Quantitative isolation and gas–liquid chromatographic analysis of total fecal bile acids

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SUMMARY A method for isolation and quantification of fecal bile acids is described which allows sterol balance studies to be made in man or in small laboratory animals without requiring the use of radioisotopes in vivo. Bile acids are purified by column and thin-layer chromatography, converted to the trimethylsilyl ethers of their methyl esters, and quantified by GLC with detection by hydrogen flame ionization. Recoveries are complete when internal standard corrections are applied, with an error $<\pm 3\%$. The claim that the final fecal bile acid fraction accounts for all the bile acids and nothing but bile acids is validated in several ways. The sensitivity is such that fecal aliquots containing as little as 50 μg of mixed bile acids can be analyzed accurately, but the procedure lends itself well to preparative scale work for more definitive study of individual bile acids.

After oral administration of cholic-24-C^{14} and chenodeoxycholic-24-C^{14} acids to one patient, 98% of the administered radioactivity was excreted in the bile acid fraction of the fecal extracts over a period of 38 days, and 2% in the urine. This experiment indicated the stability of bile acid structure during intestinal transit and provided additional evidence for the completeness of the described method of determining fecal bile acids.

KEY WORDS fecal bile acids · quantitative recoveries · thin-layer chromatography · gas–liquid chromatography · trimethylsilyl ethers · hydrogen flame detection · C^{14}-bile acids · C^{14}-cholesterol · internal standards · man · rat

IN THE STEADY STATE, the daily fecal excretion of neutral plus acidic steroids of endogenous origin should approximately equal the daily synthesis of cholesterol in any species in which the intestine is the major route of excretion, as it is in man. This statement is based on our present understanding of (*a*) the conversion of cholesterol to bile acids in the liver and the degradation of neutral and acidic sterols to a multitude of steroidal secondary products within the intestinal lumen [reviewed recently by Danielsson (1)]; (*b*) the resistance of the ring structure of cholesterol to degradation within the body or by intestinal microorganisms (2); and (*c*) the finding in the present study that the ring structure and side-chain of the two primary bile acids (in man) also remain intact during passage through the intestine or circulation within the enterohepatic system.

Accordingly a method was developed for quantitative isolation and determination of the neutral and acidic steroids[1] from feces, in order to answer certain questions concerning the mechanism whereby plasma cholesterol concentrations are altered by exchanging saturated and unsaturated fats in the diet (3). The success of such a method depended upon obtaining the steroids quantitatively, and free from the numerous contaminants in feces which have similar physical properties. This goal was first approached (4) by a combination of column and thin-layer chromatography, and the final products were weighed (neutral steroids) or titrated (acidic steroids). Applications of this procedure in sterol balance studies in man have been reported elsewhere (5).

The present and an accompanying report (6) describe refinements of this general method which afford more complete recoveries and separations, together with greater accuracy, reliability, and simplicity in performance. This paper validates a procedure for determination of the acidic steroids; its companion (6) describes a similar procedure for the neutral steroids. In both procedures the final purification and quantification depend upon the use of GLC; neither requires the administration of radioisotopes to patients. The advantages of the newer

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; PE, petroleum ether (bp 60–70°); EE, ethyl ether; TMS, trimethylsilyl.

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

procedures over our previous method and over others reported in the literature are (*a*) complete recovery of neutral and acidic steroids, without cross-contamination, and free from nonsteroidal materials, (*b*) authentic measurement of the acidic and neutral steroids by a gas–liquid chromatographic method which "sees" the cyclopentanophenanthrene ring that is common to both groups, (*c*) sufficient sensitivity to allow of the measurement of daily sterol output in individual small laboratory animals, (*d*) sufficient ease of performance to permit 12–18 determinations of both acidic and neutral steroids per week by two technicians, and (*e*) the reservation of radioisotope use for purposes other than measurement of steroid excretion, since the quantitative methods described are purely chemical.

The importance of these methods should become apparent as they are applied in various poorly defined aspects of sterol metabolism. Total daily synthesis rates of cholesterol and the effects of drugs and dietary changes on synthesis can be assessed. By concurrent use of isotope techniques it is feasible to study steroid absorption, feedback control mechanisms, pool sizes, and turnovers in intact organisms. The relative importance of excretion via pathways other than the intestine, such as skin and urine, can be evaluated, as well as the effects of intestinal microorganisms on neutral and acidic steroids. Only when dependable balance data are obtainable can the fate of dietary cholesterol be fully described, and the possibility of redistribution of plasma cholesterol into other tissues adequately explored. These and other aspects of sterol metabolism are under active study in this laboratory.

GENERAL METHODS, MATERIALS, AND APPARATUS

Solvents were distilled in all-glass apparatus before use. Solvents were removed by evaporation at temperatures below 50° on a rotary evaporator (Rinco Instrument Co., Greenville, Ill.) attached to a water aspirator.

Reference Standards. Cholic, chenodeoxycholic, and lithocholic acids labeled with C^{14} at position 24 were obtained from Nuclear Research Chemicals, Inc., Orlando, Fla. Cholic acid-H^3 and chenodeoxycholic acid-H^3, randomly labeled by exposure to tritium gas, were prepared by New England Nuclear Corp., Boston, Mass., which also supplied cholesterol-4-C^{14} and deoxycholic acid-24-C^{14}. It was essential to purify the radioactive bile acids and cholesterol by preparative TLC on Silica Gel H and G plates, with and without silver nitrate impregnation and in a variety of development systems. 5α-Cholestane (Steraloids, Inc., Pawling, N.Y.) was used as internal standard for GLC without further purification, for it gave a single peak with an area response which was 99% of the theoretical when tested against an internal standard of repeatedly crystallized cholesterol in the form of its TMS ether (see below).

Column Separations employed Florisil, 60–100 mesh (Floridin Company, Tallahassee, Fla.), activated by overnight heating at 200° and used without deactivation with water.

Thin-layer Chromatography was carried out on 0.5 mm layers of Silica Gel H (E. Merck Ag., Darmstadt, Germany; distributors, Brinkmann Instruments, Inc., Great Neck, Long Island, N.Y.) on 20 × 20 cm plates, according to Mangold (7). Contaminants were removed by preliminary development of the plates with methanol–acetic acid 9:1. The plates were then dried and activated at 120° for 1 hr.

Gas–Liquid Chromatography of bile acid derivatives was carried out on an instrument equipped with a hydrogen flame ionization detector (F and M Biomedical Gas Chromatograph, Model 400, Avondale, Pa.). Six-foot glass U-tubes, 4 mm i.d., were packed with thin-film (1–2%) coatings of DC-560 (formerly called F-60), SE-30, QF-1, XE-60, or HiEff 8B on acid-washed, silanized Gas Chrom P, 100–120 mesh (coatings and support from Applied Science Laboratories, State College, Pa.). Nitrogen was used as carrier gas at flows of 30–60 ml/min, inlet pressures 20–30 psi. Column temperatures were usually 240° and were accurately monitored with a precision mercury thermometer (Allihn type; Matheson Scientific Co., Philadelphia, Pa.); the temperature of the flash heater was about 300° and of the detector about 260°. When it was found that individual peak areas measured by triangulation checked within 1% with measurements made with a mechanically activated integrator (Disc Instruments, Inc., Santa Ana, Calif.), the latter system was used in subsequent measurements; indeed, it proved to be essential for measuring total areas of a series of unresolved peaks. All quantitative work was performed by comparison of area responses to those of known additions of 5α-cholestane as internal standard; in our hands, analyses based on volume of sample injected were grossly inaccurate.

