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Gas-Liquid Chromatographic and Mass Spectrometric Studies on Sterols in Vernix Caseosa, Amniotic Fluid and Meconium

TATU A. MIETTINEN and TAPANI LUUKKAINEN

Third Department of Medicine and Department of Clinical Chemistry, University of Helsinki, Helsinki 29, Finland

A combination of thin-layer chromatography, gas-liquid chromatography and mass spectrometry for the analysis of sterols in vernix caseosa, amniotic fluid, meconium, placenta and maternal serum allowed identification of compounds that had proved impossible by any one of the techniques alone. The following major sterols were found: cholesterol, cholestanol, Δ^7- and Δ^8-cholestenols, 7-dehydrocholesterol, desmosterol, a cholestenol with an unlocalized nuclear double bond, Δ^7- and Δ^8-methostenols, dimethyl (4,4) Δ^7- and Δ^8-cholestenols, lanosterol, Δ^7- and Δ^8-lanostenols, and a lanostenol which, according to mass spectrometric analysis, had the double bond in the 5—6 position. Differentiation between the TMSi-ethers of Δ^7- and Δ^8-isomers was based on 1) shorter retention of the latter in GLC on DC-560, 2) the presence of an intense ion of the steroid nucleus (M—90—side chain) and also A, B, and C rings (M—90— side chain—ring D) in the mass spectrum of Δ^7-sterols in contrast to Δ^8-sterols, in which the corresponding peaks were relatively small, and 3) relatively great additional loss of two hydrogens from fragments in the high mass range of Δ^8-sterols. This loss was negligible for Δ^7-derivatives. In addition, small amounts of the plant sterols β-sitosterol and β-sitostanol were identified. The sterol pattern was qualitatively almost the same in all five samples. Cholesterol comprised about 99 % of the placental and serum sterols, while other sterols, mainly cholestanol and Δ^7-cholestenol formed 10—20 % of the total in the fetal specimens. Only slight quantitative differences were found between the sterols in meconium and amniotic fluid, suggesting that the latter markedly contributes to the former. The presence of cholestanol in the fetal site may be due to synthesis of stanol by the fetal organism, although the occurrence of plant sterols suggests exchangeability of sterols between fetal and maternal tissues.

Recent studies[1] have suggested that a considerable proportion of the cholesterol precursor sterols in rat feces actually originates from skin surface lipids. During fur-licking the compounds reach the gastrointestinal tract and the unabsorbable portion is excreted with the feces. An analogous phenomenon is found in man during intrauterine life, when the fetus swallows

amniotic fluid, simultaneously ingesting sterols released from the skin into amniotic fluid with surface lipids and sloughed cells. Consequently, it seemed worth while to study the composition of these sterols and compare the pattern with that found in meconium and maternal plasma. Furthermore, plant sterols, expected to be present in the latter,[2] were assumed to be an indicator of exchange of sterols between the fetal and the maternal organism.

MATERIAL AND METHODS

Blood-free amniotic fluid, vernix caseosa and meconium were obtained from three different deliveries at term. In addition, a piece of placenta and a sample of maternal serum were taken from two of the three cases. All the specimens were handled with either steel or glass equipments in order to avoid artificial contamination with plant sterols.

A stated amount of each sample was saponified with N NaOH in 90 % ethanol. The nonsaponifiable material was extracted and its sterols submitted to successive thin-layer chromatography (TLC) on silica gel G and argentated silica gel G chromatoplate and to subsequent gas-liquid chromatography (GLC) according to the methods employed earlier for dietary and fecal neutral steroids.[1,3] The TLC procedure is illustrated schematically in Fig. 1. Three well separated fractions, designated I, II, and III in descend-

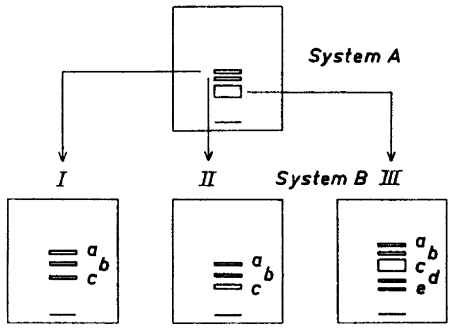

Fig. 1. Thin-layer chromatographic procedure for the fractionation of meconium sterols. System A=silica gel G chromatoplate developed with ethyl ether:heptane 1:1. System B=silver nitrate impregnated silica gel G developed with chloroform once for the whole length for Fractions I and II, and twice for Fraction III. The mobilities of the reference substances are reported in the text.

