Bile acid synthesis in man. II. Determination of 7α-hydroxycholesterol, (22*R*)-22-hydroxycholesterol, and 26-hydroxycholesterol in human meconium

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Abstract 7α-Hydroxycholesterol, (22*R*)-22-hydroxycholesterol and 26-hydroxycholesterol have been quantitated in human meconium. The method used tetrahydrofuran for extraction and solvolysis of the sulfate esters, liquid partition chromatography for the separation of the hydroxysterols, gas–liquid chromatography for quantitation, gas–liquid chromatography–mass spectrometry for identification, and tritiated and ^{14}C-labeled tracers for overall recovery standards. (22*R*)-22-Hydroxycholesterol and 26-hydroxycholesterol were present almost entirely, (>93%) in the sulfate fraction at concentrations of 3.8–6.4 and 0.4–0.8 mg per 100 g meconium, respectively. Since free tritiated (22*R*)-22-hydroxycholesterol was used as the tracer to assess recovery of this hydroxysterol, the concentrations found for this compound may be minimal. Tritiated 26-hydroxycholesterol 3,26-disulfate was used as tracer to determine the levels of this compound, and the solvolysis procedure was optimized for recovery of 26-hydroxycholesterol and least decomposition of 7α-hydroxycholesterol. No significant amounts of 7α-hydroxycholesterol were found based on the tracer-free hydroxysterol as recovery standard.

Supplementary key words hydroxysterol sulfates · solvolysis · gas–liquid chromatography · mass spectrometry

Previous studies indicated that both 7α-hydroxycholesterol and 26-hydroxycholesterol are metabolized to chenodeoxycholate and cholate, the primary bile acids of man (1). Further support for the existence of a metabolic pathway for bile acid synthesis with oxidation of cholesterol to 26-hydroxycholesterol can be derived from the findings of 3β-hydroxy-5-cholenoate (2) and 26-hydroxycholesterol (3) (both as sulfate esters) in normal human meconium. Although quantitative data exist on the proportion of 3β-hydroxy-5-cholenoate in normal meconium in comparison to other bile acids (2), the quantitation of 26-hydroxycholesterol in normal meconium, particularly in comparison to 7α-hydroxycholesterol, has not been reported. Similarly, quantitative data are lacking on the concentration of (22*R*)-22-hydroxycholesterol, also previously identified in the sulfate fraction of human meconium (3).

This report describes the quantitative estimation of 7α-hydroxycholesterol, (22*R*)-22-hydroxycholesterol, and 26-hydroxycholesterol in normal human meconium.

MATERIALS AND METHODS

Radioactive Tracers

[4-^{14}C]Cholesterol (23 mCi/mmol) (4), 7α-hydroxy-[4-^{14}C]cholesterol (1.6 mCi/mmol) (1), 26-hydroxy-[16,22-$^{3}H_2$]cholesterol (13.3 mCi/mmole) (5), (20*S*)-20-hydroxy[16-^{3}H]cholesterol (10.6 Ci/mmole) (4), and (22*R*)-22-hydroxy[1,2-$^{3}H_2$]cholesterol (22.3 Ci/mmole) (4) were purified to radiochemical purity prior to use. [4-^{14}C]Cholesterol sodium sulfate and the disulfate (sodium salt) of the tritiated 26-hydroxycholesterol were prepared according to the method of Fieser (6). The reaction mixtures were chromatographed on LH-20 according to the method of Sjövall and Vihko (7) using chloroform–methanol 1:1 containing 0.01 M sodium chloride as eluant. In this solvent system the 26-hydroxycholesterol 3,26-

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disulfate emerged in an elution volume characteristic of a disulfate whereas the cholesterol sulfate emerged where monosulfates are expected.

Solvents

All solvents were reagent grade and were redistilled in all-glass apparatus. Tetrahydrofuran was redistilled over solid KOH prior to use.