TMS Ether Formation. The TMS reaction mixture used to produce TMS ethers of bile acid methyl esters consisted of a mixture of dry pyridine (stored over Molecular Sieve 4A, Fisher Scientific Co., Fair Lawn, N.J.), hexamethyldisilazane (Peninsular Chemical Research, Inc., Gainesville, Fla.), and trimethylchlorosilane (General Electric Co., Waterford, N.Y.), mixed before use in the proportions 9:3:1. This mixture was effective for at least 6 months if protected against contamination by water vapor.

Radioactivity Measurements were performed with a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3003). Counting efficiencies were approximately

85% for C^{14} and 26% for H^3. Bile acid methyl esters were counted in PPO–POPOP–toluene solution prepared from Liquifluor (Pilot Chemicals, Watertown, Mass.). After dilution with toluene, the counting solution contained 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]-benzene per liter of toluene. Toluene-C^{14} or -H^3 (Packard Instrument Company, Inc., La Grange, Ill.) were used as internal standards to correct for quenching.

Clinical Aspects. Stools were obtained from in-patients at The Rockefeller Institute Hospital who had received either C^{14}-labeled cholesterol or bile acids by oral administration. J. F., a 39-yr-old male with familial hypercholesteremia, was given orally a mixture containing 10 μc of cholic acid-24-C^{14} (2.0 mc/mmole) and 10 μc of chenodeoxycholic acid-24-C^{14} (2.71 mc/mmole). E. K., a 45-yr-old female with familial hypercholesteremia, received 100 μc of cholesterol-4-C^{14} orally. Both patients were maintained entirely on orally administered liquid formula feedings, and total stool collections were made for several weeks. Plasma cholesterol concentrations were measured by the method of Abell, Levy, Brodie, and Kendall (8), and specific activities were determined by counting an aliquot from the PE extract used for colorimetry.

QUANTITATIVE ISOLATION OF FECAL BILE ACIDS

General Description

Fecal bile acids were obtained quantitatively after extraction of neutral steroids and saponification of taurine and glycine conjugates; free bile acids were extracted with chloroform–methanol. In most stool samples an aliquot of this extract could be subjected directly to preparative TLC after methylation of bile acids. However, if samples were particularly rich in fatty acids or acidic pigments, it was necessary to remove these contaminating acids by column chromatography before the TLC step. After preparative TLC, the bile acid methyl esters were recovered and 5α-cholestane was added as internal standard. The bile acid methyl esters were converted to TMS ethers which were quantified by GLC. Small losses of bile acids through this multistep process were corrected by incorporation of a known amount of high specific-activity internal standard, deoxycholic acid-24-C^{14}, in the original aliquot of fecal homogenate. Figure 1, a flow sheet, summarizes the various steps in the procedure.

This procedure was shown to give excellent quantification of total acidic fecal steroids, even though the mixtures obtained by trimethylsilylation and applied to GLC columns contained small amounts of methyl esters

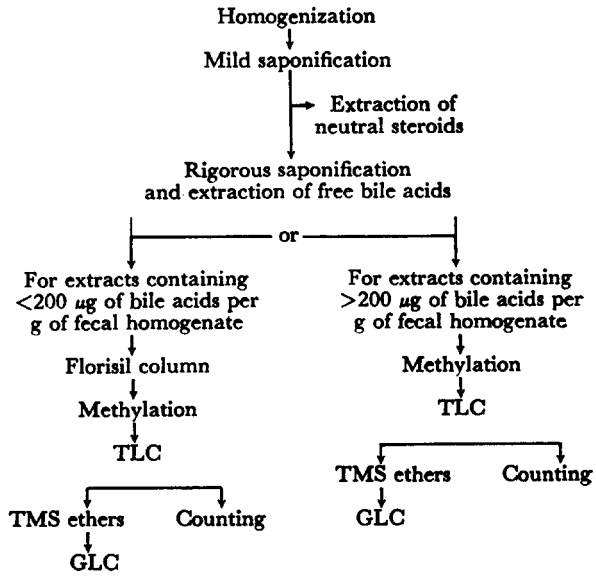

FIG. 1. Flow sheet for determination of fecal bile acids.

of acids which were not bile acids. The method was applied to fecal samples as small as 100 mg, containing as little as 0.05 mg of total bile acids. This sensitivity permitted the daily bile acid excretion by small laboratory animals to be determined.

Collection and Homogenization

In clinical experiments stools collected into clean dry weighed two-quart metal paint cans were stored at 4°. Distilled water was added in an amount (about an equal volume, in most cases) which would permit the production of a homogenate of thin enough consistency to be easily drawn up into a wide-mouthed transfer pipette. The cans were weighed again, three 2-inch metal washers were added, and the mixture was homogenized by vigorous agitation for 3 min on a $^1/_4$ hp paint mixer (Red Devil Tools, Union, N.J.). All weights were recorded to the nearest gram (Toledo Scale Co., Toledo, Ohio, model 4020). Before settling of solids could occur, aliquots of the homogenates were transferred to 125-ml glass bottles with plastic screwtops for storage at 4°; the cans were closed and discarded.

Addition of Radioactive Internal Standard for Calculation of Recovery

In order to correct for the inevitable small losses which occur in a multistep procedure, a known amount of deoxycholic acid-24-C^{14} of high specific activity was added as internal recovery standard to an aliquot of the stool homogenate from which the steroids were to be isolated. (H^3-labeled bile acids served the same purpose when fecal steroids had been labeled in vivo with C^{14}.) Radioactive cholesterol also was added as internal re-

covery standard when neutral steroids were to be isolated from the same homogenate.

Removal of Neutral Steroids

Successful measurement of fecal acidic steroids by the present procedure depended upon preliminary removal of all neutral steroids from the fecal homogenate. This removal was complete only if the conditions for saponification of sterol esters were mild; after saponification under pressure (conditions required for hydrolysis of the conjugated bile acids), the removal of neutral steroids was invariably *in*complete, as much as 5% of neutral steroids remaining with the bile acids.