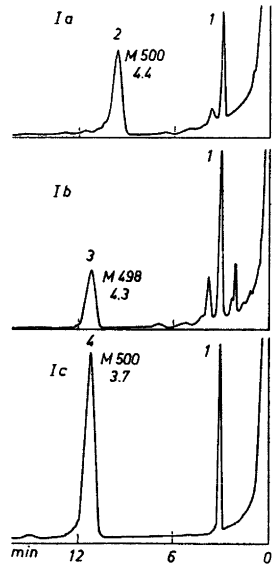

Fig. 2. GLC patterns of meconium sterols in subfractions Ia—Ic of Fig. 1. 1) 5α-cholestane, added as internal standard, 2) Δ^8-lanostenol, 3) lanosterol, and 4) Δ^6-lanostenol. The figure after M indicates the molecular weight found in mass spectrometry for the TMSi-ether of the compound. The figures below the molecular weights indicate the relative amounts (ppm) of each sterol from the total.

ing order of mobility, were obtained from the sterols of vernix caseosa, amniotic fluid and meconium. The serum and placenta gave only one visible fraction (III), which as well as the combined area of I and II, were scraped from the plate. Fraction I moved with lanosterol * and Δ^8-lanostenol, II appeared to have the mobility of methostenols,[1] and III migrated with the mobility of cholesterol, plant sterols, Δ^7-cholestenol and desmosterol. Fraction I was divided into three subfractions, Ia, Ib, and Ic, by rechromatography on argentated silica gel G. The fastest of these, Ia, now had the mobility of Δ^8-lanostenol and Ib that of lanosterol. Compounds possibly moving slower than Ic were disregarded. Fraction II was also divided into three parts, IIa, IIb, and IIc, the fastest of which had the mobility found earlier for methostenols.[1] Five subfractions were separated from Fraction III and designated, in descending order of mobility, IIIa, IIIb, IIIc, IIId, and IIIe. The mobilities of the references were as follows: cholestanol and the plant stanols corresponded to IIIa, Δ^7-cholestenol to IIIb, cholesterol and plant sterols (Δ^5) to IIIc, and desmosterol to IIIe.

For qualitative and quantitative studies, a small amount of 5α-cholestane was added to each fraction as internal standard and the sterols were analyzed by GLC as their TMSi-ethers, using both 1 % DC-560 and 1 % neopenthyl glycol succinate (both purchased from Applied Science Inc., State College, Pa.) columns. Quantitative determinations were carried out on the latter column by running fractions I and II combined, and all the subfractions of III separately. Since the peaks of mono-, di-, and trimethyl sterols tended to overlap, these compounds could not be estimated separately and were therefore quantitated as a group. In one study quantitation was also done from subfractions of both I and II. Possible losses of sterols during TLC procedures were assumed to be of the same order as for cholesterol. A correction for losses of the latter was obtained by recovery of ^{14}C-4-cholesterol (The Radiochemical Centre, Amersham, England; purified on silver nitrate-impregnated silica gel G before use) added to the saponification mixture.

Bile acids from meconium were determined as a group after extraction of sterols as presented earlier for fecal bile acids.[4] Mass spectra of sterol TMSi-ethers were recorded by an LKB model 9000 gas chromatograph-mass spectrometer (GLC—MS) equipped with a 6 ft 2 % SE-30 column at 250°C. The samples were introduced into the column in heptane, the molecular separator was maintained at 260°C and the ion source at 290°C. The ionizing energy was 70 eV.

RESULTS

Gas-liquid chromatographic (GLC) patterns obtained from different TLC fractions (see Fig. 1) of meconium are illustrated in Figs. 2—4. The following identifications were made on the basis of TLC, GLC, and GLC-MS analyses.

