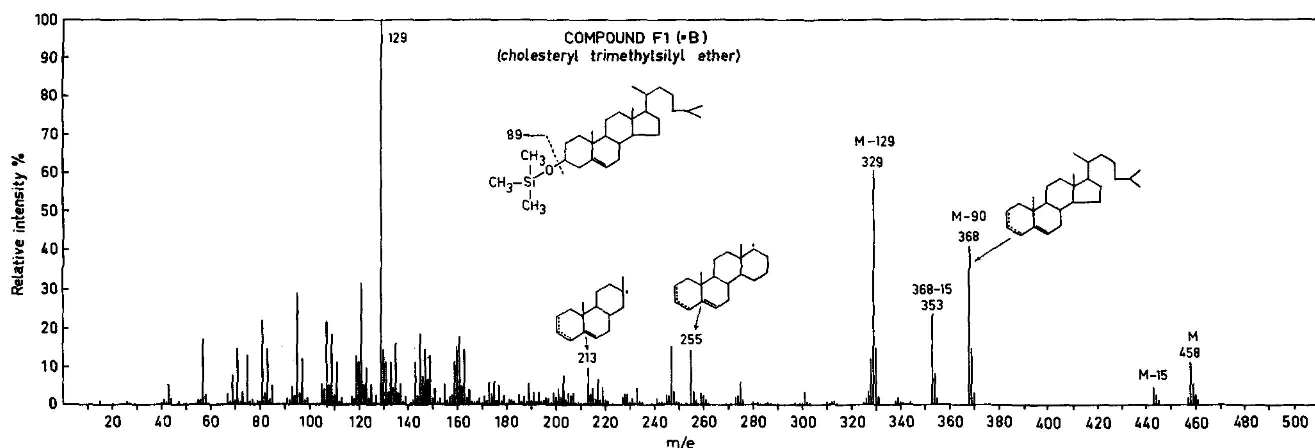

FIG. 2. Mass spectrum of the TMS ether of compound F1 (Fig. 1), subsequently identified as cholesteryl TMS ether. Conditions as in Fig. 1.

spectrum of its TMS ether were the same as those found for stigmasterol when run under the same conditions. In the mass spectrum of the TMS ether of compound F3 characteristic peaks were found at m/e 484 (parent peak), 394, 379, 355, 255, and 129. This cracking pattern was found to be in good agreement with that described by Fitche (27) for stigmasteryl acetate provided that pertinent corrections are made for the TMS group.

Compound F4

On the basis of GLC data this substance was identified as β-sitosterol (Table 2). The mass spectrum of its TMS ether is shown in Fig. 5. This spectrum has a characteristic peak in common with those shown in Figs. 2–4 (i.e. m/e 255, representing the nuclear fragment) whereas the fragments in the high mass range (> m/e 300) were increased by 28 mass units as compared with cholesteryl TMS ether. It was concluded that compound F4 was identical with cholesterol except for the side chain which contained the two extra carbons, most probably as an ethyl group. Furthermore, mass spectra obtained of β-sitosteryl TMS ether under the same conditions were identical with that shown in Fig. 5.

QUALITATIVE ANALYSES OF NEUTRAL FECAL STEROIDS

To determine whether labile compounds were destroyed during the extraction and saponification procedures, the following experiment was performed. Two fecal portions were homogenized in chloroform–methanol 1:1 (v/v). From each, four test samples were taken for further analysis. Two of the samples were extracted three times with chloroform–methanol at room temperature (group 1) and the other two in the Soxhlet extractor for 48 hr at reflux temperature (group 2). One sample from each group was then worked up according to the standard procedure whereas the other two

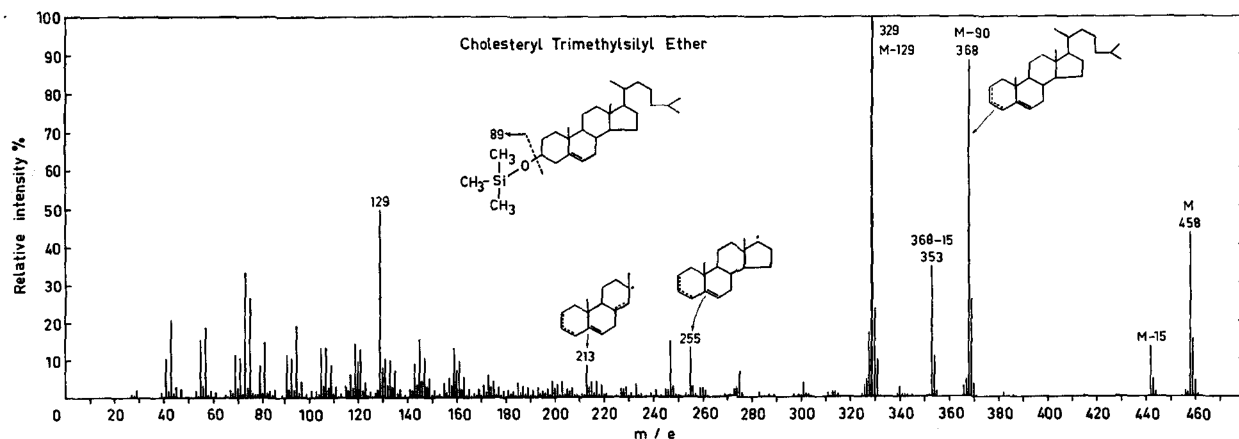

FIG. 3. Mass spectrum of cholesteryl TMS ether, analyzed with a 180° instrument. Temperature in the gas inlet system 210°.

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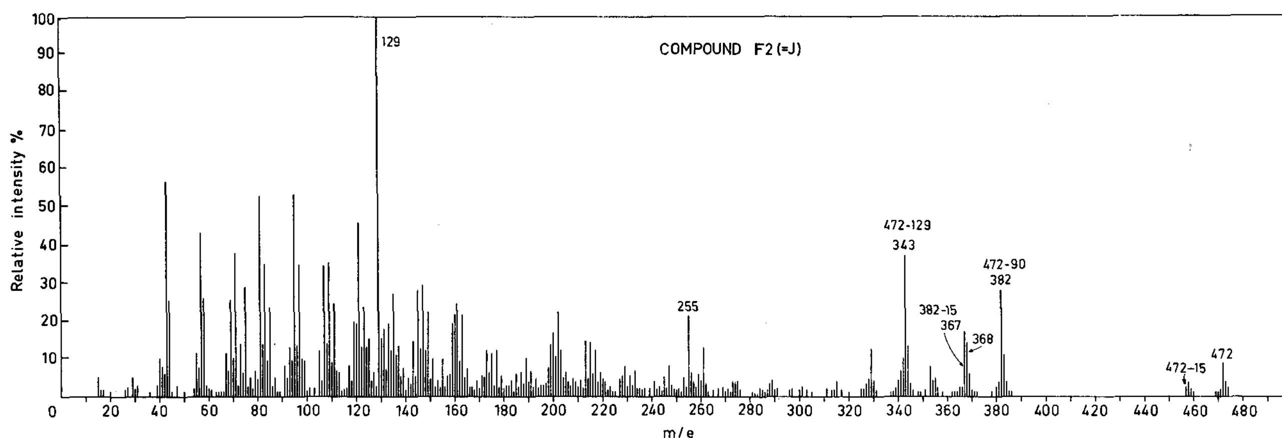

Fig. 4. Mass spectra of the incompletely separated TMS ethers of compounds F1 and F2 (Fig. 1), subsequently identified as cholesteryl TMS ether and methylcholesteryl TMS ether respectively. Conditions as in Fig. 1.

were dissolved in petroleum ether and washed with alkaline 50% ethanol in water. Exactly the same GLC pattern was obtained for the four samples of each portion.

