Characterization of trisubstituted cholanoic acids in human feces

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ABSTRACT The trisubstituted cholanoic acids in human feces have been studied by gas chromatography–mass spectrometry. The following bile acids have been identified: 3β,7α,12α-trihydroxy-, 3β,7β,12α-trihydroxy-, 3α,7α-dihydroxy-12-keto-5β-cholanoic acids and 3α,7α,12α-trihydroxy-5α-cholanoic acid. The presence in human feces of 3α,7α,12α-trihydroxy-, 3α,7β,12α-trihydroxy-, and 3α,12α-dihydroxy-7-keto-5β-cholanoic acids has been confirmed.

The composition of bile acids in human feces is summarized and possible metabolic interrelationships suggested.

KEY WORDS bile acids · trifluoroacetates · partial trimethylsilyl ethers · dimethylhydrazones · gas–liquid chromatography · mass spectrometry · identification · feces · man

An important pathway for the elimination of cholesterol from the body is the conversion into bile acids which are excreted as a complex mixture in the feces. It is well established that the complexity of this mixture is a consequence of the extensive transformations of the primary bile acids induced by the intestinal flora (1). Some of the bile acid isomers formed by the microorganisms are less available for absorption into the enterohepatic circulation than others (2). Furthermore, the effect exerted by various bile acids on the control of cholesterol and bile acid synthesis does not appear to be uniform (3–6). In view of these characteristic differences among the bile acids it is important that the composition of the final bile acid mixture be known and factors affecting the activity of bile acid-metabolizing microorganisms be studied, since this microbial activity ultimately determines the pattern of the fecal bile acids.

A few mono- and disubstituted cholanoic acids that occur in the fecal bile acid mixture have been isolated and identified by previous investigators (7–9). In the preceding paper these findings have been reviewed and a more complete appraisal of the mono- and disubstituted acids was reported (10). The present communication deals with the isolation and identification of the trisubstituted cholanoic acids.

MATERIALS AND METHODS

Isolation and Analysis of Fecal Bile Acids

Fecal samples were collected from a number of subjects consuming a standardized diet which provided 56% of the calories from butterfat. The subjects were given cholic acid-24-^{14}C prior to the collection of feces to facilitate monitoring of the isolation of the bile acids.

The feces were extracted and saponified and the crude lipids were fractionated on silicic acid columns (10). In this way two fractions were obtained: one containing predominantly the mono- and disubstituted and another the trisubstituted cholanoic acids. Fecal samples from a single subject were chosen for the present investigation because of their relatively high content of trisubstituted acids. Two consecutive samples, each collected over a 2 day period, were studied.

The fraction from the initial silicic acid chromatography which contained the polar acids (10) was dissolved in ethyl acetate–benzene 1:1 and applied to a silicic acid column providing a 50-fold excess of silicic acid. Elution of the bile acids was initiated with 50% ethyl acetate in

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Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; TFA, trifluoroacetate(s); TMSi, trimethylsilyl; RRT, relative retention time; GC–MS, mass spectrometry of GLC effluents.

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benzene and continued with progressive increments in ethyl acetate concentration up to 100%. The weight and radioactivity of the material eluted with each solvent were determined. Each fraction was analyzed by TLC and, after methylation with diazomethane, by GLC. The retention times were calculated relative to the retention time of methyl deoxycholate and will be referred to as RRT values.

The individual acids were isolated by preparative TLC of their methyl esters. The compounds were then characterized by GLC of different derivatives (partial trimethylsilyl ethers, trifluoroacetates, oxidation products, 1,1-dimethylhydrazones) and by mass spectrometric analysis of the GLC effluent (GC–MS) as described in the preceding paper (10).

Reference Compounds. Most of the reference compounds were those used in previous investigations of this series. 3α,7α,12α-trihydroxy-5α-cholanoic acid was a gift from Prof. G. A. D. Haslewood.