A 1 g aliquot of homogenized stool mixture (containing approximately 1 mg of bile acids) was added to a weighed 125 ml glass-stoppered bottle (aliquot weights were recorded to the nearest milligram). Twenty milliliters of N sodium hydroxide in 90% ethanol and a few boiling chips were added. The mixture was refluxed for 1 hr. After cooling, 10 ml of water and 50 ml of PE were added. The bottle was stoppered and shaken vigorously for 1 min and centrifuged for 5 min at 1000 × *g*. The upper phase was removed carefully, and the extraction was repeated twice more with 50 ml of PE, centrifuging each time before removal of the upper layer. The pooled upper phases contained all the neutral steroids and were essentially free of acidic steroids [and thus could be used for measurement of fecal neutral steroids as described in the accompanying report (6)]. A backwash of the PE phase with 10 ml of N NaOH in 50% ethanol removed the last traces of bile acids.

Saponification and Extraction of Bile Acids

The lower aqueous phase contained a mixture of acidic materials, including conjugated and free bile acids. Saponification under pressure was required for hydrolysis of the bile acid conjugates. To the lower aqueous phase was added 2 ml of 10 N NaOH, and the contents of the tube were saponified at 2 atmospheres (15 psi) for 3 hr in a 16 liter pressure cooker. Most of the ethanol evaporated during this procedure. The mixture was acidified to pH 2 with concentrated HCl, and 75 ml of chloroform–methanol 2:1 was added. The bottle was shaken, then centrifuged for 5 min at 1000 × *g*, and the lower phase was transferred quantitatively to a 250 ml round bottom flask. The aqueous phase was extracted twice more with 50 ml of chloroform. All lower phases were pooled, and the solvent was evaporated to dryness, leaving a dark brown residue.

TLC of Bile Acid Methyl Esters

If the stools contained large amounts of fatty acids or unusually small amounts of bile acids, or if a yield of bile acids greater than 200 μg was desired for further study of individual acids, further purification by column chromatography was required. However, in cases where there was no steatorrhea and for which only a quantitative estimation of total bile acids was needed, column chromatography was unnecessary: the sample was methylated and applied directly to a preparative TLC plate for isolation of bile acid methyl esters.

The residue obtained after extraction was taken to 25 ml in chloroform–methanol 2:1. Some (usually 5 ml) of this solution was transferred to a glass-stoppered test tube and evaporated. For complete methylation of bile acids, 4 ml of 5% HCl in super-dry methanol was added, and the contents were either refluxed for 2 hr at 100° or allowed to stand overnight. After evaporation of the solution the residue was applied along the base of a thin-layer plate with chloroform–methanol 2:1. A 20 cm plate easily accommodated the application of two samples, as well as the reference standard, methyl cholate. The plate was developed in benzene and exposed to iodine vapor to locate the fatty acid methyl ester area, a single large band with R_F about 0.65; a line was drawn immediately behind the band. The plate was then developed for a second time to the drawn line in isooctane–isopropanol–acetic acid 120:40:1 and exposed a second time to iodine vapor; the area from methyl cholate to the line contained all the bile acids. The entire bile acid zone was collected in a vacuum aspirator according to Goldrick and Hirsch (9), and the solutes were desorbed with 25 ml of methanol into a 100 ml round bottom flask or 50-ml test tube. After evaporation the bile acid methyl esters were dissolved in 10 ml of ethyl acetate containing a known amount of 5α-cholestane as GLC internal standard, and aliquots were taken for GLC and counting of radioactivity.

Trimethylsilylation for GLC

The bile acid methyl esters were exposed to silylating reagents in order to convert all free hydroxyl groups to TMS ethers, as originally reported by Makita and Wells (10). Recently Sandberg, Sjövall, Sjövall, and Turner (11) stated that, when dimethylformamide was used as solvent, TMS ethers of bile acids were formed only at the 3-position; we have found no evidence of partial derivative formation with pyridine as solvent. However, in the case of 3-keto bile acids exposed to our silylating reaction mixture, variable amounts of secondary products are formed, presumably by reaction with the enol forms of the 3-keto acids. Nevertheless, the secondary products as well as the ketonic bile acid methyl esters are quantitatively determined by the present GLC procedure and are reliably included in the total bile acid calculation.

An aliquot of the ethyl acetate solution containing bile acid methyl esters and the 5α-cholestane standard was

pipetted into a disposable stoppered glass vial of appropriate size. The solution was dried completely in an air stream with gentle warming. The silylating reagent mixture was added (100 μl/mg or less of mixed bile acid methyl esters), the vial closed, and the reaction mixture left at room temperature for 1 hr or longer.

GLC with Hydrogen Flame Detection

Linearity of response was achieved with loads varying from 0.06 to 120 μg of TMS sterols, TMS bile acids, and 5α-cholestane. In a hydrogen flame detector the ionization responses of these various compounds (whether they contained 0, 1, 2, or 3 TMS groups) were directly proportional to the actual weights (not molecular weights) of the *un*substituted parent compounds (and *not* of the complete TMS ethers). TMS derivatives of more than 20 different neutral steroids and bile acid methyl esters, as well as methyl esters of ketonic bile acids and 5α-cholestane, all showed the same ionization response per unit mass of the parent *un*substituted steroid. (This linearity was demonstrated on all the stationary phases investigated.) Besides greatly simplifying the calculation of GLC results, these findings[2] made it possible to quantify complex mixtures of acidic steroids as a group by relating their total GLC area to that of the internal standard, 5α-cholestane; no correction factors were needed for the added mass of the TMS groups, or to compensate for the different ionization responses of various steroids which have been found with other detection systems (12–16).

The TMS reaction mixture was syringe-pipetted directly into the GLC column; aliquots of 1–3 μl gave satisfactory results without giving rise to prolonged solvent peaks. The best separations of individual components were obtained on HiEff 8B and QF-1 columns, but for present purposes, the determination of total fecal bile acids as a group, GLC was routinely performed on DC-560 or SE-30 columns. On every stationary phase studied, all peaks appearing after 5α-cholestane were found to represent TMS bile acid methyl esters. Consequently, the total area of such peaks (determined by means of the disk integrator) was used to determine the total weight of bile acids relative to α-cholestane even when the separation of individual TMS bile acid methyl esters was far from complete, as on DC-560 and SE-30 columns. This weight was then corrected for losses during the procedure on the basis of the recovery of the radioactive internal standard added at the start of the isolation.

Bile acid methyl ester fractions recovered from the chromatoplate contained varying amounts of methyl esters which were not derived from bile acids. However, these contaminants all had shorter retention times than 5α-cholestane on all stationary phases used.

[2] E. H. Ahrens, Jr., T. A. Miettinen, and S. M. Grundy. Data being prepared for publication.

Florisil Column Chromatography of Bile Acids

If stools were obtained from patients with steatorrhea or if a large yield of bile acids was desired for further definition of individual bile acids, purification by chromatography on a 5-g Florisil column was necessary. After the saponification and extraction step, the substances dissolved by 25 ml of 1% propionic acid in heptane were transferred to the column, which was then eluted with a further 50 ml of the same solvent. This eluate, containing approximately 90% of all the fatty acids, was discarded. The remaining contents of the round bottom flask were transferred quantitatively to the column with a small volume of a more polar mixture: benzene–methanol–acetic acid 80:20:1. All the bile acids were eluted from the column with 100 ml of acetic acid–ethyl ether 1:9, the bulk of the colored acidic contaminants remaining on the column. The eluate containing bile acids was evaporated; remaining traces of acetic acid were removed as the heptane azeotrope, leaving a yellow residue.