Figure 6 shows a gas–liquid chromatogram of the fecal steroids from a subject on the corn oil diet. Eight peaks (A–H) are seen. By comparison with reference compounds, compound A was found to have retention times identical with those of coprostanol (Table 2). In the same manner B, E, and F were tentatively identified as cholesterol, β-sitosterol, and coprostanone respectively. The other compounds did not have retention times identical with those of any known cholesterol metabolite. To study the nature of the unknown compounds (C, D, G, and H), fractionation on aluminum oxide (Woelm, grade I) was performed. For 2 g of extract 200 g of adsorbent were used. The column was continuously eluted with benzene and the effluent was monitored by GLC. Three main fractions were collected. Fraction I contained steroid ketones, fraction II saturated sterols, and fraction III a mixture of saturated and unsaturated sterols.

GLC Analysis of Fraction I (Fig. 7)

The resemblance of compounds G and H to coprostanone in chromatographic behavior before as well as after treatment with 1,1′-dimethylhydrazine (Table 2) suggested that they might be the corresponding ketones of the dietary plant sterols. Identification of G and H by comparison with reference compounds was not possible since such substances were not available. However, knowing the retention times for cholesterol, methylcholesterol, β-sitosterol, and coprostanone, theoretical values for the retention times of methylcoprostanone and 24β-ethylcoprostanone could be calculated. The calculations applied were based on the experimental findings by several investigators that the gas chromatographic

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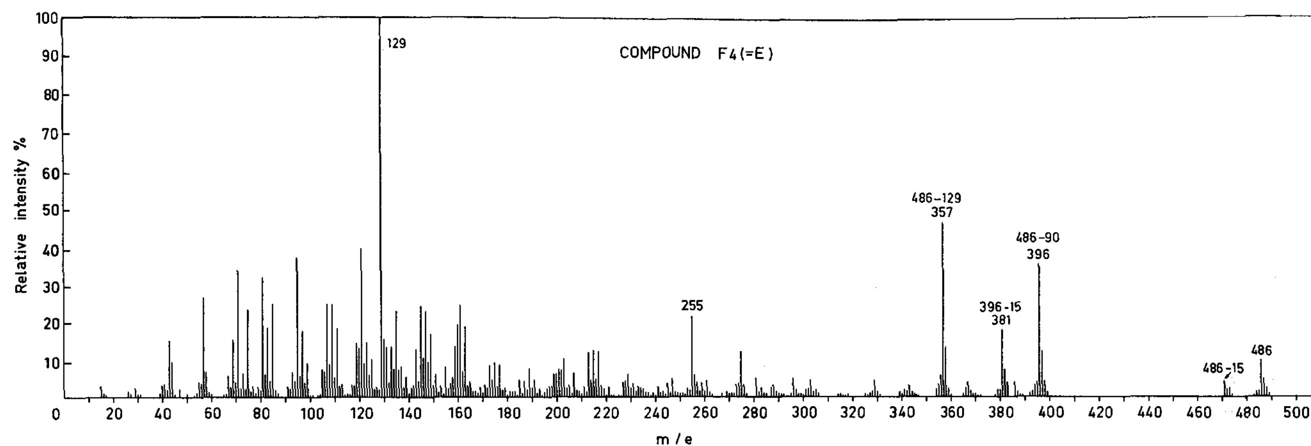

Fig. 5. Mass spectrum of the TMS ether of compound F4 (Fig. 1), subsequently identified as β-sitosteryl TMS ether. Conditions as in Fig. 1.

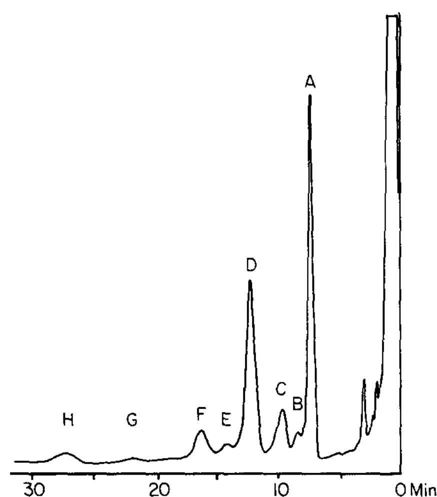

FIG. 6. Gas–liquid chromatogram of neutral steroids in a fecal sample from subject 2. Dietary fat, corn oil. Subsequent identifications were made as follows: A, coprostanol; B, cholesterol; C, methylcoprostanol; D, 24β-ethylcoprostanol; E, β-sitosterol; F, coprostanone; G, methylcoprostanone; and H, 24β-ethylcoprostanone. Conditions, 2 m x 5 mm glass column; 0.5% QF-1 on 100–140 mesh Gas Chrom P; 200°; inlet pressure 1.0 kg/cm².

behavior of most steroids is directly related to the number and nature of their functional groups (for a recent review see reference 20). Assuming that the interaction between alkyl substituents in the side chain and different functional group(s) in the steroid nucleus was negligible, the retention time for a hypothetical plant sterol metabolite could be calculated by analogy. As an example the retention time of 24β-ethylcoprostanone was calculated in the following way:

$$\frac{\text{Ret. time of cholest-5-en-3}\beta\text{-ol}}{\text{Ret. time of 5}\beta\text{-cholestan-3-one}} = \frac{\text{Ret. time of 24}\beta\text{-ethylcholest-5-en-3}\beta\text{-ol}}{\text{Ret. time of 24}\beta\text{-ethyl-5}\beta\text{-cholestan-3-one}}$$

The "theoretical" values are in close agreement with those observed for compounds G and H (Table 2).

GC–MS Analysis of Fraction I

The high mass end of the mass spectrum of compound F is shown in Fig. 8. In addition to the molecular ion at m/e 386 the following characteristic fragments are found : m/e 371 (M − 15), 353 [M − (15 + 18)], 316 (M − 70), 255 [M − (side chain + 18)], 231 [M − (side chain + ring D)], and 213 (231 − 18). The same cracking pattern was obtained for 5β-cholestan-3-one by Budzkiewicz and Djerassi (28), who also found that the formation of the ion M − 70 is characteristic for the fragmentation of steroids of the 5β-series with a keto group attached to carbon atoms 2 or 3. Except for the fragments containing the side chain essentially the same cracking pattern was obtained when compounds G and H were run in the GC–MS instrument (Fig. 8). The parent ions of these compounds, most probably the molecular ions, were found at m/e 400 and 414 respectively. In addition, peaks of high intensities (interpreted as M − 70) were recorded at m/e 330 (G) and 344 (H). Thus compounds G and H have the same nucleus as coprostanone but different side chains, and the mass spectrometric data for these steroids confirm the structures proposed on the basis of GLC analyses.

GLC Analysis of Fraction II

Compounds A, C, and D from the original petroleum ether extract (Fig. 6) appeared in this fraction (Fig. 9). Trimethylsilyl ether derivatives of these compounds were prepared and their gas chromatographic behavior, as well as that of their parent substances, was found to be in good agreement with those found or calculated for coprostanol (A), methylcoprostanol (C), and 24β-ethylcoprostanol (D) (Table 2).

GC–MS Analyses of Fraction II

The TMS ether of compound A (Fig. 9) gave a mass spectrum (Fig. 10) quite similar to that obtained for coprostanyl TMS ether. In the high mass range the peak at m/e 370 (M − 90) had the highest intensity. When compared with the cracking pattern of cholesteryl TMS ether (Fig. 2) all characteristic fragments containing the nucleus were increased by two mass units. Mass spectra of compounds C and D run as TMS derivatives contained the same nuclear ion as that of coprostanyl TMS ether (peaks at m/e 257), but different parent ions (peaks at m/e 474 and 488 respectively) were recorded.