3β,7β,12α-Trihydroxy-5β-cholanoic Acid

Methyl 3α,12α-dihydroxy-7-keto-5β-cholanoate, 6.0 g, prepared according to Fieser and Rajagopalan (11), was dissolved in 75 ml of dry acetone. Aluminum *tert*-butylate (7.2 g) in 180 ml of dry benzene was added and the mixture heated under reflux for 18 hr (9, 12). The reaction mixture was worked up in the usual manner and chromatographed on a column of 100 g of aluminum oxide (Woelm, Eschwege, Germany, grade IV). Elution with 30% ethyl acetate in benzene provided 1.879 g of methyl 3,7-diketo-12α-hydroxy-5β-cholanoate. Crystallization from ethyl acetate yielded 1.651 g of large rectangular crystals mp 191–192°C. The mass spectrum of the trimethylsilyl ether showed a molecular weight of 490. The base peak was at m/e 285, i.e., M−(90 + 115) (trimethylsilanol + side chain).

Methyl 3,7-diketo-12α-hydroxy-5β-cholanoate (750 mg) was dissolved in freshly distilled *n*-propanol (15 ml); metallic sodium (1.5 g) was added and the mixture heated under reflux for 3 hr (13). The reaction mixture was diluted with water, acidified, and extracted with ethyl acetate. The extract was evaporated to dryness and the residue was methylated and chromatographed on a column of 50 g of aluminum oxide (Woelm, grade IV). Elution with 40% ethyl acetate in benzene yielded 88 mg of impure methyl 3β,7β,12α-trihydroxy-5β-cholanoate. This material was rechromatographed on a 15 g silicic acid column. Elution with 45% ethyl acetate in benzene yielded 29 mg of methyl 3β,7β,12α-trihydroxy-5β-cholanoate, which was pure according to TLC and GLC. The methyl ester could not be crystallized and was therefore hydrolyzed. The free acid was crystallized from ethyl acetate–heptane, mp 154–157°C, $[\alpha]_D^{23} = +70.0°$ (c 1.02; ethanol).

Analysis: $C_{24}H_{40}O_5 + {}^1/_2$ ethyl acetate;

calculated: C, 69.1; H, 9.7

found: C, 68.7; H, 9.7

The mass spectrum of the methyl ester trifluoroacetate was typical of a methyl trifluoroacetoxy-cholanoate (see Results).

RESULTS

Fig. 1 shows a gas chromatographic analysis of the methylated fraction from the first silicic acid chromatography that contained the trisubstituted cholanoic acids. The RRT of the peaks are indicated in the figure. Two pairs of compounds were not resolved; the peaks with RRT 2.17 and 2.46 represent mixtures.

Table 1 summarizes the results of the silicic acid chromatography of the trisubstituted cholanoic acids. GLC of each fraction indicated that this second silicic acid chromatography had resulted in a partial separation of three major groups of bile acids: monoketo-dihydroxycholanoates, trihydroxycholanoates with a 7β-hydroxyl group, and trihydroxycholanoates with a 7α-hydroxyl group.