Fraction Ia contained one compound which fullfilled the TLC, GLC, and mass spectrometric criteria of the reference Δ^8-lanostenol. The ion at *m/e* 69, known to be characteristic [5] of Δ^{24}, was surprisingly intense (second largest after the base peak M—(90 + 15). But the other fragments found

* Common names of sterols used in this report: Cholesterol, cholest-5-en-3β-ol; cholestanol, 5α-cholestan-3β-ol; desmosterol, cholest-5,24-dien-3β-ol; 7-dehydrocholesterol, cholest-5,7-dien-3β-ol; Δ^7-cholestenol, 5α-cholest-7-en-3β-ol; Δ^8-cholestenol, 5α-cholest-8-en-3β-ol; β-sitosterol, 24β-ethyl-cholest-5-en-3β-ol; β-sitostanol, 24β-ethyl-5α-cholestan-3β-ol; methostenol, 4α-methyl-5α-cholest-7-en-3β-ol; Δ^8-methostenol, 4α-methyl-5α-cholest-8-en-3β-ol; dimethyl Δ^8-cholestenol, 4,4-dimethyl-5α-cholest-8-en-3β-ol; dimethyl Δ^7-cholestenol, 4,4-dimethyl-5α-cholest-7-en-3β-ol; lanosterol, 4,4,14α-trimethyl-5α-cholest-8,24-dien-3β-ol; Δ^8-lanostenol, 4,4,14α-trimethyl-5α-cholest-8-en-3β-ol; Δ^7-lanostenol, 4,4,14α-trimethyl-5α-cholest-7-en-3β-ol; Δ^5-lanostenol, 4,4,14α-trimethyl-5α-cholest-5-en-3β-ol.

Standards were obtained as follows: Cholesterol (Merck); Δ^7-cholestenol, cholestanol and lanosterol (including Δ^8-lanostenol), gift from Dr. E. H. Ahrens, New York; desmosterol was isolated by successive TLC on silica gel and argentated silica gel from serum of a patient treated for adrenal cancer with triparanol. β-Sitosterol and β-sitostanol were isolated from corn-sterols by the TLC procedure employed for the isolation of desmosterol.

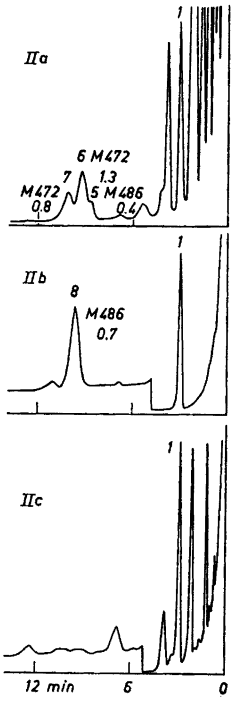

Fig. 3. GLC patterns of meconium sterols in subfractions IIa—IIc of Fig. 1. 5) dimethyl-Δ^8-cholestenol, 6) Δ^8-methostenol, 7) methostenol, 8) dimethyl-Δ^7-cholestenol. For other figures, see legend to Fig. 2.

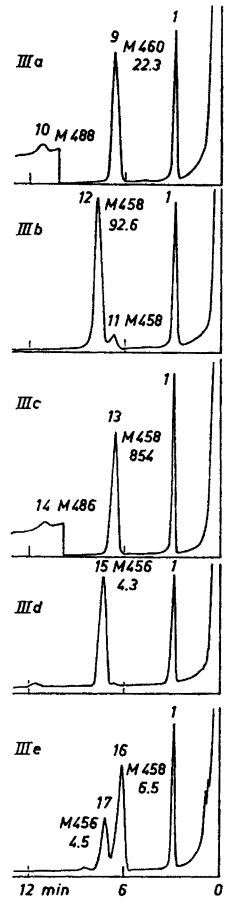

Fig. 4. GLC patterns of meconium sterols in subfractions IIIa—IIIe of Fig. 1. 9) cholestanol, 10) β-sitostanol, 11) Δ^8-cholestenol, 12) Δ^7-cholestenol, 13) cholesterol, 14) β-sitosterol, 15) 7-dehydrocholesterol, 16) cholestenol with a double bond in the unknown position in the nucleus, 17) desmosterol. For other figures, see legend to Fig. 1.

in the present study to be typical for Δ^{24} M—69, M—(69 + 90) and M—(69 + 90 + 15), were absent.