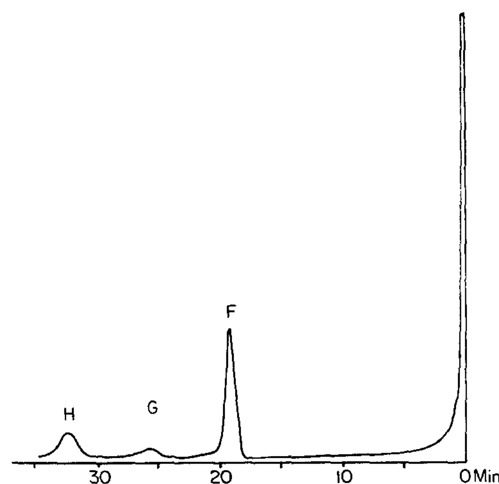

FIG. 7. Gas–liquid chromatogram of fraction I obtained by aluminum oxide chromatography of the extract shown in Fig. 6. Subsequent identifications were made as follows: F, coprostanone; G, methylcoprostanone; and H, 24β-ethylcoprostanone. Conditions as in Fig. 6.

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The side chain-containing fragments of strong intensities in the high mass range of these spectra differed from the corresponding peaks in the coprostanyl TMS ether spectrum by 14 and 28 mass units respectively (Fig. 10). Because of inadequate GLC separation of the TMS ethers, fragments from compound A contaminated the mass spectrum of compound C to a certain extent. However, this did not interfere with the reading of the specific features of the fragmentation pattern of compound C.

Additional Experiments with Fraction II

According to the GLC data, compounds C and D should belong to the 5β-series (Table 2). To study the nature of the A/B junction in more detail fraction II was oxidized according to the method of Djerassi et al. (29). GLC analyses of the reaction products revealed the presence of three compounds. The GLC and mass spectrometric data for these were identical with those described above for the keto steroids F, G, and H respectively, indicating that the starting materials (compounds A, C, and D) belonged to the same (5β) series.

Analyses of Fraction III

In addition to small amounts of the above-mentioned coprostanol analogues this fraction contained five compounds, B, E, I, J, and K (Fig. 11). B and E were already tentatively identified from the analyses of the original extract as cholesterol and β-sitosterol (Fig. 6).

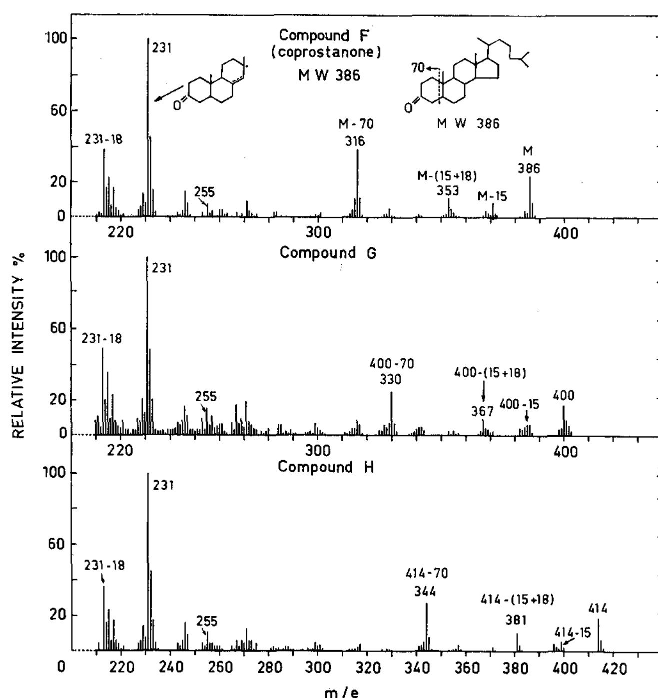

FIG. 8. High mass ends of mass spectra of compounds F, G, and H (Figs. 6 and 7). Conditions as in Fig. 1.

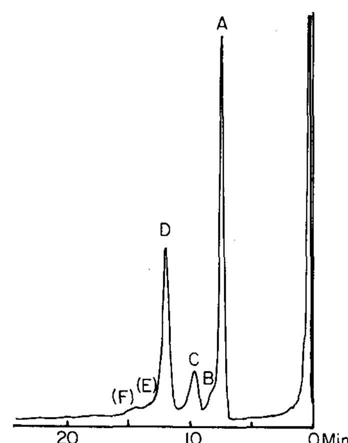

FIG. 9. Gas–liquid chromatogram of fraction II obtained by aluminum oxide chromatography of the extract shown in Fig. 6. Identifications were made as follows: A, coprostanol; B, cholesterol; C, methylcoprostanol; D, 24β-ethylcoprostanol; E, β-sitosterol; and F, coprostanone. Conditions as in Fig. 6.

The GLC behavior of compounds J and I was identical with that of methylcholesterol and cholestanol respectively. On the basis of retention time calculations as applied above, it seemed possible that compound K was 24β-ethylcholestanol (Fig. 11, Table 2).

It was not possible to analyze this complex mixture in the GC–MS instrument since no separation between compounds B and I or between E and K was obtained with the stationary phase used (SE-30). However, mass spectrometric analyses of fecal extracts containing fewer neutral steroids yielded spectra for compounds with the same retention times as B, J, and E (shown in Fig. 11) which were identical with those of cholesterol, methylcholesterol and β-sitosterol respectively (see Figs. 2, 4, and 5).

Additional Experiments with Fraction III

In order to confirm the presence of sterols with the 5α-cholestan-3β-ol nucleus, fraction III was oxidized with performic acid essentially as described by Mosbach et al. (30). The compounds thus obtained were saponified and the steroids recovered in the usual way. Since only saturated sterols should remain unchanged after the oxidation one would expect to find only compounds with 5α- or 5β-cholestane structure in this sample. A gas–liquid chromatogram of this fraction is shown in Fig. 12, in which four main peaks are seen (A, I, D, and K). The retention times of these are in close agreement with those found or calculated for coprostanol, cholestanol, 24β-ethylcoprostanol, and 24β-ethylcholestanol.

Because of the apparent similarity in the metabolism of cholesterol, methylcholesterol, and 24β-ethylcholesterol, the occurrence of methylcholestanol should be anticipated. The calculated retention time for this

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compound (Table 2) is almost the same as that found for 24β-ethylcoprostanol and a peak representing methylcholestanol (L) would be masked by peak D (Fig. 12). Analysis with the GC–MS instrument (Fig. 13) verified the existence of methylcholestanol. In the high mass end of a spectrum of the mixture of TMS ethers from compounds D and L, ions tentatively identified as M − 90 and M − (90 + 15) were recorded at m/e 398 and 383 (24β-ethylcoprostanyl TMS ether) and at m/e 384 and 369 (methylcholestanyl TMS ether).