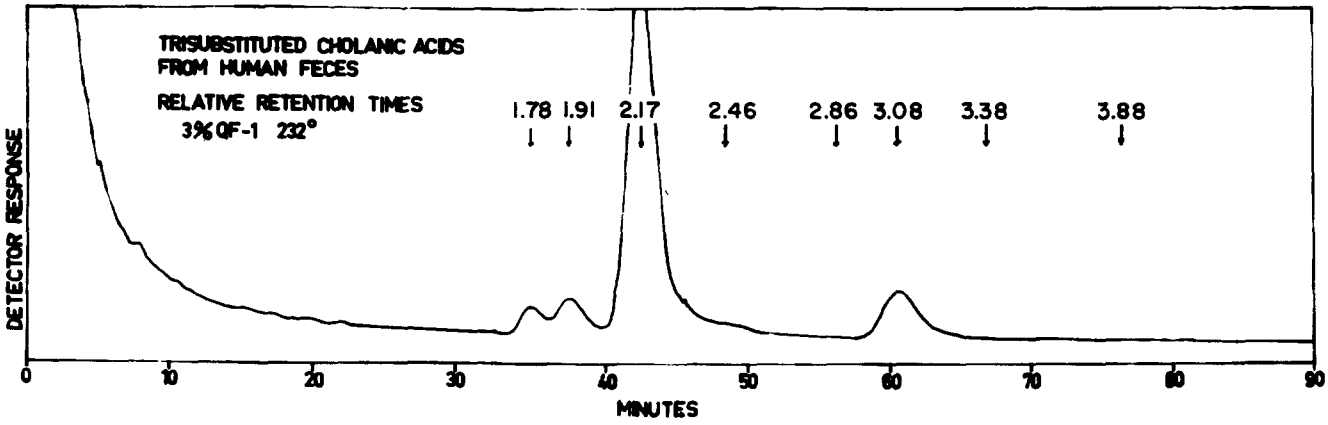

FIG. 1. Gas chromatographic analysis of the methylated fraction from the initial silicic acid column containing the trisubstituted cholanoic acids. Retention times are relative to that of methyl deoxycholate (not shown). For subsequent identifications see Table 2.

The individual methylated compounds were isolated by preparative TLC of the fraction in which they occurred in the highest concentration. Their GLC behavior and that of the different derivatives were compared with appropriate reference compounds. The trifluoroacetates were used for the GC–MS analyses.

Trihydroxycholanoic Acids

3,7,12-Trihydroxy-5β-cholanoic acids represented the major group of trisubstituted bile acids. TLC with solvent system S 7 (14) provided a separation of 3β,7α,12α- (relative mobility 0.81), 3α,7α,12α- (rel. mob. 1.00), 3β,7β,12α- (rel. mob. 1.08), and 3α,7β,12α-trihydroxycholanoic (rel. mob. 1.22) acids. The results of the GLC analyses are summarized in Table 2. The presence of a 3β- (axial) hydroxyl group is indicated by the relatively short retention times of the methyl esters of compounds having this substituent. A 7β- (equatorial) hydroxyl group is indicated by the formation of a ditrimethylsilyl ether under the selective conditions used for the reaction with hexamethyldisilazane (7α- and 12α-hydroxyl groups do not react under these conditions). The mass spectra showed essentially the same fragmentation patterns for the trifluoroacetates (TFA) of the four trihydroxycholanoates. The mass spectrum of the TFA of methyl 3β,7β,12α-trihydroxy-5β-cholanoate from feces is shown in Fig. 2.

Although some differences were noted in the relative intensities of the peaks at m/e 482 (loss of 2 moles of trifluoroacetic acid: M-2×114), m/e 368 (M-3×114), m/e 367 [M$-(2\times114+115$(side chain))], and m/e 253 [M$-(3\times114+115)$], these differences were not sufficient for the establishment of the configuration of the substituents. Furthermore, in different analyses with different instruments the relative intensities varied for each compound. In Table 2 the result of the GC–MS analysis is noted as typical for a trihydroxycholanoate. The GLC data, however, show the configuration of the hydroxyl groups (Table 2).

Minor amounts of two compounds were found (RRT 2.37 and 2.46, Table 2) and their mixture yielded oxidation products with the RRT of methyl 3,7,12-triketo-5α-cholanoate. The compounds could not be separated by TLC in any of the solvent systems used for trihydroxycholanoates (14). On GLC only an incomplete separation was obtained of the methyl esters, whereas two separate peaks were obtained after selective formation of TMSi ethers. The TFA derivatives of the compounds appeared as a single peak. The mass spectrum of the mixed TFA compounds was indistinguishable from that of the TFA of methyl cholate. This result, together with the GLC data (Table 2), shows that one of the bile acids was 3α,7α,12α-trihydroxy-5α-cholanoic acid. The result of the oxidation indicated that the other compound might also be a 3,7,12-trihydroxy-5α-cholanoate. TLC showed that it was not identical with 3α,7β,12α-trihydroxy-5α-cholanoic acid (kindly given to us by Prof. Haslewood).