Ten milliliters of water and 25 ml of ethyl acetate were added and the mixture was swirled. The lower aqueous phase was transferred to a 50 ml glass-stoppered test tube and extracted a second time with ethyl acetate. The ethyl acetate phases containing all the bile acids (plus traces of fatty acids) were pooled and evaporated. The residue was methylated, the bile acid methyl esters were isolated by preparative TLC and quantified as a group by GLC as described above.

Isolation of Individual Bile Acids

For more detailed qualitative studies of subgroups or individual bile acids, further purification of the mixed methyl esters was carried out by repeated one-dimensional TLC, using various developing systems described by Eneroth (17) and by Hofmann (18). (Two-dimensional TLC, which proved so useful in our earlier method (5), conferred no advantages here: the one-dimensional technique proved to be simpler and equally effective.) The product isolated after these steps were completely free of contaminants, and could then be identified and quantified by GLC, using QF-1 or HiEff 8B columns at 225° to obtain optimal resolution of individual TMS bile acid methyl esters; these individual components were then pure enough for study by other techniques, such as mass spectrometry.

RESULTS

Validation of the present method for determination of total fecal bile acids has been carried out from three points of view. First, the completeness of recovery, the sources of losses, and the reproducibility of the entire procedure were defined. Second, the purity of the bile acid fraction isolated by this procedure was evaluated by com-

TABLE 1 Effect of Rigorous Saponification Conditions* on Bile Acid Structure

Cholanic Acid Derivative	Recover after Saponification†
	%
3,7,12-Trihydroxy (cholic acid)	95.5
3,7-Dihydroxy (chenodeoxycholic acid)	103.5
3,12-Dihydroxy (deoxycholic acid)	100.6
3-Hydroxy (lithocholic acid)	95.5
3,12-Dihydroxy-7-keto	99.4
3,7-Dihydroxy-12-keto	94.5
3-Hydroxy-12-keto	100.0
3-Keto-12-hydroxy	101.0
3,12-Diketo	77.7
3-Keto	72.4

* Five milligrams of bile acid in 10 ml of 2 N NaOH, 115°, 2 atmospheres' pressure for 3 hr.

† As judged by quantification of TMS ether of bile acid methyl ester with same GLC characteristics as non-saponified control.

paring the specific activity of the entire bile acid mixture with that of the plasma cholesterol in a patient who had received cholesterol-4-C^{14} 18 days before stools were collected. Third, in order to show that this method is applicable to steroid balance studies in man, the stability of the bile acid structure in its passage through the human intestinal tract was established, and the major routes of excretion were determined.

These three approaches involved the administration of radioactive compounds to patients at various steps in the procedure. However, it must be emphasized that *the effectiveness of the finally developed procedure does not depend upon the in vivo administration of radioactive compounds*. Thus, the procedure can be applied in studies of steroid metabolism in patients where the use of radioactive isotopes is inadvisable, or where their use is reserved for the exploration of physiologic problems.

Recoveries and Reproducibility

Our saponification conditions were examined for deleterious effects on bile acid structure. A 5 mg sample of each of ten bile acids (Table 1) was subjected to saponification in 2 N NaOH for 3 hr at 15 psi. The mixture was acidified with HCl, 2 mg of 5α-cholestane was added to each sample, and the mixture was extracted in the routine manner, once with chloroform–methanol 2:1 and twice with chloroform. Control samples were treated identically except for omission of the saponification step, and all samples were then methylated. Bile acid methyl ester:cholestane ratios of control and saponified samples were determined by GLC of the TMS derivatives. The recovery of saponified relative to that of non-saponified bile acids was 94–101% for bile acids containing no 3-keto groups (Table 1), indicating little or no destination of most bile acids during saponification under pressure. Two of three bile acids containing 3-keto groups were partially destroyed during saponification, but, since 3-keto bile acids usually make only a small contribution to total fecal bile acids, partial losses of these compounds should not alter the quantification of total fecal bile acids significantly.

Preliminary extraction of neutral steroids after mild saponification must be quantitative in order that the subsequent extract of bile acids not be contaminated by neutral steroids: evidence that it is quantitative is presented in the accompanying report (6). After saponification of the fecal homogenate under pressure, fecal bile acids were extracted with 75 ml of chloroform–methanol 2:1 followed by two extractions with 50 ml of chloroform.

TABLE 2 Completeness of Bile Acid Extraction from Various Fecal Homogenates after Saponification Step

Successive Extractions	Sample 1		2		3		4	
	*cpm/g**	%†	*cpm/g*	%	*cpm/g*	%	*cpm/g*	%
First Saponification (2 N NaOH, 2 atm., 3 hr)								
1. Chloroform–methanol 2:1	5881	91.0	2975	90.0	3980	91.3	5065	92.2
2. Chloroform	410	97.0	215	96.8	290	97.9	390	99.2
3. Chloroform	50	97.8	40	98.0	20	98.4	30	99.7
4. Chloroform	30	98.2	30	98.6	20	98.9	0	99.7
5. Chloroform	25	98.5	10	99.0	10	99.2	10	99.9
Second Saponification (2 N NaOH, 2 atm., 3 hr)								
6. Chloroform–methanol 2:1	30	99	20	99.6	10	99.4	0	99.9
7. *n*-Butanol	55	100	2	100	30	100	5	100
Total	6470		3300		4360		5500	

Feces samples from patient J. F., given cholic-24-C^{14} and chenodeoxycholic-24-C^{14} acids orally 4–10 days prior to stool collections, to produce maximum labeling of these primary bile acids and their metabolic products without labeling the neutral steroids.

* Radioactivity in each extract (per gram of stool homogenate).

† Cumulative percentage recovery for successive extractions, based on total counts recovered after seventh step.

TABLE 3 REPRODUCIBILITY OF BILE ACID EXTRACTION AFTER SAPONIFICATION STEP

Replicates	Radioactivity	Difference from mean
	*cpm/g**	%
1	5731	+2.10
2	5575	−0.68
3	5589	−0.42
4	5550	−1.12
5	5609	−0.07
6	5625	+0.21
Mean	5613	

Feces sample from patient J. F., given cholic-24-C^{14} and chenodeoxycholic-24-C^{14} acids orally 4 days prior to stool collection. Routine extraction method: 75 ml of chloroform–methanol 2:1 and 2 × 50 ml of chloroform.

* Per gram of stool homogenate.

Table 2 shows that the recovery of bile acids, labeled in vivo, from various samples of feces was essentially complete in these three extractions; the routine method uses these three extractions only. A fourth and fifth extraction with 50 ml of chloroform, resaponification of the aqueous phase, and two final extractions (with chloroform–methanol 2:1 and with butanol) yielded negligible additional radioactivity. In other experiments the extracted residue was analyzed by combustion (5); insignificant amounts of $C^{14}O_2$ indicated that the bile acids had been completely extracted.