Chromatography, quantitation, and mass spectrometry

Partition chromatography was done using Celite 545 as inert support in 60 × 2 cm glass columns. The lower phase of the solvent system, isooctane–methanol–water 10:9:1, 0.5 ml/g Celite, was used as the stationary phase. The columns were packed dry and washed with mobile phase until 2–3 fractions emerged. The sterols were introduced into the column by dissolving them in 2.0 ml of stationary phase followed by washing with 10–20 ml of mobile phase. Fractions of 10 ml each were collected. In this system cholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, (22*R*)-22-hydroxycholesterol and 26-hydroxycholesterol typically emerge in fractions 8–12, 25–31, 33–40, 48–60, and 62–75, respectively. No separation between 7α-hydroxycholesterol and (20*S*)-20-hydroxycholesterol is achieved in this system. In this system up to 500 mg of meconium extract could be processed without loss of resolution. However, with the heavier extracts it was necessary to wash the tip of the column and the siphon free from adhering cholesterol crystals, after the cholesterol fractions emerged, so that no contamination with cholesterol of the other fractions would occur.

Thin-layer chromatography was done on precoated silica gel G plates 20 × 20 cm, 250 μm thick, purchased from Analtech, Newark, DE. The plates were dipped in a methanolic solution of Rhodamine 6 G (180 mg/liter) and dried first at room temperature and then at 110°C for 30 min. The sterols were then visible under 366 nm light (8). In the solvent system benzene–ethyl acetate 3:7, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 26-hydroxycholesterol, (22*R*)-22-hydroxycholesterol, (20*S*)-20-hydroxycholesterol, and cholesterol had the following R_f values: 0.39, 0.48, 0.65, 0.67, 0.75, 0.84, and 0.90, respectively. Elution from the thin-layer plates was done by scraping off the zone into a fine sintered glass funnel and washing with 10 ml of acetone. Separation from the dye was accomplished by partition chromatography on Celite 545 in the solvent system hexane–benzene–methanol–water 1:1:1:1 on a 7 × 0.5 cm column. The sterols emerge with the front (1–2 ml) while the dye remains on the column.

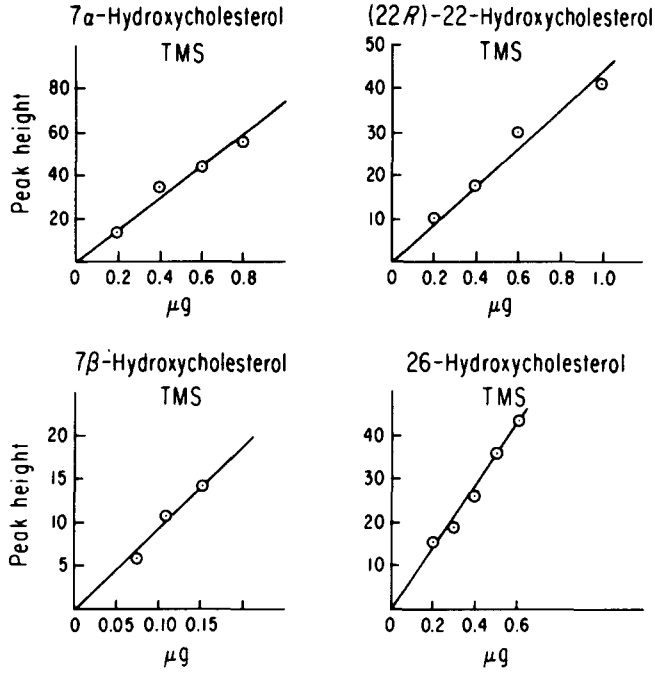

Fig. 1. Gas–liquid chromatographic peak height–mass relationship for the di(trimethylsilyl)ethers of 7α-, 7β-, (22*R*)-22- and 26-hydroxycholesterol.