Dihydroxy-monoketo-5β-cholanoic Acids

The predominant bile acid in this group was 7-ketodeoxycholic acid (3α,12α-dihydroxy-7-keto-5β-cholanoic acid). The amount of this compound relative to that of the other trisubstituted bile acids is indicated in Fig. 1 (peak with RRT 3.08). The mass spectrum of the TFA showed a base peak at m/e 269 [M$-(2\times114+115)$],

TABLE 1 SILICIC ACID CHROMATOGRAPHY OF THE PARTIALLY PURIFIED TRISUBSTITUTED CHOLANOIC ACIDS FROM FECES

Eluting Solvent (% Ethyl Acetate in Benzene)	50	55	60	65	70	75	80	100
Fraction: Weight, *mg*	9	7.3	4.4	12.6	15.3	10.4	12.0	8.0
Radioactivity, *cpm*	2,300	6,650	3,400	17,800	29,100	15,150	4,700	1,670
GLC Retention Times Relative to Methyl Deoxycholate								
1.81				tr.	+	+	tr.	
1.93				+	tr.			
2.14			tr.	+++	tr.			
2.17		tr.	tr.	tr.	+++	++++	++	+
2.37						+	+	
2.46						+	+	
2.52							tr.	
2.86				tr.				
3.07		+++	++					
3.33		tr.	+					
3.88		tr.						

The notations + and tr. refer to a rough estimation of the proportions between the compounds in each fraction as judged from GLC.

TABLE 2 RESULTS OF GLC AND GC–MS ANALYSES OF COMPOUNDS ISOLATED FROM HUMAN FECES

Silicic Acid Fraction (% Ethyl Acetate in Benzene)	Solvent Systems Used in TLC (ref. 14)	Retention Times Relative to Methyl Deoxycholate of Different Derivatives*				GC–MS Results	Identification*
		Methyl Ester	TFA	TMSi	Oxidation Product		
50	S 14, S 7	2.52	1.45	1.72	5.72	—	See text
55	S 7	3.10 (3.07)	1.99 (1.99)	1.86 (1.88)	5.65 (5.60)	Identical	3α,12α,7-keto (ref. 3α,12α,7-keto)
	S 7	3.88† (3.94)‡	1.98 (2.44)	2.29 (3.94)	5.52 (5.60)	—	See text (ref. 3-keto-7α,12α)
60	S 7	3.33 (3.33)	2.37 (2.42)	2.05 (2.09)	5.49 (5.60)	Identical	3α,7α,12-keto (ref. 3α,7α,12-keto)
65	S 7	1.91 (1.93)	1.00 (1.00)	0.65 (0.65)	5.59 (5.60)	Trihydroxy-cholanoate	3β,7β,12α (ref. 3β,7β,12α)
	S 7	2.14 (2.17)	1.19 (1.18)	0.74 (0.74)	5.62 (—)	Trihydroxy-cholanoate	3α,7β,12α (ref. 3α,7β,12α)
	S 7	2.86	1.58	—	§	See text	See text
70	S 7	1.80 (1.81)	0.95 (0.95)	1.13 (1.13)	5.56 (5.60)	Trihydroxy-cholanoate	3β,7α,12α (ref. 3β,7α,12α)
	S 7	2.17 (2.14)	1.30 (1.29)	1.23 (1.23)	5.60 (5.60)	Trihydroxy-cholanoate	3α,7α,12α (ref. 3α,7α,12α)
80	S 6, S 2	2.37 (2.37)	1.35 (1.37)	1.27 (1.31)	7.18 (7.25)	See text	3α,7α,12α(5α) [ref. 3α,7α,12α(5α)]
	S 6, S 2	2.46	1.35	1.69	7.18	See text	See text

TFA, trifluoroacetate; TMSi, partial trimethylsilyl ether. GLC was on 3% QF-1 at 232 °C.
* Substituted 5β-cholanoic acids unless otherwise noted. Greek letters denote configuration of hydroxyl groups on carbon atoms 3, 7, or 12.
† RRT of dimethylhydrazone 1.98.
‡ RRT of dimethylhydrazone 1.89.
§ Several products formed.

pronounced peaks at m/e 383 [M−(114 + 115)] and m/e 384 (M−2 × 114), and smaller peaks (relative intensity 10–15%) at m/e 498 (M−114) and m/e 251 [M−(2 × 114 + 115 + 18)].