GLC occasionally revealed another small peak with a longer retention time than Δ^8-lanostenol, having as the TMSi-ether a molecular weight of 500. The retention data and the fragmentation pattern suggested that the compound was probably Δ^7-lanostenol.

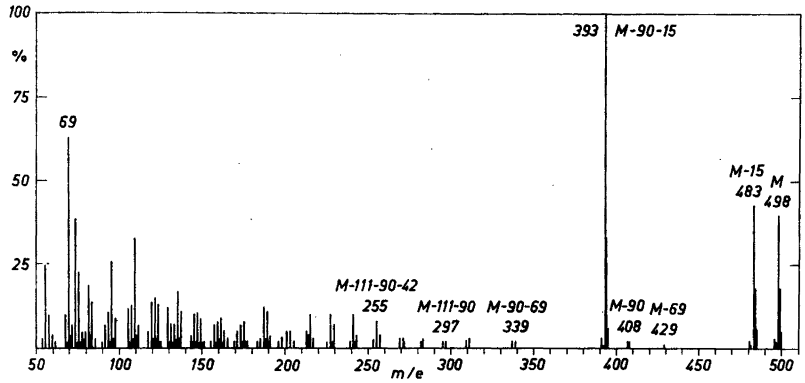

Fig. 5. Mass spectrum of lanosterol isolated from meconium (see Fig. 2; middle panel).

Fraction Ib gave one peak on GLC, which had the same retention time as lanosterol. The mass spectrum of the compound, presented in Fig. 5, was identical with authentic lanosterol. Lanosterol and Δ^8-lanostenol, like other Δ^8-cholestenols, gave a markedly weak peak of M—(90+side chain).[5] Further, the fragments of the high mass range are seen to lose two additional hydrogens. This loss of hydrogens was found in this study to be characteristic of Δ^8-sterols. However, this process is of relatively low intensity for C-14-methyl sterols, as seen in Fig. 5. The elimination of the unsaturated side chain is also accompanied by loss of two hydrogens, as in the case of other sterols with unsaturated side chains.[5-7]

Fraction Ic contained one major compound, the TMSi-ether of which had a molecular weight of 500. The major peaks on the mass spectrum in descending order of intensity were at *m/e* 395 (M—90—15), 69, 485 (M—15), 129, 500 (M), 371 (M—129), 410 (M—90), 282 (M—113—90—15), 255 (M—133—90—42), and 297 (M—113—90). No significant losses of additional hydrogens were seen here in the high mass range. In analogy to lanosterol, the compound appears to contain three methyl groups in the nucleus, probably 2 at carbon 4, one at carbon 14, and in addition one double bond. The presence of the ions at *m/e* 129 and 371 (M—129)[8] suggests that the latter was in the 5—6 position. Thus, the compound could be called Δ^5-lanostenol. This structure also apparently explains its marked retention on silver nitrate-impregnated silica gel G.

Fraction IIa, on GLC, gave several peaks, of which the last two had the respective retention times found earlier[1] for Δ^8- and Δ^7-methostenols. A third peak, preceding these two, gave the mass spectrometric fragmentation of a sterol. Its TMSi-ether had the molecular ion at *m/e* 486, suggesting that the sterol contained two methyl groups and one double bond. The peak at *m/e* 283 (M—113—90) had a relatively low intensity and, furthermore, the main fragments in the high mass range lost two hydrogens rather readily, suggesting that the double bond was in the 8—9 position.

The next GLC-peak consisted of a compound with a molecular weight of 472 for the TMSi-derivative. The fragmentation pattern was almost identical with that of methostenol, but the main fragments, particularly that at *m/e* M−90−15, readily lost two hydrogens. In addition, the relatively low intensity of the ions at *m/e* 269 (M−113−90) and 227 (269−42) associated with a shorter retention time on GLC as compared with that of methostenol,[1] suggested that the compound was Δ^8-methostenol.

The last compound had the retention time and mass spectrometric fragmentation pattern reported [9] for the TMSi-derivative of methostenol. The base peak of the spectrum was at *m/e* 472. In contrast to the TMSi-ethers of Δ^8-sterols, the fragments at *m/e* 269 (M−113−90) and 227 (M−113−90−42) were of high intensity, and no major losses of two additional hydrogens from the fragments were observed in the high mass range.