The reproducibility of the bile acid extraction routinely employed was tested on replicate samples of feces from the same patient. Total bile acids extracted from six replicate 1-g stool homogenates were assayed in terms of radioactivity per gram of homogenate. As seen in Table 3, percentage differences from the mean were small.

Although the extraction step was found to be nearly quantitative, small amounts of bile acids were lost in subsequent steps of the complete procedure (including column chromatography on Florisil). These losses were variable and sometimes amounted to as much as 14% (specific losses 4–5%, nonspecific losses 9–10%, see below). Table 4 describes an experiment in which a radioactive internal standard mixture of cholic-, deoxycholic- and lithocholic-24-C^{14} acids (1:1:1) was added to seven replicate unlabeled fecal homogenates. Percentage recoveries of radioactivity after neutral steroid extraction, saponification, bile acid extraction, Florisil column chromatography, methyl ester formation, and preparative TLC were 86–94% (Table 4, column 3). The yield of bile acids in each sample was then determined by GLC and corrected according to the recoveries of internal standards. The standard deviation of the recoveries thus calculated from the mean recovery was only ±3%.

Certain bile acids in feces are even more nonpolar than lithocholic acid and might be preferentially lost during neutral steroid extraction or chromatography. The extent of these *specific losses* was measured in a study of stools (J. F.) collected 4–10 days after oral administration of radioactive cholic and chenodeoxycholic acids. Table 5 shows that the initial PE extraction of neutral steroids was essentially uncontaminated by bile acids. However, a very small fraction (approximately 2%) of bile acids was so nonpolar that it was eluted in the fatty acid fraction from the Florisil column, and another 2% was retained on the column after elution of bile acids. If TLC was carried out *after* Florisil column chromatography, the fatty acid area contained essentially no bile acids, but if preparative TLC was carried out without prior Florisil column chromatography, 2% of the labeled bile acids was found in the fatty acid area.

In addition to these specific losses, amounting to as much as 4–5% (Table 5), *nonspecific losses* of as much as 9–10% may occur at any of the several steps of the isolation procedure, owing to incomplete transfers or spillage. The data presented in Tables 4 and 5 indicated the necessity for correcting final GLC data by means of an internal recovery standard. When it was unnecessary to subject the extract to column chromatography on Florisil, the total losses (specific and nonspecific) were usually less than 5%, but were nevertheless corrected for by this means.

Specific Activities of Fecal Bile Acids and Serum Cholesterol

After administration of radioactive cholesterol to patients, the specific activity of biliary bile acids approximates that of plasma cholesterol after some days, and the two activities are almost the same for the next several weeks

TABLE 4 RECOVERY OF BILE ACIDS THROUGH COMPLETE ISOLATION AND QUANTIFICATION PROCEDURE; REPRODUCIBILITY OF METHOD

Replicates	Recovery of Radioactive Standard*		Corrected GLC Value for Fecal Bile Acids†	
	Total Radioactivity Recovered	Recovery	Per Gram of Fecal Homogenate	Difference from Mean
	cpm	%	*μg*	%
1	5,177	88.3	679	+0.44
2	5,054	86.5	719	+6.25
3	5,433	92.5	670	−0.89
4	5,380	91.7	662	−2.07
5	5,270	89.9	660	−2.36
6	5,482	93.6	669	−1.05
7	5,242	89.3	676	0.00
				SD ±3%

* Cholic-24-C^{14}, chenodeoxycholic-24-C^{14}, and lithocholic-24-C^{14} acids in equal proportions of C^{14}; 5860 cpm added to each replicate sample of nonradioactive feces.

† Corrected according to recovery of added radioactive standard.

TABLE 5 Analysis of Specific Losses of Bile Acids at Various Stages of Isolation Procedure

(GLC quantification taken as 100% in each sample, after correction for recoveries of internal standard)

Procedural Step	Radioactivity Recovered		
	Sample I	Sample II	Sample III
	cpm; % of total recovered activity in parentheses		
1. Neutral steroid extraction by PE	12 (0.24)	42 (0.96)	38 (0.65)
2. Florisil column chromatography			
(*a*) 1% propionic acid in heptane (fatty acid fraction)		81 (1.86)	103 (1.76)
(*b*) Ethyl ether–acetic acid (bile acid fraction to TLC)		—	—
(*c*) 10% acetic acid in methanol (after elution of bile acids)		86 (1.97)	110 (1.88)
3. Preparative TLC after Florisil column			
(*a*) Bile acid area		4138 (94.82)	5574 (95.31)
(*b*) Area ahead of bile acids		6 (0.13)	13 (0.22)
(*c*) Area behind bile acids		12 (0.26)	7 (0.12)
4. Preparative TLC, not preceded by Florisil column			
(*a*) Bile acid area	4800 (97.82)		
(*b*) Area ahead of bile acids	90 (1.83)		
(*c*) Area behind bile acids	5 (0.11)		

Fecal homogenates from patient J. F., given cholic-24-C^{14} and chenodeoxycholic-24-C^{14} acids orally 4–10 days prior to stool collections.

(19, 20). Yet it is not generally appreciated that fecal bile acids from stools collected on any given day may be discernibly more radioactive than plasma cholesterol obtained the same day. Table 6 presents comparisons of specific activities of simultaneously collected serum cholesterol and fecal bile acids (isolated by the present procedure) from the stools of patient E. K. who had received cholesterol-4-C^{14} orally 18 days prior to the first stool collection. It is seen that the specific activity of fecal bile acids on any given day was consistently higher than that of serum cholesterol on the same day; plots of the two slopes showed a mean difference of 11.4%. These differences can probably be explained entirely on physiological grounds, in part by the prolonged half-life of bile acids in formula-fed patients (21) and in part by retention of feces for appreciable periods of time in the large intestine.

TABLE 6 Comparisons of Specific Activities of Plasma Cholesterol and Fecal Bile Acids on Corresponding Days

Time after Isotope Administration	Specific Activities	
	Plasma Cholesterol	Total Fecal Bile Acids
days	*dpm/mg*	
15	2630	—
19	—	2300
20	2050	—
22	1972	—
23	—	1820
24	—	2172
25	1762	—
27	1680	1860
29	1650	1750
32	1600	1810
34	1495	—
36	1460	1660
41	—	1620
43	1260	1405
46	—	1350
47	1172	1310

Cholesterol-4-C^{14}, 100 μc, given orally to patient E. K., 15 days prior to first analyses.