Only small amounts of 3α,7α-dihydroxy-12-keto-5β-cholanoic acid were present. The typical mass spectrum of its TFA is shown in Fig. 3. Because of the presence of a 12-keto group, the loss of a fragment of mass 155 [M−155, M−(114 + 155), and M−(2 × 114 + 155)] is a prominent feature. This corresponds to the loss of the side chain and the D-ring with capture of one hydrogen by the fragment containing the 12-keto group (15).

Unidentified Cholanoic Acids

Two compounds present in trace amounts possibly also belonged to the dihydroxy-monoketo-5β-cholanoic acids. Oxidation of the compound with RRT 3.88 (as the methyl ester) yielded a derivative with the RRT of methyl 3,7,12-triketo-5β-cholanoate. A dimethylhydrazone with an RRT of 1.98 could be prepared from the fecal bile acid; this indicates the presence of a 3-keto group (also indicated by the high RRT value of the parent compound). Since a TMSi ether was formed, the compound could not be methyl 3-keto-7α,12α-dihydroxy-5β-cholanoate, the GLC data for which are also shown in Table 2. It might therefore have a 7β- or a 12β-hydroxyl group.

The behavior of the compound with an RRT of 2.52 on silicic acid column and thin-layer chromatography and the RRT of its oxidation product indicated that this compound was a ketonic bile acid. The short RRT of the methyl ester might be due to the presence of a 3β-hydroxyl group and it can be speculated that the compound was 3β,12α-dihydroxy-7-keto-5β-cholanoic acid methyl ester, which can be calculated to have an RRT of 2.54.

One compound did not yield a triketocholanoic acid methyl ester on oxidation. This compound, the methyl ester of which had an RRT of 2.86, gave a mass spectrum (as the TFA) with a base peak at m/e 299. Apart from this feature the mass spectrum showed pronounced peaks at m/e 482, 368, and 253, typical of a trisubstituted methyl cholanoate.[1] It is therefore possible that this trace component represents a bile acid not belonging to the 3,7,12-substituted class.

DISCUSSION

The studies presented in this and the preceding paper (10) have shown that the human fecal bile acids are a

[1] Eneroth, P., B. Gordon, R. Ryhage, and J. Sjövall, to be published.