According to mass spectrometric analyses compound K seemed to be pure (Fig. 13). As could be expected for

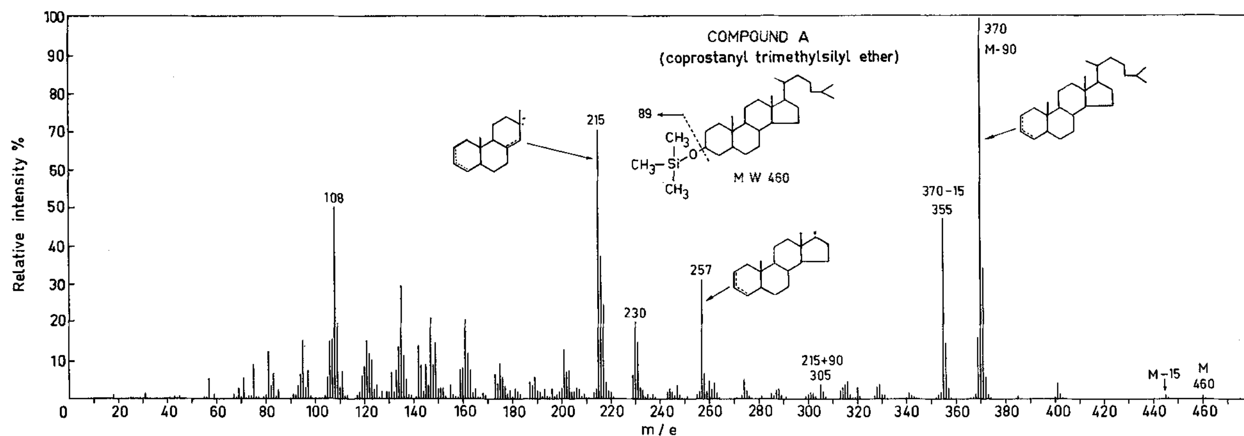

FIG. 10a

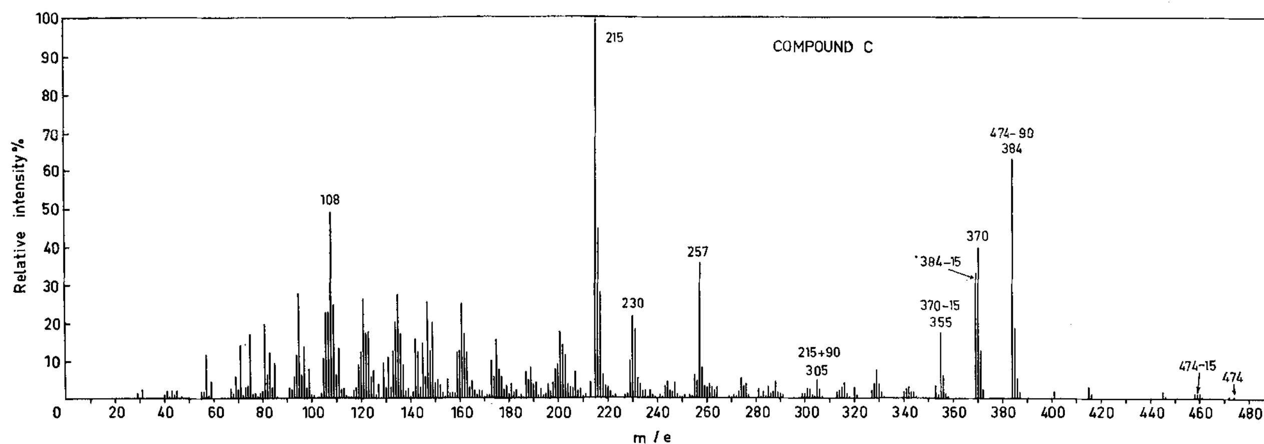

FIG. 10b

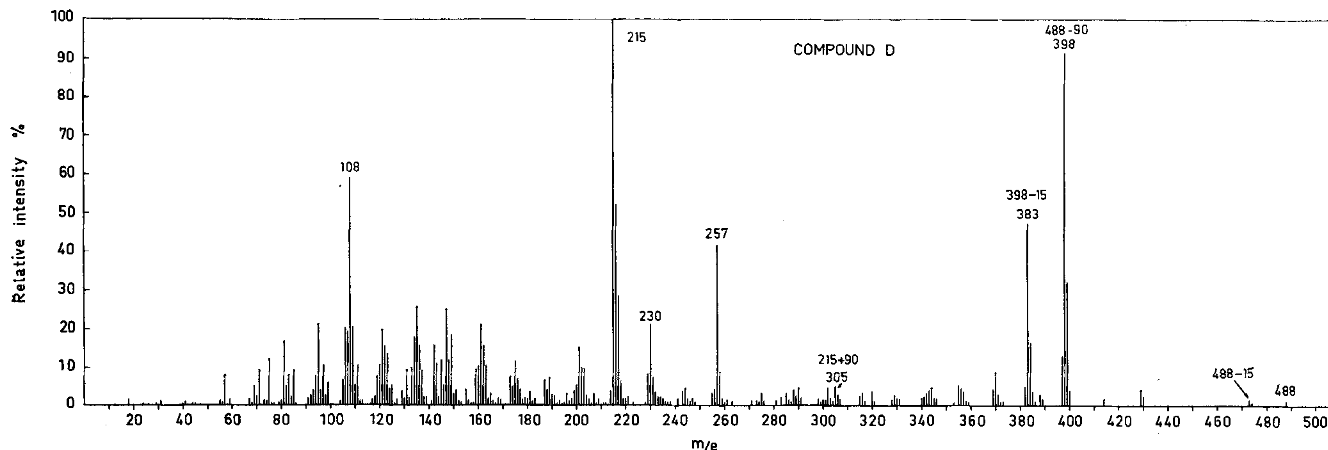

FIG. 10c

FIG. 10. Mass spectra of TMS ethers of compounds shown in Fig. 9. Conditions as in Fig. 1.

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a 24β-ethylcholestanyl TMS ether, fragments were found at m/e 398 (M − 90), 383 [M − (90 + 15)]. The mass spectra recorded for compounds A and I were identical with those of coprostanol and cholestanol.

The above-mentioned mixture of 5α- and 5β-steroid alcohols was subsequently oxidized according to the method of Djerassi et al. (29). A gas–liquid chromatogram of the steroid mixture thus obtained is shown in Fig. 14. Six peaks are seen: F, M, G, N, H, and O. With the column used (4% QF-1) compounds F, M, G, and H had the same retention times as coprostanone, cholestanone, methylcoprostanone, and 24β-ethylcoprostanone respectively. The ratio between the retention times for reference samples of cholestanone and coprostanone was the same (1.09) as that obtained for compounds M and F, N and G, and O and H. Furthermore, mass spectrometry of compounds M, N, and O failed to reveal a significant M−70 peak characteristic of the ketones of the 5β-series, indicating that they (as well as their corresponding parent substances I, L, and K) belonged to the 5α-series (28).

In Fig. 12 a small peak (U) appears at the place of cholesterol. This could represent cholesterol, epicholesterol (if the oxidation had been incomplete), or epicoprostanol, all of which have the retention time 1.00 on QF-1. When this material was run on SE-30 four main peaks with retention times 0.89, 1.00, 1.54, and 1.73 were seen. The retention time of epicoprostanol on this phase is 0.90 (i.e. almost identical with coprostanol) and those of epicholesterol and cholestanol 0.94 and 1.00 respectively. Mass spectrometric analyses of the compound(s) from the performic-oxidized extract

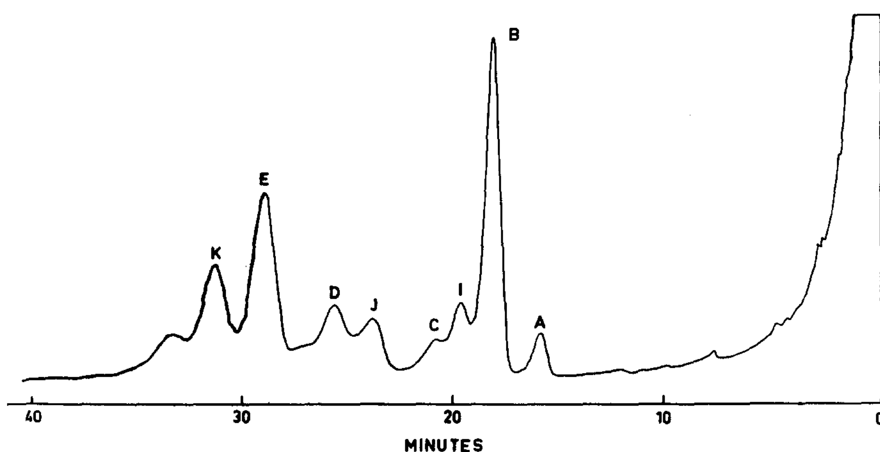

Fig. 11. Gas–liquid chromatogram of fraction III obtained by aluminum oxide chromatography of the extract shown in Fig. 6. A, coprostanol; B, cholesterol; I, cholestanol; C, methylcoprostanol; J, methylcholesterol; D, 24β-ethylcoprostanol and methylcholestanol; E, β-sitosterol; K, 24β-ethylcholestanol. Conditions, 2 m x 5 mm glass column; 3% QF-1 on 100–140 mesh Gas Chrom P; 210°; 1.2 kg/cm².

