The steroids of 2000-year-old human coprolites

Don S. Lin, William E. Connor, L. Kyle Napton, and Robert F. Heizer

Department of Medicine and Clinical Research Center, University of Oregon Health Science Center, Portland, OR 97201; Institute for Archeological Research, California State College, Stanislaus, Turlock, CA 95380; and Department of Anthropology, University of California, Berkeley, CA 94704

Abstract Six samples of human coprolites, some more than 2,000 years old, were analyzed for fecal steroid composition. Despite this very lengthy period of storage, the fecal steroids of coprolites were remarkably similar to those of stool samples collected today. The sterol nucleus was clearly rather stable under the dry environmental conditions of the Nevada Caves. The steroid content (μg/g dried weight) of coprolite was low in comparison to that of modern man. The bile acid/cholesterol and plant sterol/cholesterol ratios of the coprolite, however, were similar to these ratios of the stools of modern man. In the six coprolites, an average 73% of the neutral steroids was digitonin-precipitable. This precipitate was composed of cholesterol and three plant sterols (campesterol, stigmasterol, and β-sitosterol) and their bacteria-modified products. A portion of the neutral steroids had been converted to products tentatively identified as epimers of these steroids. Individual bile acids were identified in the coprolite. The bile acid composition of the coprolite was similar to that of the stool of modern man.

Supplementary key words fecal steroids · plant sterols · bile acids · epimers · digitonin-precipitable sterols · Tarahumara Indians · bacterial degradation

Under certain environmental conditions, organic material, including feces, may be preserved for very long periods of time. These conditions include, among others, freezing in permafrost, chemical action in peat bogs, continuous immersion in sea water, and desiccation in arid environments. Study of prehistoric human feces, or coprolites, has already provided important information about the dietary patterns and food preparation practices of prehistoric peoples (1, 2). Little, however, is known about the chemical components of coprolites, especially their content of fecal steroids (cholesterol, plant sterols, and bile acids).

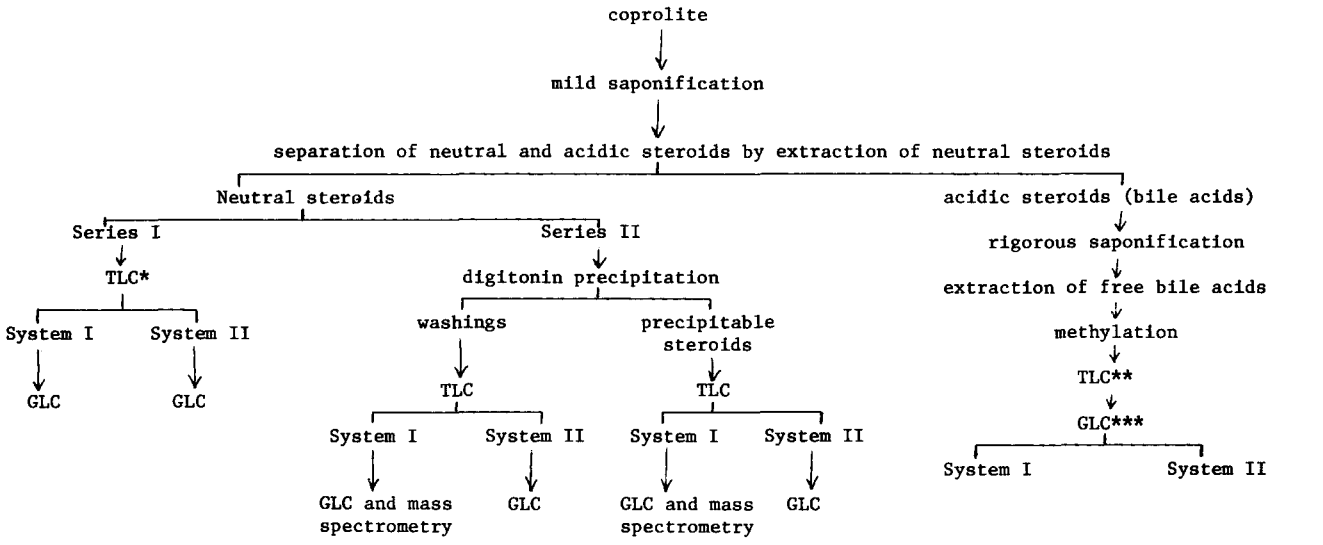
In 1968–1970, two of us (LKN and RFH) obtained from dry deposits in Lovelock Cave, Nevada, numerous specimens of desiccated human excrement, some more than 2,000 years old as indicated by radiocarbon dating. Studies of samples of these specimens enabled us to obtain extraordinary types of information from the past that can be derived with such conciseness in no other way and that are uniquely capable of giving us detailed knowledge of the diet of individuals who lived centuries ago. In an earlier published report (1) we discussed the macroanalyses to which we were able to subject the Lovelock Cave coprolites. These studies centered mainly on analysis of the gross diet and food acquisition and preparation practices of prehistoric inhabitants of the cave and adjacent sites.