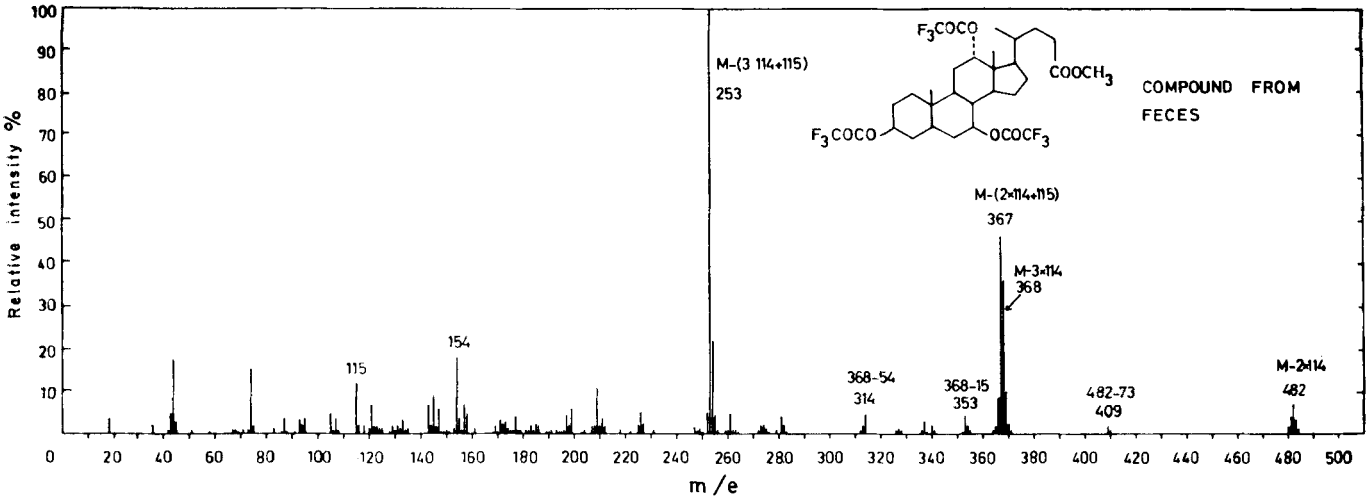

FIG. 2. Mass spectrum of the trifluoroacetate of methyl 3β,7β,12α-trihydroxy-5β-cholanoate isolated from feces.