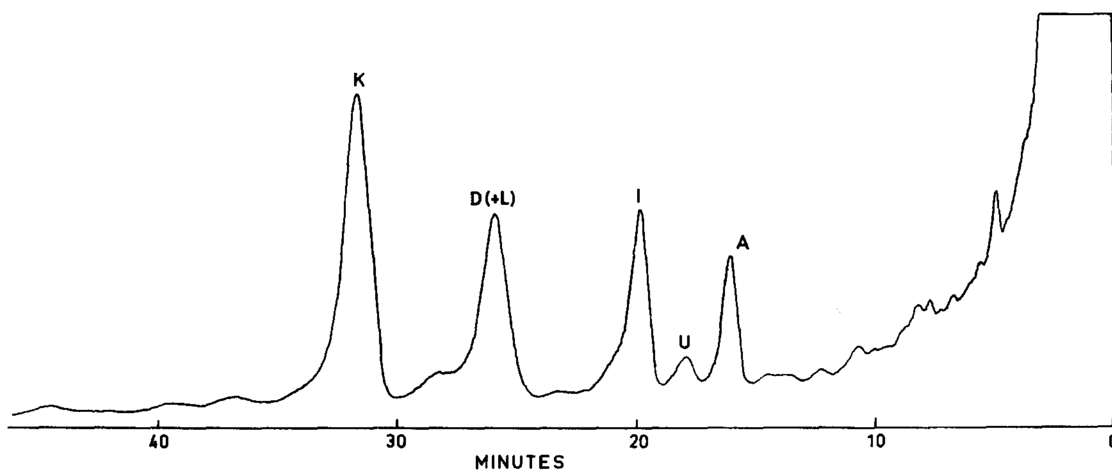

Fig. 12. Gas–liquid chromatogram of sterols remaining after performic acid oxidation of the fraction shown in Fig. 11. Subsequent identifications were made as follows: A, coprostanol; U, probably epicoprostanol; I, cholestanol; D (+L), 24β-ethylcoprostanol (+ methylcholestanol); and K, 24β-ethylcholestanol. Conditions as in Fig. 11.

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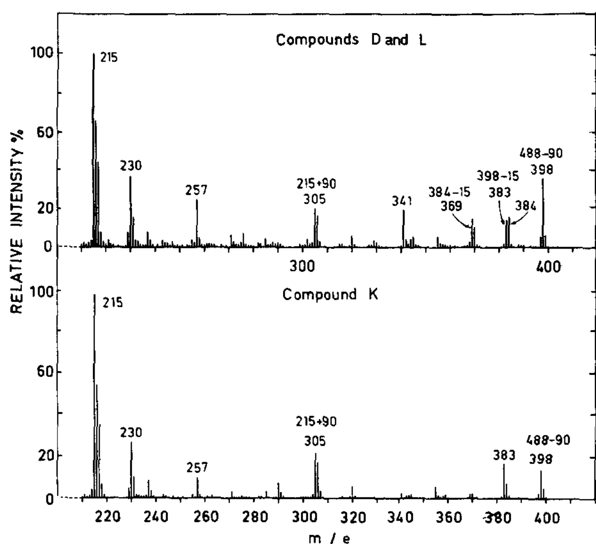

Fig. 13. High mass ends of mass spectra of the TMS ether of compound K (24β-ethylcholestanol) and the mixed spectra of compounds D + L (24β-ethylcoprostanol + methylcholestanol). Conditions as in Fig. 1.

with the retention time 1.00 on SE-30 did not reveal any signs of cholesterol or epicholesterol. This indicated that the peak U in Fig. 12 represented epicoprostanol. The direct demonstration of epicoprostanol in the coprostanol peak on SE-30 cannot be made by mass spectrometry.

In summary, the findings indicate that cholesterol and the two main plant sterols, methylcholesterol and β-sitosterol, undergo similar molecular transformations in the intestinal lumen. As is obvious from Fig. 6 the coprostanol analogues are the predominant metabolites and the corresponding 3-keto derivatives and the unsaturated sterols are less important quantitatively. Peaks corresponding to the 5α-cholestan-3β-ol nuclear structure were not observed in the "primary" gas chromatograms (e.g. Fig. 6) and the amount of these sterols was negligible compared with the total amount of neutral steroids present in feces.

Quantitative Analysis

Extraction and Saponification

The reliability of the standard procedure for quantitative analysis was tested in the following ways:

(*a*) The recovery of cholesterol added to fecal homogenates prior to extraction in the Soxhlet apparatus was examined with the aid of cholesterol-C^{14}, which was completely recovered in the petroleum ether extracts in two separate experiments.

(*b*) Cholesteryl acetate was heated at 60° for 1 hr in an atmosphere of nitrogen with the same amount of KOH as was used for biological extracts. According to GLC data complete hydrolysis occurred.

(*c*) Two equal samples were taken from the chloroform–methanol extract of three different fecal portions. One sample from each portion was treated under nitrogen at 60° as described for cholesteryl acetate, while the other was hydrolyzed at reflux temperature without nitrogen. After extraction of the steroids with petroleum ether all samples were analyzed quantitatively by means of GLC. The results are presented in Table 3.

GLC Response; Peak Area Determination

All calculations of peak areas were made by triangulation. In spite of a slight tailing of some steroids all compounds studied gave a linear response (Fig. 15) within the ranges used for determination of biological samples. Under the conditions used in this investigation (0.5% QF-1) the response relative to cholesterol was found to be 1.03, 0.98, 0.95, and 0.90 for cholestanone, cholestanol, coprostanone, and coprostanol respectively.

In some analyses there was a slight overlapping between the peaks of cholesterol and coprostanol. Model experiments with known mixtures of these compounds showed that the triangulated area of the cholesterol peak could be overestimated by 10–40% if the same baseline was used as that drawn for the larger coprostanol peak. It was found that this error could be satisfactorily corrected for, as long as the coprostanol/cholesterol ratio did not exceed 7:1, by measuring the area as illustrated in Fig. 16. This ratio was rarely exceeded in biological

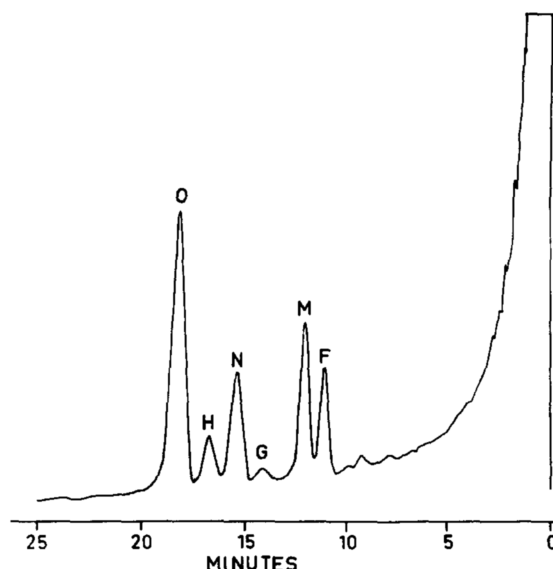

Fig. 14. Gas–liquid chromatogram of steroid ketones obtained after chromic acid oxidation of the fraction shown in Fig. 12. Subsequent identifications were made as follows: F, coprostanone; M, cholestanone; G, methylcoprostanone; N, methylcholestanone; H, 24β-ethylcoprostanone; and 0, 24β-ethyl-cholestanone. Conditions: 2 m x 5 mm glass column; 4% QF-1 on 100–140 mesh Gas Chrom P; 230°; 1.5 kg/cm².