A more detailed comparison, in which fecal bile acids were quantified both by the comparison of specific activities and by isolation as described above, is shown in Table 7. After extraction of neutral steroids in the routine manner, fecal samples were saponified under pressure, the free bile acids were extracted, aliquots of these extracts were counted, and the weight of fecal bile acids was calculated on the basis of plasma cholesterol specific activities. Fecal bile acids were also determined from

TABLE 7 Comparisons of Quantification of Fecal Bile Acids by Isotopic Calculation and by Present Isolation Procedure

Time after Isotope Administration	Isotope Method			Isolation Method
		Fecal Bile Acids		Fecal Bile Acids
	Plasma Cholesterol Specific Activity	Dpm/mg of Fecal Homogenate	Mg/g of Fecal Homogenate	Mg/g of Fecal Homogenate
days	*dpm/mg*			
23	1972	3130	1.55	1.40
24	1900	3300	1.74	1.56
27	1680	2050	1.22	0.95
29	1650	1860	1.11	0.97
33	1600	1550	0.97	0.86
41	1300	1635	1.02	1.15

Both analyses carried out on fecal samples collected from patient E. K., given cholesterol-4-C^{14}, 100 μc, orally 23 days prior to first analyses.

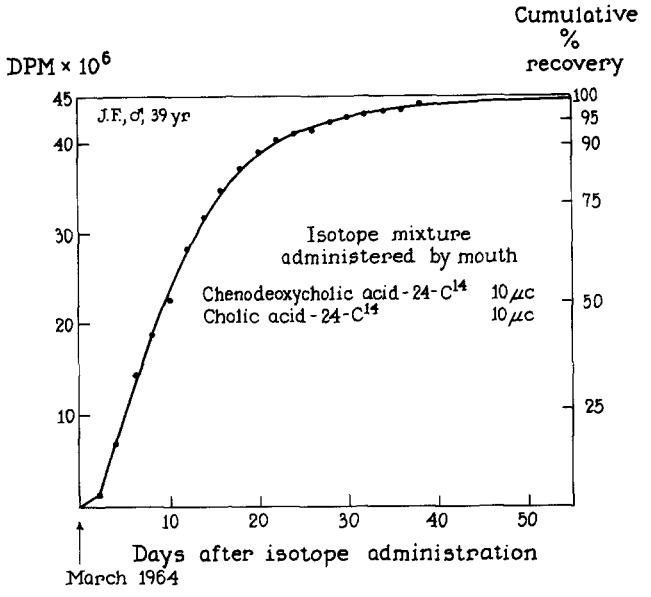

FIG. 2. Cumulative recovery of total radioactivity in fecal bile acid fraction of present procedure, after oral administration of two primary C^{14}-bile acids to 39-yr-old hypercholesteremic male.

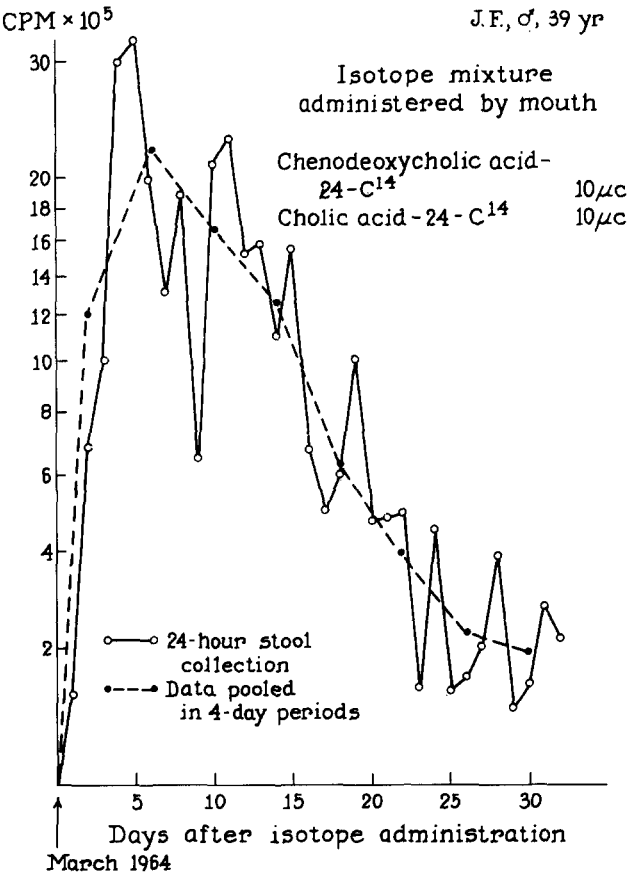

FIG. 3. Daily excretion of radioactivity in fecal bile acid fraction after oral administration of two primary C^{14}-bile acids to 39-yr-old hypercholesteremic male.

other aliquots by the complete isolation procedure described in this report: Florisil column, TLC, and GLC. As shown in the last two columns of Table 7, the results of GLC analysis were lower in all cases (except one) than those obtained by the radioactivity method.

The consistency with which we have been able to quantify accurately 11 different bile acids in 25 reference mixtures containing known weights of 5α-cholestane as internal standard was taken as strong evidence that bile acids were not being *under*estimated by our GLC procedure (thus causing falsely high specific activities in the experiments described in Tables 6 and 7). The bile acids used in our reference mixtures represented a wide range of polarity (methyl cholanate to methyl cholate) and many combinations of hydroxyl and keto functions at positions 3, 6, 7, and 12 on the cholanic acid ring.[3] Therefore, the above results can be explained only by assuming a delay of several days between the formation of bile acids from cholesterol and their excretion in the feces. It cannot be assumed that this delay is the same for all patients, or even for any one patient at different times.

Validation of the Purity of the Final Bile Acid Fraction

The data presented in Tables 6 and 7 support the notion that our GLC analysis measured bile acids only, for the presence of GLC-detectable contaminants in the finally isolated bile acid fraction would have resulted in specific activities for fecal bile acids *lower* than those of plasma cholesterol on the corresponding day.

In further studies of these labeled extracts, the total bile acid mixtures were subdivided by preparative TLC (17, 18) into seven subgroups. The specific activities of these subfractions, calculated from radioactivity measurements and mass analyses by GLC, were then compared. The specific activity of the total mixture was 2105 dpm/mg; activities of the seven subgroups ranged from 2040–2430 dpm/mg with a mean of 2185 and a standard deviation of 140 dpm/mg (coefficient of variation, 6.4%). Although the specific activities of the various individual bile acids would not be expected to be identical, the close correspondence of the specific activity of the total mixture with those of its component parts suggested that the solutes in the subfractions were either entirely composed of bile acids or that all were almost equally contaminated by impurities other than bile acids. The latter possibility seems extremely remote.

Further indication of the accuracy of the final GLC analysis of fecal bile acids was afforded by studies of feces

[3] The bile acids so tested included methyl cholanate and the following derivatives of it: 3αOH; 3,12 diketo; 3αOH, 12-keto; 3αOH, 7βOH; 3αOH, 6αOH; 3αOH, 7αOH; 3αOH, 12αOH; 3αOH, 7,12-diketo; 3αOH, 7 keto, 12αOH; and 3αOH, 7αOH, 12αOH.