In 1970, when we completed the major phase of the Lovelock Coprolite Analysis project, we expressed hope that the "biochemical" aspects of these unusual specimens would be investigated, and recently this profitable area of inquiry has been pursued (by DSL and WEC) with an analysis of samples of six Lovelock Cave coprolites. These specimens were analyzed for fecal steroids using modern analytical techniques to provide answers to the following questions. Over this long period, to what extent had bacterial degradation of the ring structure of the steroid nucleus occurred? Did the pattern and quantity of the fecal steroids differ from those of fresh human feces? Finally, further information about the diet of these ancient people and their sterol metabolism might well be obtained.

MATERIALS AND METHODS

We analyzed samples of coprolites whose dates of origin as determined by radiocarbon dating ranged between 50 A.D. and 100 B.C. These coprolites were found in Lovelock Cave, Churchill County, Nevada (1). One sample was obtained from the abdomen of a desiccated human body. The others were found in the stratified deposits of the cave at different depths. Each sample was analyzed at least twice and the same results were obtained in repeated

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; TMS, trimethylsilyl.



* TLC of neutral steroids: System I, Florisil plate; solvent- heptane:ethyl ether 45:55
System II, $AgNO_3$ plate; solvent - chloroform:methanol 97:3

** TLC of bile acids: silica gel H plate; first solvent - benzene; second solvent - isooctane:isopropanol: acetic acid, 120:40:1

*** GLC of bile acids: System I, SE-30 column, TMS derivative of bile acid methyl ether
System II, QF-1 column, TFA derivative of bile acid methyl ether

Fig. 1. Flow chart for separation and identification of steroids in coprolites.

runs. A flow chart indicating the analytical procedure is shown in **Fig. 1.** Analyses of the neutral steroids and bile acids of the coprolites were mainly based on methods previously described (3–5). Coprolites were ground and dried to constant weight in a vacuum desiccator. A 0.2–0.4 g portion of this powder was weighed out in a 125-ml reagent bottle. It was refluxed for 1 hr at 100°C with 20 ml of 1 N NaOH. The neutral and acidic steroids were then separated by the extraction of neutral steroids with petroleum ether. For purification and identification, the neutral steroid fraction was subjected to the analysis of two thin-layer chromatographic systems (TLC) with and without prior precipitation with digitonin. The first TLC system (I) involved Florisil plates and heptane–ethyl ether 45:55 as solvent. The second TLC system (II) was thin-argentation chromatography using silver nitrate impregnation with chloroform–methanol 97:3 as solvent. Neutral steroids were also purified by precipitation with digitonin (6). The precipitate was washed with diethyl ether and dried. The free sterols were recovered from the digitonide by dissolving the precipitate in pyridine and extracting the free sterols with diethyl ether (7). The ether extract was dried under vacuum over concentrated H_2SO_4. The free sterols were redissolved in chloroform for TLC.