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samples. Two "baselines" were drawn for the cholesterol peak so that two triangles ABC and ABD were obtained. The mean of their areas was used to calculate the amount of cholesterol. As shown in Table 4 the errors involved in these calculations, when evaluated by recovery studies, did not result in a less accurate quantification of cholesterol than of coprostanol. Data demonstrating the reproducibility of peak area measurements are shown in Table 4.

Pure reference samples of β-sitosterol and methylcholesterol were not available and the amounts of these compounds were read from the cholesterol standard curve. The accuracy of these determinations is difficult to evaluate. However, the sum of the peak areas obtained for crystalline commercial β-sitosterol (containing 30% methylcholesterol) equaled the peak area (±5%) obtained for corresponding amounts of cholesterol. Coprostanol and coprostanone were used as standards for the corresponding plant sterol metabolites. Excluding 24β-ethylcoprostanol the amounts of plant sterols and plant sterol metabolites in feces were small and the values for these compounds, given in Table 5, are therefore subject to greater methodological errors than is the case with cholesterol and its metabolites.

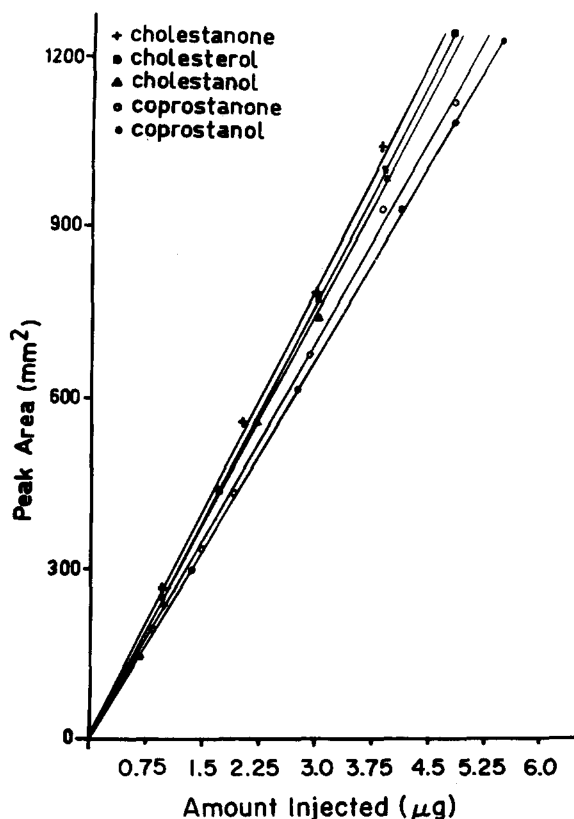

FIG. 15. Relationship of varying amounts of steroids to area recorded. Column and conditions as in Fig. 6.

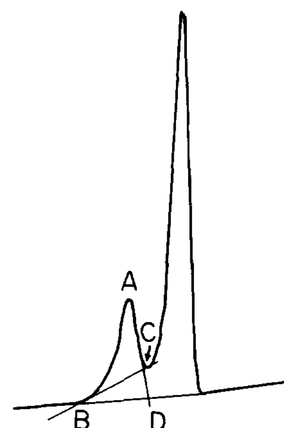

FIG. 16. Construction of baselines where peaks of cholesterol and coprostanol overlap.

Quantitative Determination of Dietary Sterols and Fatty Acids

The butter and corn oil diets differed in sterol content as well as in fatty acid composition. The dishes supplemented with butter contained more cholesterol but no significant amounts of plant sterols. The daily sterol intakes from the standard dishes are given in Table 6. Unfortunately, no representative samples of the fruits and bread were saved, so that figures are not available for the total plant sterol intake. The fecal methylcholesterol and β-sitosterol encountered during the butter period (see below) probably derive from the bread, fruit, and marmalade consumed.

The fatty acid composition was similar in dishes supplemented with the same type of fat. The amount of each fatty acid was determined by GLC as the percentage of the total amount of even numbered C_{10}–C_{20} fatty acids. The following mean values for capric, lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, arachidic, and arachidonic acids were found: butter diets: 2.8, 4.0, 14.5, 39.3, 2.7, 12.1, 19.9, 3.0, —, —; corn oil diets: —, —, 1.4, 25.8, 3.8, 6.9, 34.8, 21.4, 0.6, 3.3.

Quantitative Determination of Neutral Fecal Steroids

Data summarizing the results of the quantitative analyses are given in Tables 5 and 6. The analyses were made

TABLE 3 COMPARISON BETWEEN TWO DIFFERENT PROCEDURES FOR SAPONIFICATION OF FECAL EXTRACTS

Extract	Coprostanol		Cholesterol		Coprostanone	
	Under Reflux	60° under N_2	Under Reflux	60° under N_2	Under Reflux	60° under N_2
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
1	5.23	5.19	0.87	0.89	3.31	3.25
2	7.88	8.02	0.80	0.78	1.34	1.32
3	36.6	35.0	4.50	4.25	9.10	9.25

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TABLE 4 Recovery of Cholesterol, Coprostanol, and Coprostanone Added to Human Fecal Extract

	Coprostanol		Recovery of Added Compound	Cholesterol		Recovery of Added Compound	Coprostanone		Recovery of Added Compound
	Calculated	Found		Calculated	Found		Calculated	Found	
	mg	*mg*	%	*mg*	*mg*	%	*mg*	*mg*	%
Sample 1 (S1)	—	1.76	—	—	0.18	—	—	0.45	—
S1 + 0.15 mg cholesterol	—	1.78	—	0.33	0.34	107	—	0.49	—
S1 + 0.29 mg cholesterol	—	1.76	—	0.47	0.46	97	—	0.43	—
S1 + 0.64 mg cholesterol	—	1.79	—	0.82	0.86	106	—	0.48	—
S1 + 1.02 mg cholesterol	—	1.68	—	1.20	1.19	99	—	0.50	—
S1 + 1.46 mg cholesterol	—	1.81	—	1.64	1.70	104	—	0.50	—
Sample 2 (S2)	—	0.74	—	—	0.40	—	—	0.44	—
S2 + 0.23 mg coprostanol 0.50 mg coprostanone	0.97	0.98	104	—	0.41	—	0.94	0.90	92
S2 + 0.45 mg coprostanol + 0.25 mg coprostanone	1.19	1.23	109	—	0.42	—	0.69	0.64	80
S2 + 0.50 mg coprostanol + 0.40 mg coprostanone	1.24	1.23	98	—	0.38	—	0.84	0.80	90

either of single or of pooled fecal portions. Every subject showed a considerable irregularity in the frequency of the defecations and one fecal portion often had to be considered equivalent to the feces produced during 1–5 days. In consequence the steroid content differed makedly in the different fractions analyzed and a wide range for the calculated daily excretion was found.

With one exception (Subject 3), coprostanol was the main neutral steroid regardless of type of diet consumed. The mean ratios between coprostanol, cholesterol, and coprostanone were found to be approximately 15:3:1 for the normal subjects on the butter fat diet. When the butter was replaced by corn oil the excretion of coprostanone and/or cholesterol increased and the above-mentioned ratio was changed to about 7:2:1. The relative proportions between the plant sterols and their respective metabolites in feces were similar to those found for cholesterol and its metabolites.