Gas–liquid chromatography for quantitation of the sterols was done using a Packard model 7300 gas chromatograph (Packard Instruments, Downers Grove, IL) equipped with a hydrogen flame detector and U-shaped columns 3 ft. × 2 mm filled with 3% SE-30 on Supelcoport 80/100 mesh (Supelco, Inc., Bellefonte, PA). The following conditions were maintained for the quantitation of the trimethylsilyl derivatives of the sterols studied (with the exception of the 26-hydroxycholesterol): injection port, 255°C; oven, 225°C; detector, 245°C; nitrogen flow, 35 ml/min; air flow, 350 ml/min; hydrogen flow, 35 ml/min. With the 26-hydroxycholesterol, the oven was kept at 235°C. Typical retention times for 7α-hydroxycholesterol, 7β-hydroxycholesterol, (22*R*)-22-hydroxycholesterol, and 26-hydroxycholesterol di(trimethylsilyl)ethers were: 10.5, 16.5, 19.0, and 18.7 min, respectively. Trimethylsilylation was done with 5 μl TRI-SIL 'TBT' (Pierce Chemical Co. Rockford, IL) in 15 μl of ethyl acetate (dried over sodium sulfate) at room temperature for 5–10 min. Typical calibration curves obtained with the di(trimethylsilyl)ethers of 7α-hydroxycholesterol, 7β-hydroxycholesterol, (22*R*)-22-hydroxycholesterol and 26-hydroxycholesterol are given in **Fig. 1.**

Scintillation counting was done in a Packard model 3320 Tri-Carb scintillation spectrometer. The free

sterols were counted in 10 ml of toluene containing 0.4% 2,5-diphenyloxazole with efficiencies of 58% for ^{3}H and 60% for ^{14}C. The sulfates were counted in mixtures of scintillation fluid–methanol 1:1 from which the sulfates did not come out of solution.

Mass spectrometry was done with an LKB 9000 instrument (LKB Instruments, Rockville, MD) equipped with a 6 ft. × 0.25 in. column containing 1% SE30 on 100–120 mesh Supelcoport. Column, flash heater, and separator temperatures were set at 215°C, 230°C, and 260°C, respectively, and the electron energy during scans was 70 eV.

Determination of total (free and sulfated) 7α-hydroxycholesterol, (22*R*)-22-hydroxycholesterol and 26-hydroxycholesterol

Meconium pooled from 4–6 healthy newborn babies (15–30 g) was kept at −20°C until analyzed. The meconium was thawed and homogenized in a Waring blender with 10–15 ml of water and 25–30 ml of tetrahydrofuran. Tracer 7α-hydroxycholesterol (7800 dpm, 0.9 μg), (22*R*)-22-hydroxycholesterol (344,000 dpm, 2.8 ng), and 26-hydroxycholesterol 3,26-disulfate (130,000 dpm, 1.8 μg) were added followed by 400 ml of tetrahydrofuran. The mixture was stirred for 30 min; the protein precipitate and other solid material was filtered through a Buchner funnel, and the tetrahydrofuran was evaporated under reduced pressure at 30°C. To the aqueous suspension sufficient sodium chloride was added to make a 20% solution which was then extracted three times with 40 ml of tetrahydrofuran. To the combined tetrahydrofuran extracts an equal volume of dry tetrahydrofuran was added followed by 0.1 ml per 100 ml of solvent of 70% perchloric acid. The mixture was allowed to solvolyze for 72 hr at room temperature (23–25°C). After concentrated ammonium hydroxide was added so as to show an alkaline response to litmus paper, the solution was evaporated under reduced pressure. The residue was transferred with benzene to a separatory funnel, washed with 5% aqueous sodium hydroxide and with distilled water to neutrality. The residue was then subjected to partition chromatography. The fractions containing the radioactive peaks of the respective compounds were combined and chromatographed on thin-layer plates. Aliquots of the spot eluates corresponding to the respective sterols (after separation from dye) were taken for quantitation by gas–liquid chromatography and for counting, from which the specific activity (dpm/μg) was obtained. The total amount of sterol present in meconium was calculated by dividing the radioactivity added at the beginning of the analysis by the specific activity. The remainder of the eluate from the thin-layer chromatography was used for mass spectrometry.