TLC bands with the same R_f values as reference standards of cholesterol, cholestanol, coprostanol, and coprostanone and all of the remaining bands were scraped off the TLC plate. The sterols of each band were extracted with ethyl ether and derivatized to trimethylsilyl ethers (TMS) before gas–liquid chromatographic analysis (GLC). Cholestane was used as the internal standard.

The aqueous layer left from neutral steroid extraction contained bile acids which were saponified in a pressure cooker at 15 psi. The free bile acids were extracted and methylated with diazomethane. The methyl esters of bile acid were chromatographed using two solvent systems on the thin-layer silica gel H plate. The first was benzene and the second was isooctane–isopropanol–acetic acid 120:40:1. The area including the bands from cholic acid to lithocholic acid was scraped off and extracted with methanol.

These bile acids were derivatized to trimethylsilyl ethers and trifluoroacetates and then subjected to GLC using a less polar SE-30 column (silicon gum, methyl) as well as a more polar liquid phase column QF-1 (silicon gum, trifluoropropyl, methyl). The gas–liquid chromatographic analyses were performed on an instrument equipped with a hydrogen flame ionization detector (Hewlett-Packard model 7610A, Skokie, IL). The conditions used for the neutral steroid and bile acid analyses as TMS derivatives

were as follows. The column was a glass U tube 4 ft × ¼ in OD packed with 3.8% SE-30 on Diatoport S (80/100 mesh) with a helium flow rate of 75 mg/min, 40 psi head pressure. Column temperature was 230°C, injection port 250°C, flame detector 280°C. Conditions used for the analysis of bile acids as TFA derivatives were as follows. The column was a glass U tube, 4 ft × ¼ in OD packed with 1% QF-1 gas chrom P (100/120 mesh). Column temperature was 205°C, injection port 210°C, flame detector 240°C. A Hewlett-Packard model 3370B integrator was used to obtain the retention time and peak area of each compound for identification and quantitation.

In the feces of modern man, the fecal steroids can be separated into three groups of compounds according to their structure by TLC. The cholesterol band contains three plant sterols (campesterol, stigmasterol, and β-sitosterol) in addition to cholesterol (3, 5). A small amount of ring-saturated 5α-derivatives of these four sterols will be in the cholesterol band in TLC system I and separated from the cholesterol band in TLC system II. In the coprostanol band, there are coprostanol and ring-saturated 5β-homologs of the three plant sterols. The coprostanone and 3-keto homologs of the three plant sterols are in the coprostanone band. In the acidic steroid fraction, lithocholic acid and deoxycholic acid are the two main fecal bile acids found in man. Based on these established facts, we have identified the steroids in coprolite by their characteristics on TLC and GLC in comparison with those of reference standards[1] and of compounds already known to be present in the feces of modern man (3, 5).

GLC quantitation was done with cholestane and hyodeoxycholic acid as internal standard (3, 5). The loss during the process was monitored by the radioactive standards of [4-^{14}C]cholesterol (New England Nuclear Corp., Boston, MA) for neutral steroids and [24-^{14}C] deoxycholic acid (Tracer Lab., Waltham, MA) for bile acids. Radioactivity was measured by Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, IL) with an absolute activity analyzer.

For further identification, the steroids in coprolites were analyzed by combined gas–liquid chromatography–mass spectrometry. The analyses were made using a DuPont 21-491B mass spectrometer interfaced with a Varian 2700 gas chromatograph. The ion source was maintained at 250–270°C and the ionization energy was 70 eV. Samples were separated using a 6 ft × 2 mm (ID) glass column packed with 3% OV-101 on 100/120 Gas Chrom Q maintained at 235°C.