TABLE 8 DAILY FECAL BILE ACID EXCRETION IN MAN, RATS, AND RABBITS

Comparison of results obtained since 1957 in various laboratories by several methods

Authors	Method of Measurement	Number and Clinical Status	Diet and Exptl. Variables	Daily Fecal Bile Acid Excretion
I. Human Subjects				
				mg/24 hr
Gordon et al. (1957) (24)	$Zn(OH)_2$ pptn.; titration (46)	4 with various illnesses	Basal = low-fat solid foods	142
			Plus coconut oil (100 g)	127
			Plus sunflower seed oil (100 g)	258
Lindstedt (1957) (25)	Cholic acid decay	8 normals	Ordinary solid foods (uncontrolled)	360 (cholic and cholic products only)
Haust and Beveridge (1958) (26)	Spectrophotometry	10 normals	Formula feeding	
			Butter (60% of calories)	180
			Corn oil (60% " ")	250
			Fat-free	277
Curran et al. (1959) (27)	$Zn(OH)_2$ pptn; titration (46)	5 normals	Solid foods (controlled repetitive menu)	
			Control	247–570
			Plus vanadium	118–725
Engelberg (1959) (28)	$Zn(OH)_2$ pptn; titration (46)	3 normals	Ordinary solid foods (uncontrolled)	
		1 hypercholesteremic	Control period	220–456
			Plus heparin(intraven.)	143–580
Goldsmith et al. (1960) (29)	Silicic acid column; titration	2 hypercholesteremic	Solid foods (controlled)	
			50 g of mixed fats	550–950
			90 g of butter fat	500–1200
			90 g of cornoil	1100–1600
			90 g of butter fat plus neomycin	2000–4000
Lindstedt and Ahrens (1961) (20)	Cholic acid decay	2 hypercholesteremic	Formula feeding 40% fat calories	87–93 (cholic products)
Antonis and Bersohn (1962) (30)	Titration	58 normals (29 white, 29 Bantu)	Solid foods (controlled) 40% fat calories	
			Butter	343–380
			Sunflower seed oil	546–570
			15% fat calories	320–430
Rosenfeld and Hellman (1962) (31)	Isotopic balance (47)	2 patients (diagnosis not stated)	Not stated	290
Moore et al. (1962) (32)	Isotopic balance (47)	5 normal	Solid foods (controlled: 40% fat calories)	
			Butter	473
			Safflower oil	557
Miller et al. (1962) (33)	Silicic acid column (29); titration	1 hypercholesteremic	Solid foods (115 g of animal fats)	
			Control	550–750
			Plus nicotinic acid	500
Roels and Hashim (1964) (34)	Ion-exchange (45) and glass paper chromatography and charring (50)	1 hypercholesteremic	Formula feeding	
			Coconut oil	50–550
			Corn oil	275–550
			Medium chain glycerides	50–450
		1 normal	Coconut oil	500–1200
			Corn oil	900–1900
Powell et al. (1962) (35)	Spectrophotometry (45)	8 normals	Solid foods (controlled: 33% fat calories)	
		5	Control	843–871
			Plus neomycin	1410–2114
			Post-treatment	454–738
		3	Control	687–704
			Plus polymixin B and bacitracin	776–1710
			Post-treatment	404–1137
Danielsson et al. (1963) (22)	Cholic and chenodeoxycholic decay (25)	2 normal	Solid foods (controlled)	
			40% butter fat calories	190–200 (cholic products)
				290–390 (cheno products)
Spritz et al. (1965) (5)	Florisil column, TLC, and titration plus isotopic balance	1 normocholesteremic	Formula feeding (40% fat calories)	
		2 hypercholesteremic	Saturated	101–247
		1 hyperglyceridemic (CHO-type)	Unsaturated	134–271

(*Continued*)

TABLE 8 *Concluded*

Authors	Method of Measurement	Number	Diet and Exptl. Variables	Daily Fecal Bile Acid Excretion
II. Rats				*mg/kg body wt per 24 hr*
Portman and Murphy (1958) (36)	Cholic acid decay (25)	4	Rat chow	36.4
		4	Synthetic—starch	10.3
		3	Synthetic—sucrose	7.7
		4	Synthetic—sucrose plus fiber	23.4
Eriksson (1960) (37)	Cholic acid decay (25)	21	Oats and barley	22
Strand (1963) (38)	Cholic acid decay (25)	22	Rat chow	22.8
Roscoe and Fahrenbach (1964) (39)	Charcoal adsorption and titration	10	Rat chow (Purina)	52.5
Wilson (1964) (21)	Isotopic equilibrium after implantation of cholesterol-4-C^{14}	6	Synthetic (16% fiber)	
		6	Sterol-free	11
		7	0.5% cholesterol	31
Miettinen and Ahrens (1965) (unpub.)	Present method (isolation and GLC)*	5	Rat chow	40.5
		10	Synthetic (10% fat calories; sterol-free coconut oil)	17.3
III. Rabbits				
Mosbach et al. (1956) (40)	Diatomaceous earth column; titration (51)	6	Rabbit pellets (Purina)	24
Hellström and Sjövall (1962) (41)	Deoxycholic acid decay (25)	26	Pellets (3.5% fat calories)	27
Hellström et al. (1962) (42)	Deoxycholic acid decay (25)	10	Pellets (3.5% fat calories)	27.5
			Synthetic (26% fat calories)	
		10	Coconut oil	10.9
		8	Corn oil	11.1

* Identical results obtained by isotopic equilibrium technique after cholesterol-4-C^{14} implantation (21).

from rats implanted with cholesterol-4-C^{14} according to Wilson (21); these studies (T. Miettinen and E. H. Ahrens, Jr.) will be reported separately. The purpose of this procedure was to produce a plateau of plasma cholesterol specific activity, at which time the input of newly formed unlabeled cholesterol molecules was in equilibrium with the absorption of labeled molecules from the implant. When that plateau was attained, it was found that the specific activity of the fecal acidic steroids remained identical with that of plasma cholesterol for many subsequent weeks. Physiological considerations would lead one to expect this correspondence; that it was in fact demonstrated is further evidence for the accuracy of the mass analyses of fecal bile acids by our GLC procedure.

Stability of Bile Acid Structure during Intestinal Transit

The cumulative recovery of bile acid metabolites following oral administration of cholic-4-C^{14} and chenodeoxycholic-4-C^{14} acids to patient J. F. is shown in Fig. 2. All feces were collected in 24-hr pools for 2 months; aliquots were saponified and extracted according to the present procedure, and the total radioactivity of each extract was determined. Approximately 98% of administered radioactivity was excreted in the stools within 38 days. The radioactivity in 24-hr urine samples also was measured in 4 samples collected in the second week after administration of the radioactive bile acids: daily urinary radioactivity totaled 1–2% of daily fecal radioactivity. Daily variations in excretion of labeled fecal bile acids were large (Fig. 3), but when the data were pooled in 4-day periods a smooth excretion curve was obtained.