RESULTS

Optimization of conditions for extraction and solvolytic cleavage of sterol sulfates

To determine total (free and sulfated) 7α-hydroxycholesterol, (22*R*)-22-hydroxycholesterol, and 26-hydroxycholesterol, mild conditions had to be established for sulfate ester cleavage under which the acid-sensitive 7α-hydroxycholesterol would not be destroyed. The optimization experiments were done using [4-^{14}C]cholesterol sulfate and tritiated 26-hydroxycholesterol 3,26-disulfate as model compounds. Tetrahydrofuran was chosen as solvent because of its previously demonstrated excellent properties for both the extraction and solvolytic cleavage of steroid sulfates (9). Both the model sulfated sterols and the free sterols were extracted quantitatively with equal volumes of tetrahydrofuran from aqueous media containing 20% sodium chloride. During these studies it was observed that, whereas cholesterol sulfate was readily soluble in dry tetrahydrofuran, 26-hydroxycholesterol 3,26-disulfate was not taken up by this solvent, but was soluble in tetrahydrofuran containing 2.5% water.

The effect of acidity, time of reaction, temperature, and water content on the solvolytic cleavage of the model sterol sulfates is given in **Table 1.** The results demonstrated that 26-hydroxycholesterol 3,26-disulfate was more resistant to solvolysis than cholesterol sulfate. The mildest conditions for the quantitative cleavage of both sterol sulfates in the model aqueous system were 24 hr in tetrahydrofuran containing 2.5% water and 0.01 M perchloric acid at room temperature. Under these conditions 70% of added 7α-hydroxycholesterol was recovered, as determined by thin-layer chromatography and confirmed by gas–liquid chromatography–mass spectrometry. However, further experiments indicated that these optimal conditions did not lead to quantitative ester sulfate cleavage in the presence of meconium, contrary to the results obtained in the model experiments in aqueous systems. Solvolysis experiments in the presence of meconium extracts under various water and acid concentrations are presented in **Table 2.** It is evident from this table that quantitative sulfate ester cleavage in the pres-

TABLE 1. Solvolytic cleavage of cholesterol sulfate and 26-hydroxycholesterol 3,26-disulfate in tetrahydrofuran

Sterol Sulfate	Radioactivity Added	Temperature	Perchloric Acid Concentration	Water Content	Reaction Time	Free Sterol Recovered	
	cpm	°C	*M*	%	*hr*	*cpm*	%
Cholesterol sulfate	35,000	23	0	5	72	420	1.2
	35,000	23	0.01	5	24	36,000	103
	35,000	50	0.01	5	2	29,000	83
26-Hydroxycholesterol 3,26-disulfate	26,000	23	0	5	72	200	<1
	26,000	23	0.01	5	24	10,500	41
	26,000	23	0.01	5	48	20,000	79
	25,000	23	0.01	5	72	22,500	90
	26,000	50	0.01	5	2	19,500	75
	25,500	23	0.01	2.5	24	27,000	106

The tetrahydrofuran containing 5% water was the upper layer obtained after equilibration of equal volumes of solvent with 20% aqueous sodium chloride (9). The 2.5% aqueous solvent was obtained by further dilution with dry tetrahydrofuran. The specified perchloric acid concentration was obtained by adding the appropriate volume of 70% perchloric acid. The sterol sulfate was dissolved in 5 ml of solvent in glass-stoppered test tubes. At the end of the reaction, a few drops of concentrated ammonium hydroxide was added and the solvent was evaporated to dryness under a stream of nitrogen. The residue was partitioned between 5 ml of benzene and 2 ml of water, and aliquots of the benzene layer were counted. Since the sulfates are not extracted into benzene, the total counts obtained represented the "free sterol recovered" given in the last column of the table. More than 90% of the radioactivity in the benzene layer was associated with the respective free sterol as determined by thin-layer chromatography following the addition of carrier sterol.

ence of meconium extract was obtained at a water concentration of 2.5% and 0.01 M perchloric acid when the reaction was carried out for 72 hr. Under these conditions, however, only approximately 36% of added 7α-hydroxycholesterol remained unaltered. We were unable to find solvolysis conditions under which no decomposition of 7α-hydroxycholesterol took place.