The total excretion of neutral steroids was higher on the corn oil than on the butter fat diet. Although much of the difference was due to the presence of the large amount of plant sterols in the corn oil, four out of the six normal subjects excreted more cholesterol and neutral cholesterol metabolites during the corn oil period.

In all the three subjects from whom it was possible to collect feces without interruption an elevated excretion of cholesterol and its metabolites was observed after the change of diet. In one subject this increase was pronounced during the first week on the corn oil diet. However, it occurred gradually over the course of the dietary period in the other two, and for this reason it was considered justified to include the results of subjects 4–7, for whom feces collection had been interrupted.

The serum cholesterol was lower in all subjects on the corn oil regimen. This change was established within the first 2 weeks.

DISCUSSION

It is apparent from the previous section that GLC permits tentative identification of unknown compounds present in unpurified biological extracts even when only a limited number of reference substances is available. An indispensable prerequisite is the use of different stationary phases for the analyses of pertinent derivatives of the compounds. Once the fecal neutral steroids had been identified, quantitative studies could be performed. For this purpose QF-1 was chosen as stationary phase, since it permits determination of keto steroids in the presence of corresponding hydroxyl compounds.

Somewhat conflicting results have been reported on the specific GLC response (defined as peak area/microgram of compound injected) of cholesterol and coprostanol. Thus Rosenfeld et al. (14) found a higher response for coprostanol than for cholesterol with an ionization detection system connected to a SE-30 column, whereas Wilson (13) reported that the reverse is true. Since it has been shown that oxygenated steroids might be adsorbed and/or destroyed on the columns, it seems likely that the discrepancies mentioned might be attributable to differences in column preparation, retention times (i.e. column temperatures), carrier gas contaminants, etc. The type of detector and operating conditions are also of importance for the specific response (31). From the work of Simmonds and Lovelock (32) with an absolute detection system, it is apparent that with SE-30 and QF-1 as stationary phases a higher specific response should be obtained for coprostanol than for cholesterol. With our QF-1 columns we obtained a 10% lower response for coprostanol than for cholesterol. This unexpected result was found to be valid even after repeated checks on the purity of the coprostanol standard. Our data on coprostanol and coprostanone support the concept that steroid

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ketones give higher responses than corresponding hydroxyl compounds (12, 31, 32). This response difference was of the same order of magnitude for the 5α- and 5β-series.

Prior to the identification of the neutral fecal steroids it was essential to establish the nature of the dietary sterols. The corn oil diet used in this investigation contained four main sterols, among which cholesterol and β-sitosterol were predominant. Although our analytical approach did not permit us to draw conclusions about the configuration of the side chain, it appears reasonable to assume that the ethylcholesterol found is identical with β-sitosterol (24β-ethylcholest-5-en-3β-ol), a substance of widespread occurrence in the plant kingdom. The 24α-ethyl isomer is known to contaminate β-sitosterol only to a minor extent (33). The only known plant sterol with properties consistent with the GLC and mass spectrometric data obtained for the methylcholesterol si campesterol. This sterol was originally isolated from soybean oil (25) and was found to have the methyl group in the 24α-position. We have found a methylcholesterol in corn oil and in two commercial preparations of β-sitosterol. This is compatible with the finding of Thompson et al. (34), who recently prepared campesterol of 91% purity from a commercial preparation of β-sitosterol. The GLC analyses reported in that paper yielded a ratio between the retention times of β-sitosterol and campesterol of 1.24 on 0.5% QF-1 and 1.25 on 1% SE-30. Our corresponding values were 1.25 and 1.26 respectively. It is not known whether a 24β-methylcholesterol exists in nature and it is likely that the methylcholesterol encountered in this investigation has the 24α-methyl

TABLE 5 Excretion of Fecal Neutral Steroids (Mg/Day)

Subject	Dietary Fat*	Sampling Period†	Number of Samples	5β-3β-ol	5-en-3β-ol	5β-3-one	24α-CH_3-5β-3β-ol	24α-CH_3 5-en-3β-ol	24α-CH_3-5β-3-one	24β-C_2H_5-5β-3β-ol	24β-C_2H_5-5-en-3β-ol	24β-C_2H_5-5β-3-one
		days										
1	B	14–27	3	301 (359–220)	85 (118–54)	42 (55–27)	19 (26–7)	—	—	80 (168–30)	9 (20–0)	—
	C	1–29	5	340 (457–184)	98 (134–24)	53 (64–35)	53 (84–36)	—	—	166 (336–146)	74 (114–40)	10 (27–0)
2	B	14–32	3	352 (359–354)	45 (47–45)	20 (22–8)	10 (17–3)	—	—	66 (69–57)	10 (11–9)	—
	C	1–32	5	435 (537–317)	31 (43–18)	73 (86–57)	90 (106–73)	—	17 (20–12)	252 (279–164)	27 (38–10)	43 (51–13)
3	B	14–31	3	348 (539–347)	163 (229–136)	34 (62–20)	15 (19–0)	—	—	52 (70–34)	37 (60–31)	—
	C	1–25	4	228 (536–128)	468 (615–317)	29 (38–21)	27 (44–0)	48 (57–40)	—	89 (102–62)	219 (301–132)	—
4	B	14–26	3	350 (447–129)	19 (30–10)	16 (22–10)	26 (48–9)	—	—	44 (59–20)	8 (10–0)	—
	C	14–32	5	294 (424–160)	43 (97–19)	35 (51–25)	57 (93–52)	—	—	135 (198–131)	23 (46–13)	10 (15–0)
5	B	15–26	2	509 (644–376)	93 (165–55)	29 (39–20)	23 (31–15)	—	—	66 (85–46)	13 (18–8)	—
	C	16–24	5	666 (1305–235)	93 (176–37)	70 (170–20)	147 (242–59)	—	13 (25–0)	347 (462–201)	62 (152–24)	25 (37–0)
6	B	15–26	2	662 (704–620)	70 (71–68)	21 (25–17)	37 (37–36)	—	—	78 (81–75)	—	—
	C	14–31	5	539 (616–462)	76 (103–49)	118 (137–67)	66 (78–59)	—	21 (33–0)	238 (271–125)	15 (23–9)	38 (43–6)
Mean	B			420	79	27	22	—	—	97	13	—
	C			417	135	63	73	—	—	205	70	29
7	B	14–23	6	383 (633–187)	50 (86–33)	96 (165–131)	14 (24–12)	—	—	21 (40–12)	—	—
	C	14–24	7	343 (688–92)	118 (227–29)	109 (300–35)	56 (118–12)	—	16 (31–0)	150 (307–46)	73 (90–11)	21 (47–0)

* B, butter. C, corn oil.
† The figures on the left refer to the 1st day of feces collection after the beginning of the dietary period.