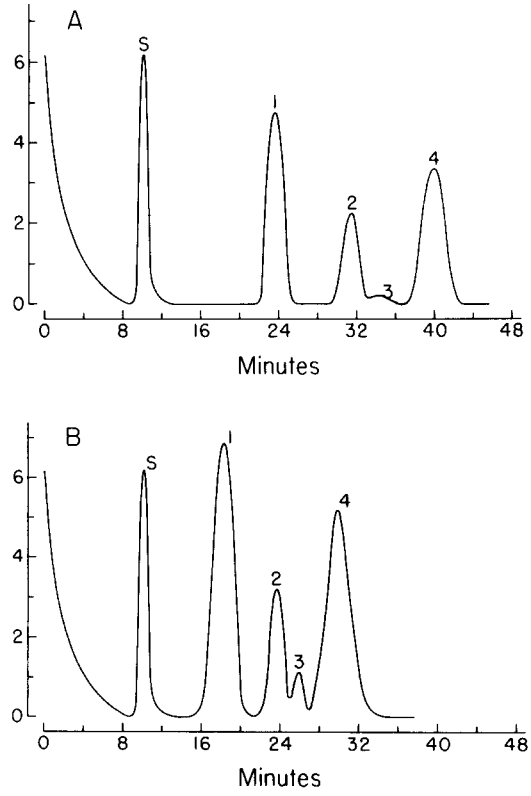


Fig. 2. Gas–liquid chromatography of the digitonin-precipitable neutral steroids in coprolite as trimethylsilyl ethers. (*A*) Δ^5-sterols. Peak identification: 1, cholesterol; 2, campesterol; 3, stigmasterol; 4, β-sitosterol. (*B*) 5β-Stanols. Peak identification: 1, coprostanol; 2, coprocampesterol (5β-cholestan-24b-methyl-3β-ol); 3, coprostigmastenol (5β-cholestan-22-ene-24b-ethyl-3β-ol); 4, coprostigmastanol (5β-cholestan-24b-ethyl-3β-ol). In both, peak *S* represents internal standard, 5α-cholestane; the 3-keto homologs of these 5β-stanols have the same pattern but are present in small quantities.

RESULTS AND DISCUSSION

The patterns of the coprolite steroids by gas–liquid chromatography were similar to those obtained from fresh human stool in that four major sterol peaks occurred: cholesterol and the three plant sterols campesterol, stigmasterol, and β-sitosterol (**Figs. 2*A*** and **2*B***). In modern man and animals, the cholesterol molecule may be hydrogenated by the intestinal

[1] Reference standards used for identification were: 5α-cholestane, cholesterol (5-cholesten-3β-ol), campesterol (5-cholesten 24b-methyl-3β-ol), stigmasterol (5,22-cholestadien-24b-ethyl-3β-ol), β-sitosterol (5-cholesten-24b-ethyl-3β-ol), coprostanol (5β-cholestan-3β-ol), coprostanone (5β-cholestan-3-one), lithocholic acid (5β-cholanic acid-3α-ol), chenodeoxycholic acid (5β-cholanic acid 3α,7α-diol), hyodeoxycholic acid (5β-cholanic acid 3α,6α-diol), and cholic acid (5β-cholanic acid 3α,7α,12α-triol), from Applied Science Laboratories, Inc., State College, PA. Epicoprostanol (5β-cholestan-3α-ol), cholestanol (5α-cholestan-3β-ol), and deoxycholic acid (5β-cholanic acid, 3α,12α-diol) were obtained from Steraloids, Inc., Pawling, N.Y.

bacteria to form coprostanol and coprostanone (5, 8–10). This phenomenon occurred in the coprolites as well. An average of 78% (range 41–94%) of their neutral steroids were in the form of stanols and stanones derived from both cholesterol and the plant sterols (**Table 1** and Figs. 2*A* and 2*B*). Of great interest was the large amount of unmodified cholesterol found, ca. 22% of the total neutral steroids, even after 2,000 years of opportunity for bacterial alteration. This attested to the well-preserved character of the specimens. The fecal plant sterols and cholesterol were modified similarly.