7α-, (22*R*)-22- and 26-Hydroxycholesterol in human meconium

The results of the determination of these sterols in human meconium are given in **Table 3.** As may be seen, both (22*R*)-22- and the 26-hydroxycholesterol were consistently found. No significant 7α-hydroxycholesterol was found in any of the meconium pools. The identity of the (22*R*)-22-hydroxycholesterol and of the 26-hydroxycholesterol in each sample was conclusively established by gas–liquid chromatography–mass spectrometry and typical mass spectra of authentic (22*R*)-22- and 26-hydroxycholesterol (as the di(trimethylsilyl)ethers) as compared to material isolated from human meconium are given in **Figs. 2** and **3.** The mass spectra of the (22*R*)-22-hydroxycholesterol and 26-hydroxycholesterol presented here agreed well with those previously described (10, 11). In view of the insignificant amounts of 7α-hydroxycholesterol found in human meconium, additional experiments were done on the recovery of this compound added to meconium. The results are given in **Table 4.**

Both 7α- and 7β-hydroxycholesterol were recovered and were conclusively identified by gas–liquid chromatography–mass spectrometry. It is apparent from Table 4 that *a*) the recovery of the added sterol after correction for procedural loss was satisfactory, and *b*) the specific activities of both 7α- and 7β-hydroxycholesterol were close to that of the added tracer (8840 dpm/μg), indicating no significant dilution with endogenous material.

The question as to what extent the hydroxysterols determined in human meconium were in the sulfated form was studied by separating the free from the sulfated sterols prior to solvolysis. The results are given in **Table 5**, from which it is obvious that practically all of the (22*R*)-22- and 26-hydroxycholesterol of meconium were present in sulfated form.

TABLE 2. Solvolysis of tritiated 26-hydroxycholesterol 3,26-disulfate in the presence of meconium

Sterol Disulfate Added	Water Content	Perchloric Acid Concentration	Recovery of Free Sterol	
cpm	%	*M*	*cpm*	%
11,000	5	0.01	6,200	56
11,000	5	0.02	7,200	65
13,000	2.5	0.01	12,500	97

Tritiated 26-hydroxycholesterol 3,26-disulfate was added to 5 ml of meconium extract in tetrahydrofuran (prepared as described in Materials and Methods). Reaction was allowed to proceed for 72 hr and the recovery of free sterol determined as described in the legend of Table 1.

TABLE 3. (22*R*)-22-, 26-, and 7α-Hydroxycholesterol in human meconium

Meconium Sample No.	Weight of Meconium Sample Analyzed	(22*R*)-22-Hydroxycholesterol		26-Hydroxycholesterol		7α-Hydroxycholesterol
	g	*μg in sample*	*mg/100 g*	*μg in sample*	*mg/100 g*	*μg in sample*
1	18.0	680	3.8	N.D		<2
2	31.0	2,000	6.5	205	0.66	<3
3	15.0	N.D		60	0.40	<1
4	25.4	N.D		160	0.63	N.D
5	20.0	850	4.3	137	0.69	<1
6	14.3	720	5.0	115	0.80	<1

The meconium samples were each the pool from 4–6 babies. The values determined represent total sterol, free and sulfated combined.

DISCUSSION

The results of the present study have shown that 26-hydroxycholesterol (as a sulfate ester) is consistently found in normal human meconium in concentrations of 0.4–0.8 mg%. Since tritiated 26-hydroxycholesterol 3,26-disulfate was used as recovery standard, and since no free sterol was found, the concentrations given would appear to represent the total 26-hydroxycholesterol present as sulfate ester in meconium. From our results it is, however, not possible to tell whether this sterol was present as a monosulfate (sulfurylated at either C-3 or C-26) or as the 3,26-disulfate, or both. 26-Hydroxycholesterol (present largely in the monosulfate fraction) appears to have been found in some but not in all of the meconium samples studied by Eneroth and Gustafsson (3). The failure by these workers to find this sterol consistently may have been because no tracer was used for recovery estimates or for determination of optimal conditions for the solvolysis of this sterol sulfate. As shown in our study the quantitative solvolysis of 26-hydroxycholesterol 3,26-disulfate requires a longer reaction time than that required for the cleavage of cholesterol sulfate.