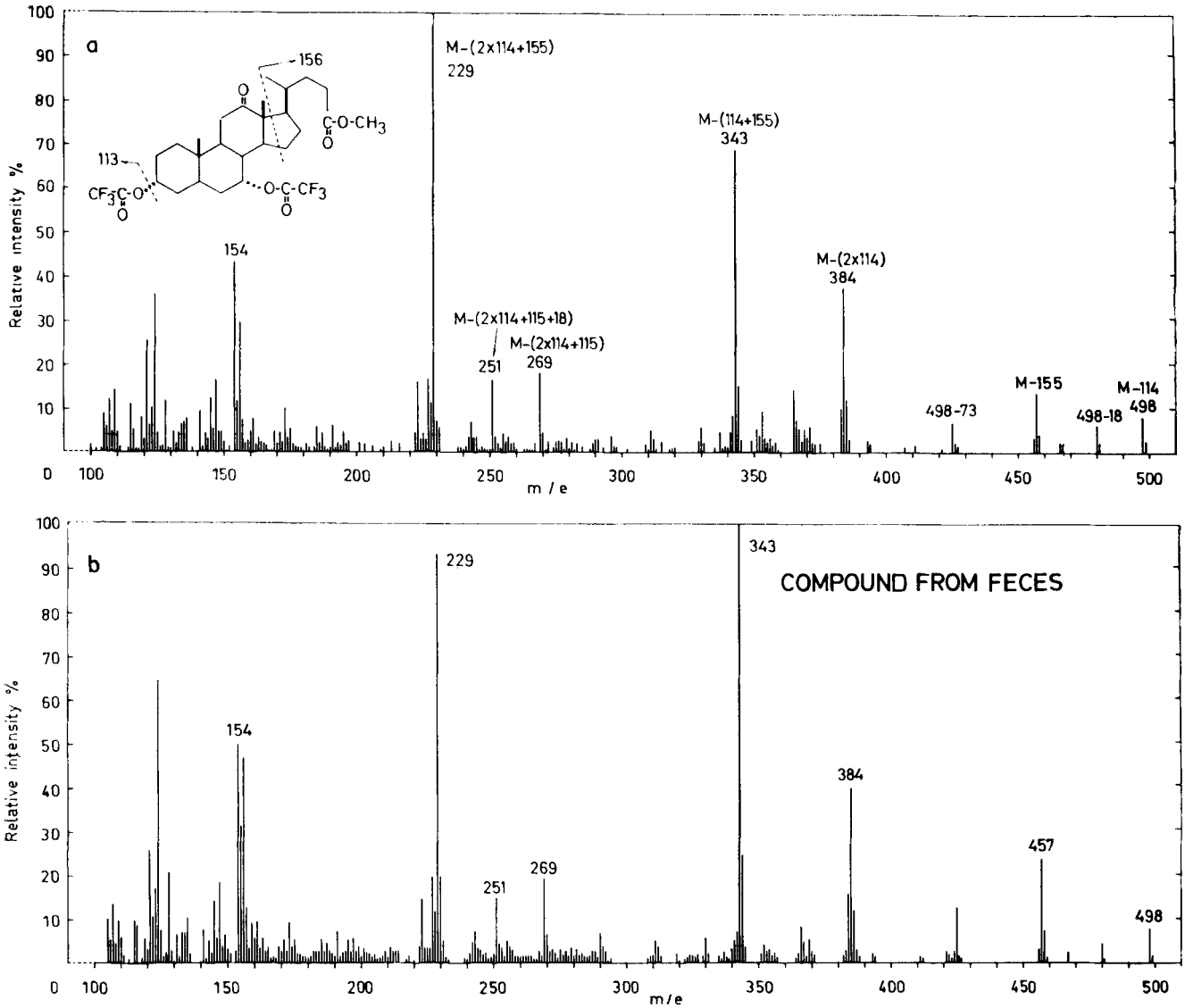

FIG. 3. Comparison between the mass spectra of the trifluoroacetates of authentic methyl 3α,7α-dihydroxy-12-keto-5β-cholanoate (Fig. 3a) and of the compound isolated from feces (Fig. 3b).