This experiment indicated that for the one patient so tested these primary bile acids and their metabolic by-products were almost quantitatively excreted in the stools. In addition to supporting one of the premises on which sterol balance studies must be based, this experiment further validated the quantitative aspects of the present procedure.

DISCUSSION

Bile acids are excreted in the feces in a wide range of molecular forms. The primary bile acids of man (1), cholic and chenodeoxycholic acids, are synthesized from cholesterol and conjugated with taurine or glycine in the liver. The conjugates are excreted into the intestine where partial deconjugation and transformation into a series of secondary products are caused by bacterial action. The numerous secondary bile acids found in feces, some of which have been identified (22, 23), result from a series of reductions, oxidations, and steric inversions of hydroxyl groups on the steroid nucleus of the primary acids.

The complexity of the mixture of fecal bile acids makes it impossible to approach their determination by methods based on the presence of any specific functional groups other than the side-chain carboxyl group. Our earlier efforts to estimate fecal bile acids by titration depended upon devising methods for avoiding titratable contaminants. While these efforts were sometimes successful (5), continuing use of these procedures showed that in some samples of feces we were not able to obtain the bile acids free from these contaminants. Thus we turned to GLC for measurement of the cyclopentanophenanthrene nucleus (which is the only feature other than the -COOH group that is shared by all bile acids). This method proved to be quantitative and also simpler: it was no longer necessary to isolate the bile acids from all the acidic contaminants, since these proved to have shorter retention times during GLC than any of the bile acids. Thus, in the present procedure the quantitative analysis of bile acids is carried out simultaneously with final chromatographic purification. This report presents the evidence for our claim that we are measuring essentially all the fecal bile acids and nothing but bile acids.

Table 8 lists the various procedures which have been applied to the estimation of fecal bile acids in other laboratories, and results obtained in man, rats, and rabbits. This summary is presented for completeness' sake, even though we have considerable reservations about the validity of some of the results obtained. For instance, in on-going experiments in six hypercholesteremic patients we have found by the present method that the daily excretion of fecal bile acids ranged from 120 to 225 mg; these patients were maintained at constant body weight on formula diets, with cholesterol intakes lower than 100 mg/day and with fat intakes equivalent to 40% of total calories. While the observations of some of the laboratories listed in Table 8 are in accord with these findings, others report considerably higher values. Some part of the discrepancy seems to be due to methodologic differences. Thus, the method of Mosbach, Kalinsky, Halpern, and Kendall (43) for specific analysis of di- and trihydroxy bile acids by spectrophotometry has been used in some laboratories (26, 35); in our view it is inappropriate for the measurement of the multiple forms of bile acids in feces, and indeed it neglects the mono-substituted cholanic acids which form a large proportion of the fecal bile acid mixture. Lewis (44) described a method for titration of fecal bile acids after zinc hydroxide precipitation. In our hands this method failed to yield complete recoveries when tested with fecal samples from patients whose bile acids had been labeled in vivo with C^{14}-cholesterol or C^{14}-bile acids, nor were all titratable contaminants removed. Chromatography on ion-exchange columns according to Kuron and Tennent (45) provided quantitative recovery of bile acids, but acidic pigments were incompletely removed; despite repeated washings of the columns, acidic materials which were difficult to separate from the bile acids continually bled from the ion-exchange resin. The method of Goldsmith, Hamilton, and Miller (46), which depends upon separating the bile acids from sterols and fatty acids on silicic acid columns and determining them by titration, failed in our hands due to incomplete removal of titratable contaminants. In studies of bile acids in rat feces, Roscoe and Fahrenbach (39) utilized activated charcoal to remove acidic pigments, prior to titration of the bile acids. However, human feces generally contain much larger quantities of acidic pigments, and charcoal proved to be ineffective for their complete removal. Thus, it appears that most titration methods run the danger of overestimating the fecal bile acids, but in some procedures this overestimation may be concealed by the incompleteness of fecal bile acid extractions and recoveries.

Several laboratories have measured total fecal bile acids by determining the radioactivity in appropriate extracts of the feces of patients to whom radioactive cholesterol had previously been administered. This approach was first used by Hellman, Rosenfeld, Insull, and Ahrens (47), and is based on the assumption that fecal bile acid specific activity is equal to that of plasma cholesterol in samples collected simultaneously. As shown in Tables 6 and 7, a correction factor must be applied in order to compensate for the appreciable interval between the formation of bile acids and their eventual excretion in the stools; the magnitude of this factor probably is variable from day to day and from patient to patient. However, when some such correction is made, the radioactive technique provides values for total fecal bile acids which are comparable with those obtained by GLC analysis. The method of quantification from isotope data has the advantage of technical simplicity and speed, but certain disadvantages other than that already mentioned should be noted. First, the technique depends upon the prior administration of radioactive cholesterol or precursors, and in many clinical situations this may not be permissible because of radiation hazard. Second, the use of isotopes for purely methodologic purposes may deprive the investigator of the opportunity to employ them for exploration of dynamic physiological questions. And third, a time period of considerable length must ensue between the administration of the isotope and the beginning of blood, tissue, and fecal collections, owing to the relatively slow rate of equilibration of cholesterol and bile acids in plasma and other tissue pools. In our experience with five formula-fed patients the crossover of the specific activity curves of plasma cholesterol and fecal bile acids occurred at a time between 7 and 21 days, whereas in normal young males eating solid foods Lindstedt (48) found crossovers at 4–5 days.

Procedures for quantification of fecal bile acids by GLC or isotopic methods depend upon complete removal of the neutral from acidic steroids. Lewis and Myant (49) noted that extraction of neutral fecal steroids from an alkaline saponification mixture by hexane was incomplete. We have observed that after saponification under pressure the extraction of neutral steroids, especially coprostanol, by PE from fecal extracts containing bile acids was incomplete. This separation problem was solved by carrying out a preliminary mild saponification under reflux, followed by PE extraction from a 60% alkaline ethanol mixture to obtain quantitative separation of neutral from acidic steroids (6). Then, in order to complete the hydrolysis of conjugated bile acids, the acidic fraction was saponified at a pressure of 2 atmospheres.

The present method of quantitative isolation of fecal bile acids, when carried out in parallel with a procedure for isolating and quantifying the neutral steroids [see the accompanying report (6)], makes it possible to carry out sterol balance studies in man or in small laboratory animals. In addition, it is feasible also to measure the rate of conversion of cholesterol to bile acids in the intact organism, which in the steady state is directly reflected in the daily excretion of bile acids. Lindstedt and his associates (20, 22, 25) have published valuable data bearing on this question which are based on decay curves of specific biliary bile acids after administration of radioactive bile acids. This technique, which in man requires 4 or 5 duodenal aspirations of bile at 2- to 3-day intervals, permits the measurement of the pool size, turnover, and synthesis rates of the particular bile acid administered in radioactive form. Although the procedure does not lend itself to studies of sterol balance or total conversion of sterol to bile acids, it should prove a valuable adjunct to any satisfactory sterol balance technique for more detailed studies of specific bile acids.

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