(22*R*)-22-Hydroxycholesterol (as a sulfate) has been found by us in relatively large concentrations (3.8–6.4 mg%). Because we used the free sterol as tracer to assess recovery yields, and not the sulfated sterol, these values may be minimal. Our results are in agreement with those of Eneroth and Gustafsson (3) who found the (22*R*)-22-hydroxycholesterol, in the monosulfate fraction, as the most important $C_{27}O_2$ sterol of meconium.

Our failure to observe any significant amounts of 7α-hydroxycholesterol in meconium was not due to our inability to recover this sterol under the solvolysis conditions used. However, the possibility that 7α-hydroxycholesterol 3,7-disulfate may not be cleaved under these conditions cannot be eliminated at present, and further studies on the preparation of this sulfate and its solvolytic cleavage are indicated.

The occurrence of free 7α-hydroxycholesterol in human meconium (0.4 mg%) has been described by

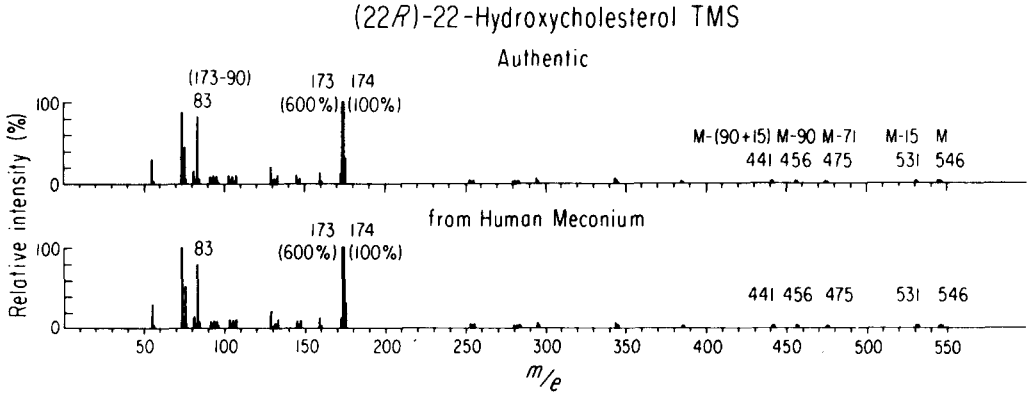

Fig. 2. Mass spectra of authentic and isolated (22*R*)-22-hydroxycholesterol di(trimethylsilyl)ether.

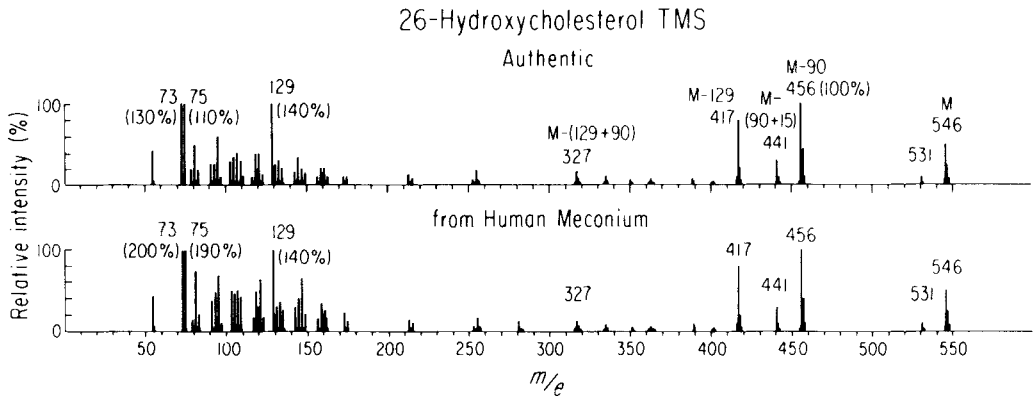

Fig. 3. Mass spectra of authentic and isolated 26-hydroxycholesterol di(trimethylsilyl)ether.