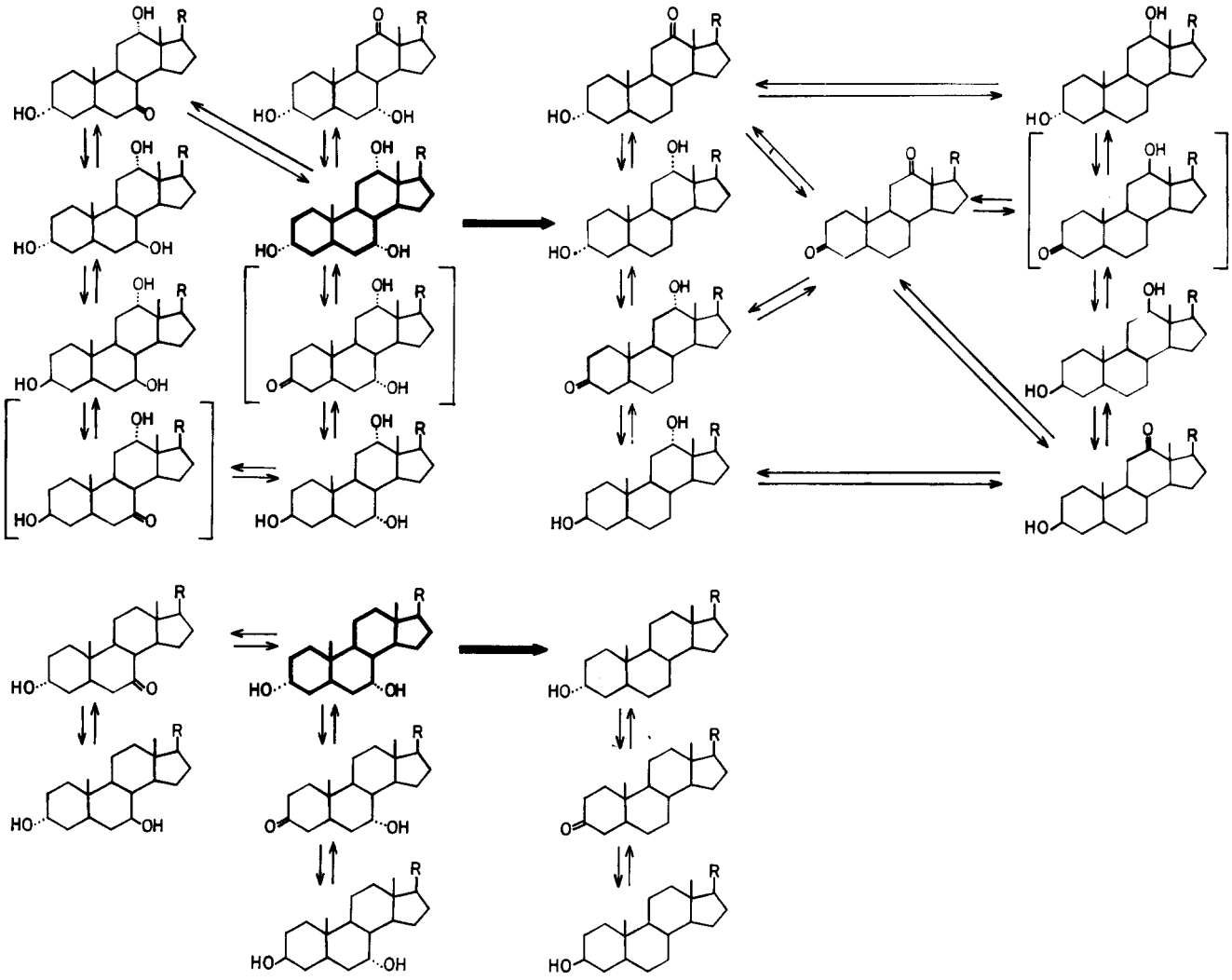

FIG. 4. Possible metabolic interrelations among bile acids isolated from human feces. The primary bile acids, cholic acid and chenodeoxycholic acid, are indicated by heavy lines. The compounds within brackets have not been positively identified. Allocholic acid is not included in this scheme.

mixture of a large number of isomeric hydroxy- and ketocholanoic acids substituted at one or several of the positions 3, 7, and 12. This is in agreement with previous studies of the metabolism of labeled bile acids (16). In addition to those previously isolated, several new isomers have now been identified. The bile acids found in human feces have been summarized in Fig. 4.

Although the present studies were not intended to show the quantitative relations among the various compounds, it is obvious that the predominant bile acids are nonketonic. In separate studies[2] (16) it has been found that the bile acids lacking a 7-hydroxyl group and having a 3α- or a 3β-hydroxyl group either alone or in combination with an oxygen function at C_{12} usually are the predominant bile acids in human feces. The fecal samples analyzed in the present investigation contained an unusually large amount of trisubstituted bile acids (30–40% of the total bile acids as compared to <10% usually) and were therefore chosen for a study of these compounds.

The metabolic interrelations among the various bile acids cannot be evaluated by the present studies. Some possible interrelations among the different metabolites, based on previous observations of the metabolism of labeled bile acids in the liver and intestine of various animals and man (1), have been suggested in Fig. 4. It should be realized, however, that many of the compounds can probably be formed from several precursors.

Our knowledge of the redox equilibria between keto- and hydroxycholanoic acids is limited. However, we can say that the diet might bring about changes in the pH of the intestinal contents, which is one important factor governing the redox equilibrium (17). Gustafsson, Midtvedt, and Norman (18) have isolated a single microorganism that is capable of carrying out a number

[2] Eneroth, P., K. Hellström, and J. Sjövall, to be published.

of oxidations and reductions of the various hydroxyl groups in the bile acid nucleus, as well as removal of the 7-hydroxyl group. When the complex mixture of metabolites of cholic and chenodeoxycholic acids produced in vitro by this microorganism was studied by TLC, spots were detected that corresponded to most of the compounds identified in human feces. It is therefore possible that this microorganism is responsible for the presence of most of the bile acid metabolites found in feces, although it should be realized that other bacteria have also been shown to oxidize hydroxyl groups in bile acids (19, 20).

Although we have been aware of the possible presence of bile acids with more or less than 24 carbon atoms (21), of bile acids with oxygen functions in positions other than 3, 7, or 12 (22), of unsaturated bile acids (23), and of esterified cholanoic acids like those described by Norman (2), we could detect only trace amounts of compounds which might represent unsaturated bile acids or bile acids substituted at positions other than 3, 7, or 12. Since the fecal extracts were hydrolyzed, the presence of esters could not be assessed. Traces of a 5α-cholanoic acid, allocholic acid (22, 24), were found, but in spite of specific attempts to isolate allodeoxycholic acid (25) this acid could not be found in the same sample.

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