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TABLE 6 Excretion of Fecal Neutral Steroids, Serum Cholesterol, and Amount of Sterols Consumed

Subject	Serum Cholesterol*		Fecal Cholesterol						Fecal Methylcholesterol				Fecal β-Sitosterol				Total Output of Neutral Steroids	
			Intake†		Output‡		Output − Intake		Intake†		Output‡		Intake†		Output‡			
	B§	C§	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C
	mg/100 ml		mg/day						mg/day				mg/day				mg/day	
1	207	167	250	130	428	491	178	351	—	103	19	53	—	313	89	250	536	794
2	183	139	250	130	417	539	167	409	—	103	10	107	—	313	76	322	503	968
3	285	194	250	130	545	717	295	587	—	103	15	75	—	313	84	308	644	1100
4	193	146	250	130	385	372	135	242	—	103	26	61	—	313	52	168	463	601
5	189	133	375	195	631	829	256	634	—	152	23	160	—	450	79	434	733	1434
6	234	157	250	130	753	733	503	603	—	103	37	87	—	313	78	291	868	1111
Mean	215	156	271	141	527	614	256	471	—	111	22	91	—	336	76	296	625	1001
7	601	436	188	99	529	570	341	471	—	76	14	72	—	235	21	260	564	902

* Mean of determinations made weekly.
† The sterols in bread, fruit, and marmalade have not been included.
‡ Calculated as the sum of the sterol and its neutral metabolites. The values given represent the mean of samples analyzed during the period.
§ B, butter fat diet. C, corn oil diet.

configuration. It might be worth mentioning in this connection that Kuksis and Huang (35), when analyzing a commercial sample of β-sitosterol, found a compound which they suggested could be a C_{28} sterol because of the gas chromatographic behavior of the substance. The fourth sterol found in the diet containing corn oil was stigmasterol. It was present in an amount only one-third that of methylcholesterol. This explains why no metabolites of this compound were detected in the feces.

All fecal extracts examined contained coprostanol, cholesterol, coprostanone, and in some instances traces of cholestanol. These steroids are known to be present in feces from man and animals (1). Minor amounts of other metabolites might have escaped detection since individual peaks with an area less than approximately 5% of the major peak in the chromatogram (usually coprostanol) could not be observed. Fractionation on aluminum oxide of a representative extract yielded significant amounts of cholestanol but no evidence for the presence of any additional cholesterol metabolites except for epicoprostanol, as discussed below. The presence in fecal extracts of other neutral steroids than those found in this study has been reported. Cook et al. presented evidence for the presence of Δ^4-cholestenone (cholest-4-en-3-one) in human feces (36). Rosenfeld and Hellman on the other hand could not confirm this (37). Sterols involved in cholesterol metabolism such as Δ^7-coprostenol, lathosterol, 7-dehydrocholesterol, and methostenol have been found in rat feces (38–40). In human feces we could not detect any of these compounds by GLC analysis of fecal extracts prepared at room temperature and subsequently handled under nitrogen.

It has also been reported that the amount of a non-digitonin-precipitable, nonketonic component in the fecal neutral lipid fraction from rat feces increased when diets supplemented with corn oil were fed (41). It was suggested that this substance might be a 3α-hydroxylated sterol. Epicoprostanol has been identified in ambergris (42) and dog feces (43). The contribution of this sterol, if present, to the total amount of fecal cholesterol metabolites in man seems to be negligible according to our data. Thus, digitonide precipitation of a cholesterol-rich extract gave rise to a more than 98% reduction of the "cholesterol-epicholesterol-epicoprostanol" peak on QF-1. Furthermore, the experiment shown in Fig. 12 also indicated that only minor amounts of epicoprostanol were present.

It has been established that coprostanol is formed from cholesterol by the action of intestinal microorganisms (44). Since plant sterols and cholesterol are converted into analogous metabolites, the action of the enzyme(s) involved in this transformation seems to be independent of the structure of the steroid side chain.

Two metabolic schemes have been outlined for the formation of coprostanol from cholesterol. Anchel and Schoenheimer (45) suggested that Δ^4-cholestenone and coprostanone are intermediates in this transformation whereas Rosenfeld et al. (44) have demonstrated the formation of coprostanol by a direct microbial reduction of the double bond. A recent report by Rosenfeld et al. (46) furthermore showed that coprostanol and coprostanone are to some extent interconvertible.

It has been noted by Goldsmith et al. (3) that various dietary fats may influence the transformation of cho-

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lesterol differently. In the present investigation we have made the same general finding. Thus, after the change of diet proportionally larger amounts of coprostanone were found in feces from five out of six normal subjects whereas the sixth subject excreted comparatively more cholesterol. The accumulation of coprostanone and/or cholesterol in the feces of subjects on the corn oil diet might indicate interference with the transformation of cholesterol to coprostanol via the pathway shown by Anchel and Schoenheimer.

One purpose of the present investigation was to compare the effect of corn oil- and butter-supplemented diets on the excretion of fecal neutral steroids. It was considered of interest to use a diet more similar to the ordinary Swedish diet than synthetic formula diets often used in experiments of this kind (5). For this purpose a series of four standardized meals were prepared and to these a supplement of butter and corn oil was added. Although basic dietary constituents such as meat and fish contained some fat, there was a marked difference between the two diets in sterol and fatty acid composition. In agreement with the results of similar investigations (2–4) the total fecal excretion of neutral steroids (including the plant steroids) increased when butter was replaced by corn oil in the diet. This increase was statistically significant ($0.01 > P > 0.005$).[4] The mean excretion of cholesterol and cholesterol metabolites also increased from 527 to 614 mg/day, but this change was not statistically significant ($0.08 > P > 0.07$).

Because of the lack of reliable figures for the absorption of cholesterol in the intestines it was not possible to determine the excretion of "nondietary" cholesterol; i.e., the daily contribution from the body cholesterol pool to the neutral steroid fraction in feces. Since part of the fecal neutral steroids represents dietary cholesterol that had never been absorbed, the difference in excretion of nondietary cholesterol as neutral fecal steroids on the two diets may be greater than the figures for the total excretion of cholesterol and its neutral metabolites indicate. The cholesterol intake was higher when the butter fat diet was fed, thus, provided that the absorption of cholesterol was similar on both diets, the excretion of "nondietary" cholesterol would be greater on the corn oil diet. If, now, the corn oil diet induced a greater absorption of cholesterol than the butter diet this difference would be further increased. On the other hand the difference would be decreased if cholesterol were absorbed to a greater extent on the butter diet. In this connection it is of interest to note that Spritz et al. (5), using a cholesterol-free diet of formula type, found the same absorption of a tracer dose of C^{14}-labeled cholesterol whether the dietary fat was corn oil or coconut oil. The values presented in the same paper on the absorption of ingested labeled cholesterol varied between 62 and 86%. These values are in good agreement with earlier reports (47).

Spritz et al. (5) found no correlation between type of fat consumed and the excretion of nondietary fecal sterols and fecal bile acids, when sterol-free diets were given. When corn oil and coconut oil were compared, there was an increased excretion of total fecal neutral steroids on the corn oil regimen, as in the present study. However, by labeling body cholesterol pools with C^{14}-cholesterol, they showed that the increased fecal excretion of sterols on corn oil was due to corn oil sterols and not to an increased excretion of nondietary steroids.

When the above-mentioned assumptions concerning the absorption of cholesterol are made, results from the present investigation might indicate that there was an increased elimination of cholesterol from the body cholesterol pool as neutral fecal steroids during the corn oil period. However, it should be emphasized that nothing can be said as to whether this could be the result of increased synthesis, altered excretion of cholesterol as bile acids, or a decrease of the body cholesterol pool. A reduction of the serum cholesterol level may or may not be related to the changes described. It was not the aim of the present investigation to study the mechanism for the serum cholesterol-lowering effect of a diet supplemented with corn oil but only to study the effect of the diet on the excretion of fecal neutral steroids. A study on the mechanism of the influence of diet on the serum cholesterol level should include measurements of the size and distribution of the body cholesterol pool as well as determinations of the total excretion of cholesterol and its metabolites including the excretion of bile acids.

The excretion of fecal bile acids under similar experimental conditions as used in this investigation will be the subject of a subsequent report.

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[4] The probability P was arrived at by calculating t from the expression

$$t = M_d \Big/ \sqrt{\frac{\Sigma(d - M_d)^2}{n(n-1)}}$$

where M_d is the mean difference within n pairs and d is the difference within each pair.

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