Kinsella and Francis (12), but the possibility of its artifactual formation by autoxidation of cholesterol was not discounted by these workers.

The physiological meaning of the hydroxycholesterols in meconium is difficult to assess. The compounds isolated may represent merely excretory end products accumulated during intrauterine life with no other important role. The relatively high levels of the (22*R*)-22-hydroxycholesterol may be related to the hypertrophic adrenal cortex of the fetus, as pointed out by Eneroth and Gustafsson (3). The intermediary role of this sterol in the formation of pregnenolone from cholesterol has been the subject of numerous studies (4, 11, 13–15). More recently, the formation of (22*R*)-22-hydroxycholesterol from cholesterol has been shown to be the first identifiable event in the side-chain cleavage of cholesterol to pregnenolone (16, 17). The nature of the sulfate ester found in human meconium (esterified at C-3 or C-22) as well as the site of conjugation (liver, adrenal) remains to be established.

3β-Hydroxy-5-cholenoic acid (as the sulfate ester of the glycine or taurine conjugate) in human meconium (1.5 mg%) represented approximately 10% of the total bile acid content and appeared to be higher in premature infants (2). This relatively high concentration of the monohydroxy bile acid is consistent with our observation in human meconium of 26-hydroxycholesterol, a probable precursor of this bile acid.

It appears that there are multiple pathways leading to bile acid synthesis from cholesterol in man (1, 18). The presence of 26-hydroxycholesterol and 3β-hydroxy-5-cholenoate in meconium may be analogous to the accumulation of 25-hydroxylated bile alcohols in bile and feces in patients with cerebrotendinous xanthomatosis (19) and represent the excretion of intermediates in bile acid synthesis because of incomplete conversion to chenodeoxycholic and cholic acids.

TABLE 4. Recovery of 7α-hydroxycholesterol added to human meconium

Exp. No.	7α-Hydroxycholesterol Added	7α-Hydroxycholesterol Recovered		7β-Hydroxycholesterol Recovered
	μg	*μg*	*Sp act dpm/μg*	*Sp act dpm/μg*
1	40	43	7,420	6,670
2	6.2	6	7,920	N.D
3	19	16.3	9,170	N.D

In experiments 1 and 3, 7α-hydroxy[4-^{14}C]cholesterol was added to meconium and taken through the extraction and solvolysis procedure described under Materials and Methods. In experiment 2, the 7α-hydroxycholesterol was added after solvolysis.

TABLE 5. Occurrence of (22*R*)-22- and 26-hydroxycholesterol in human meconium in the sulfate and free fractions

Fraction	(22*R*)-22-Hydroxycholesterol		26-Hydroxycholesterol	
	μg	*mg/100 g meconium*	*μg*	*mg/100 g meconium*
"Sulfate"	322	1.3[a]	160	0.64
"Free"	19.4	0.1	<3	<0.012

Human meconium (25.4 g) was extracted with tetrahydrofuran (which extracts both the free and sulfated sterols) as described in Materials and Methods. After the addition of tracer 26-hydroxycholesterol 3,26-disulfate the solvent was evaporated under reduced pressure. The residue was partitioned between benzene and water. The extract of the benzene phase (containing the "free" sterol fraction) after addition of tracer (22*R*)-22- and 26-hydroxycholesterol was analyzed in the usual manner. The aqueous phase (which contained most of the 26-hydroxycholesterol 3,26-disulfate radioactivity) after the addition of tracer (22*R*)-22-hydroxycholesterol was solvolyzed and analyzed for the hydroxysterols to give the content of the "sulfate" fraction.

[a] Tracer (22*R*)-22-hydroxycholesterol was added in this case only before the partition chromatographic step. This may account for the lower concentration observed as compared to the results of Table 3, in which the tracer was added at the beginning of the analysis.

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