Ninety-five per cent of the nonsaponifiable material contained in the coprolites had the characteristics of the steroid nucleus and could be identified by their TLC and GLC behavior as one or the other of the fecal steroids. Their GLC patterns were similar to those of fresh human stool, but a few minor unidentified peaks were detected and the individual peaks were less sharply defined. The greatest aberration was represented by the stanol fraction of coprolite samples no. 5 and no. 6. Overlapping peaks were observed. These minor and overlapping peaks disappeared after digitonin precipitation (Fig. 2*B*). Concurrently, the steroidal content of the coprolite decreased, the average recovery being 73% of the initial value (range of 53–95%). This loss seemed related to the age of the coprolite. The oldest coprolites (no. 5 and no. 6) had the greatest loss with recoveries of only 53 and 54%, respectively.

To check the reliability of the methodology, we analyzed fresh human feces in tandem with the coprolites; 95% recovery was obtained after digitonin precipitation. The washings of the digitonin-precipitable material of these two samples were recovered and subjected to the same TLC and GLC procedures. They had similar TLC and GLC behavior as the digitonin-precipitable steroids; however, the retention times of these steroids on GLC were slightly longer.

Since the majority of the steroids in the washing were from the stanol fraction, we added epicoprostanol as a standard in the mixture of these coprolite steroids. On the GLC peak of the standard was superimposed a peak that overlapped the coprostanol peak and had a slightly longer retention time before digitonin precipitation. Thermodynamically, epicoprostanol is more stable than coprostanol (11, 12). The in vitro interconversion of these alcohols proceeds by initial conversion to ketones (13). It is certainly conceivable that modification of the steroid molecules to form epimers occurred during the long period of storage.

The identities of the steroids in coprolite were further confirmed by combined gas–liquid chromatography–mass spectrometry. The principal ions of fragmentation of the TMS derivatives of the steroids in the Δ^5-sterol fraction of coprolite were identical with those of authentic cholesterol and the plant sterols campesterol, stigmasterol, and β-sitosterol. The mass spectrograms, of 5β-stanol and the 3-epimer of 5β-stanol fractions from coprolites also confirmed the presence of the saturated form of steroids from these four sterols (cholesterol and three plant sterols).

Thus, the nonsaponifiable materials of the neutral

TABLE 1. Neutral and acidic steroids (μg/g of coprolite) of human coprolites

Specimen No. and Date	Cholesterol and Metabolites						Plant Sterols and Their Metabolites			
	Neutral Steroids				Bile Acids[a]	Total Neutral Steroids plus Bile Acids	Neutral Steroids			
	Sterol	Stanol	Stanone	Total			Sterol	Stanol	Stanone	Total
No. 1. Feces from pelvic cavity. c. 1750 A.D.	602	861	65	1258	250	1508	178	535	160	873
No. 2. At 0–6 in. below surface. c. 50 A.D.	270	2092	110	2472	1665	4137	510	1956	103	2569
No. 3. At 0–6 in. below surface. c. 50 A.D.	4883	3198	204	8285	850	9135	441	440	38	919
No. 4. 10 ft. below surface. c. 50 A.D.	354	5043	380	5777	1465	7242	253	1904	121	2278
No. 5. 11–12 ft. below surface. c. 100 B.C.	733	7450	392	8575	1743	10318	769	2029	107	2905
No. 6. 11–12 ft. below surface. c. 100 B.C.	615	6059	252	6926	2336	9262	251	3036	127	3414

[a] Individual bile acids are listed in Table 2.

steroids of coprolites were usually composed of four fractions: (*1*) 73.0% was digitonin-precipitable and had TLC, GLC, and mass spectrometry behavior identical to that of the fecal steroids of modern man; (*2*) 0.5% was digitonin-precipitable steroids but with different TLC and GLC behavior than modern fecal steroids; (*3*) 22% was tentatively identified as epimers of cholesterol and plant sterols on the basis of not being digitonin-precipitable, of having the same TLC and mass spectrometry behavior, and of having similar GLC behavior but with slightly longer retention times than modern fecal steroids; and (*4*) 4.5% was not digitonin-precipitable and had TLC and GLC behavior different than modern fecal steroids.

With TLC, the greater part of the acidic steroid fraction of the coprolites (95%) was confined to the bile acid region. This area included the bands from cholic acid to lithocholic acid. The steroids in one-half of the lipid extract from this bile acid region were converted to TMS derivatives and chromatographed in a SE-30 column. Those in remaining extract were chromatographed through a QF-1 column as TFA derivatives. The same quantitative results were obtained from both procedures. These comparisons strengthened the point that the compounds analyzed actually were bile acids. The GLC patterns of the coprolite bile acids were similar to those of modern human stool (Fig. 3). Individual bile acids were identified by their retention times relative to methyl hyodeoxycholate-TFA on the QF-1 column (14, 15) (**Table 2**). Lithocholic acid and deoxycholic acid were the two major bile acids. The total combined percentage of these two bile acids in coprolites

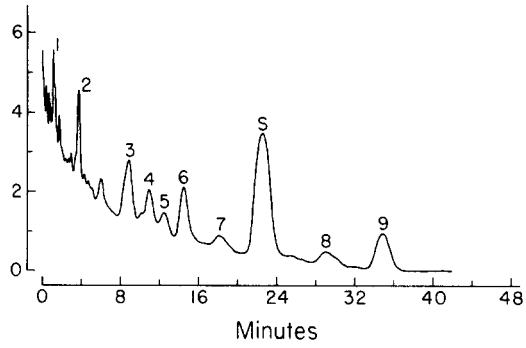


Fig. 3. Gas–liquid chromatography of the bile acids in coprolite as trifluoroacetate in QF-1 column. Peak identification: 1 and 2, unknown (nonbile acid zone); 3, lithocholic acid; 4, unknown; 5, 3β,12α-dihydroxycholanic acid; 6, deoxycholic acid; 7, chenodeoxycholic acid; 8, 3β-hydroxy-12-ketocholanic acid; 9, 12-ketolithocholic acid; *S*, standard, hyodeoxycholic acid.

was 61.1% (51–92%), somewhat lower than the 82.5% (74–89%) found in four modern human stools analyzed simultaneously. Chenodeoxycholic acid, 3β,12α-dihydroxycholanic acid (5β-cholanic acid 3β,12α-diol), 3β-hydroxy-12-keto cholanic acid (5β-cholanic acid 3β-ol-12-one), and 12-keto-lithocholic acid (5β-cholanic acid 3α-ol-12-one) were 5.4% (0–10%), 4.8% (0–12%), 2.0% (0–6%), and 11.5% (0–22%), respectively, in the coprolites and only 1.0% (0.1–2.5%), 8.1% (6.3–9.6%), 1.3% (0.5–2.5%), and 3.6% (0.8–7.6%), respectively, in modern human stool.

The average cholesterol-derived steroid content of five coprolites (Table 1 and excluding sample no. 1 from the pelvic cavity) was 8,019 ± 2.436 (mean ± SD) μg/g of dry weight of coprolite (the neutral

TABLE 2. Individual bile acids[a] (μg/g of coprolite) of human coprolites

Specimen No. and Date	Litho-cholic	3β,12α-Dihydroxy-cholanic	Deoxy-cholic	3α,12β-Dihydroxy-cholanic	Cheno-deoxy-cholic	3β-Hydroxy-12-keto-cholanic	12-Keto-litho-cholic	3-Keto-7α-hydroxy-cholanic	Un-identified	Total
No. 1. Feces from pelvic cavity. c. 1750 A.D.	95		135						20	250
No. 2. 0–6 in. below surface. c. 50 A.D.	433	200	500		167	100	167		98	1665
No. 3. 0–6 in. below surface. c. 50 A.D.	177	11	257	80	80		110	9	126	850
No. 4. 10 ft. below surface. c. 50 A.D.	467	110	296		63	47	147	31	304	1465
No. 5. 11–12 ft. below surface. c. 100 B.C.	715	93	342		47	31	256	62	197	1743
No. 6. 11–12 ft. below surface. c. 100 B.C.	911	56	456		152	23	505		233	2336

[a] Each individual bile acid was identified by its retention time relative to hyodeoxycholate ME-TFA on gas–liquid chromatography with QF-1 column.

TABLE 3. Bile acid:cholesterol and plant sterol:cholesterol ratios in stool samples and coprolites

	Indians		Coprolite				
	Low Cholesterol	High Cholesterol	No. 2	No. 3	No. 4	No. 5	No. 6
Bile acid:cholesterol	0.72 ± 0.16	0.32 ± 0.09	0.67	0.10	0.25	0.20	0.34
Plant sterol:cholesterol	0.98 ± 0.19	0.48 ± 0.15	1.04	0.11	0.39	0.34	0.49

steroids, including the usual fecal steroids cholesterol, coprostanol, and coprostanone, the 3-epimers, and cholestanol averaged 6,407 ± 2,468 μg/g of dry weight and the bile acids averaged 1,611 ± 535 μg/g of dry weight). The cholestanol content of these coprolites was found to be 125 ± 72 μg/g. Plant sterols, their bacteria-modified products (saturated 5β- and 3-keto homologs), and their 3-epimers amounted to 2,417 ± 938 μg/g of dry weight.

For comparison, we have calculated the steroid content of the stools of eight Tarahumara Indians[2] who received either low cholesterol (<50 mg/day) or high cholesterol (about 1000 mg/day) diets with 400–500 mg of plant sterol per day. The steroidal content per g of dry weight of stool was calculated with the assumption that the water content of the stool was 75% (16). For the low cholesterol diet, the total of cholesterol-derived steroids was 15,474 ± 4,037 μg/g of dry weight (cholesterol and bacteria-modified products averaged 9,071 ± 2,609 μg/g of dry weight; bile acids averaged 6,381 ± 1,776 μg/g of dry weight); and for plant sterols the total was 8,996 ± 3,471 μg/g of dry weight. For the high cholesterol diet, the total of cholesterol-derived steroids was 24,046 ± 5,735 μg/g of dry weight (cholesterol and bacteria modified products 18,443 ± 4,987 μg/g of dry weight, bile acids 5,603 ± 1,337 μg/g of dry weight) and plant sterols was 8,736 ± 2,884 μg/g of dry weight.

The steroid content of the coprolites was low in comparison to that of the Tarahumara Indians. However, certain soil microorganisms have been found to be capable of completely oxidizing steroids to carbon dioxide (17, 18). It is conceivable that, during its long period of storage, some steroid in the coprolite was oxidized to products that are not detectable by our analytical system. Furthermore, the addition of such materials as ash and minerals to the coprolite during this long period of time could also affect the steroidal content expressed per unit weight. Therefore, these possibilities must be taken into consideration in interpreting the results in terms of absolute quantity.

[2] The Tarahumara Indians live in the mountains of northern Mexico. Recently, we had the opportunity to carry out some metabolic balance studies with them.

When we compare the ratios of bile acids to cholesterol and plant sterols to cholesterol between coprolite and stool samples of the Tarahumara Indians, the ratios of the coprolite were found quite variable. The ratios of coprolite no. 2 are close to those of the stools of Indians consuming a low cholesterol diet with 400–500 mg daily intake of plant sterol. Coprolites no. 4, no. 5, and no. 6 had similar ratios to those of the stools of Indians consuming a high cholesterol diet. Coprolite no. 3 had the lowest ratios. These figures are listed in **Table 3.**

The diet of the inhabitants of Lovelock Cave and of other prehistoric peoples included a variety of foods such as seeds, plants, fish, and meat (1, 2). The supply of food was, however, uncertain (19). Therefore, their dietary intake of sterol may have been subjected to quite a fluctuation. This seems to coincide with the variable ratios we found in these coprolites.

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