The compounds emerging from the column before and immediately after 5α-cholestane are probably aliphatic alcohols.

Fraction IIb on GLC showed one major peak with the molecular ion of TMSi-ether at *m/e* 486. The intensity of the peak on the mass spectrum at *m/e* 129 was low, no ion was found at *m/e* M−129, the fragments at *m/e* 283 (M−113−90) and 241 (M−113−90−42) were of high intensity and no significant losses of two hydrogens, such as are typical of Δ^8-sterols, were observed. Thus, the compound had two methyl groups in the nucleus, probably at carbon 4, and one double bond, most likely in the 7−8 position. The corresponding Δ^8-isomer was found in Fraction IIa, although other Δ^7- and Δ^8-sterols, *e.g.* methostenols, were not separated from each other by TLC on argentated chromatoplate.

Fraction IIc contained a large number of compounds, probably aliphatic alcohols, that emerged from the column before or immediately after 5α-cholestane. These compounds were followed by very small peaks, the mass spectra of which had such a low intensity that exact identifications were impossible. The fragmentation pattern of the TMSi-ethers appeared to be due to sterols with the following molecular weights, in increasing order of retention times: 458, 480, 470, 482, and 486.

Fraction IIIa was expected to contain cholestanol and plant stanols. The presence of the former was confirmed with GLC and GLC—MS. In addition, a small peak with the retention time of β-sitostanol was found on GLC. On GLC—MS, the TMSi-ether of the compound gave a molecular ion at 488, suggesting that β-sitostanol was present.

Fraction IIIb, according to TLC, GLC and GLC—MS criteria, contained Δ^7-cholestenol as the major component. The minor peak preceding that of Δ^7-cholestenol on GLC showed, on GLC—MS, a fragmentation pattern resembling that of Δ^7-cholestenol. However, the peaks at *m/e* 255 (M−113−90) and 213 (M−113−90−42) had relatively low intensities as compared with the mass peak, and here again additional hydrogens were lost from the main fragments, of which M−(90 + 15 + 2) was almost as intense as M−(90 + 15). This fragmentation and the calculated retention time suggest that the compound was Δ^8-cholestenol.

Fraction IIIc contained cholesterol and traces of another compound that had the characteristics of β-sitosterol. The fragmentation of its TMSi-

ether was identical with that of authentic β-sitosterol and was in good agreement with other reports.[7,8]

Fraction IIId consisted of one compound, the TMSi-ether of which had a molecular weight of 456. The major peaks on the mass spectrum, in descending order of intensity, were at *m/e* 129, 253 (M—113), 327 (M—129), 343 (M—113), 351 (M—90—15), 366 (M—90), and 441 (M—15). Accordingly, the compound was probably 7-dehydrocholesterol.

Fraction IIIe was expected to contain desmosterol. Two peaks were found on GLC, however, the latter one having the retention time of desmosterol. The TMSi-ether of the compound in the first peak had a shorter retention time than cholesterol and a molecular weight of 458, suggesting, surprisingly, that it had only one double bond. According to descending intensity, the major peaks on the mass spectrum were at *m/e* 458 (M), 353 (M—90—15), 213 (M—113—90—42), 443, 255 (M—113—90), 368 (M—90), and 345 (M—113). Thus, the double bond was probably in the A, B, or C ring in a position resulting in a short retention time on GLC and a marked affinity for argentated silica gel G. It is clear from the behavior in TLC, GLC, and GLC—MS that Δ^5, Δ^7, and Δ^8, and an unsaturated side chain are excluded.

The other peak gave mass spectrometric characteristics obtained for the reference desmosterol. As reported by others,[5-7] the cleavage of the unsaturated side chain was indicated in the mass spectrum by the appearance of fragments M—111, M—(111 + 2), M—(111 + 90), M—(111 + 90 + 2), M—(111 + 90 + 42), and M—(111 + 90 + 42 + 2). In addition to the characteristic presence of the ion at *m/e* 69 [5] and the typical fragmentation due to Δ^5 (the ion at *m/e* 129 and M—129),[8] the following characteristic peaks were seen on the mass spectrum of the TMSi-ether of this sterol at *m/e* M—69, M—(69 + 15), M—(69 + 90), and M—(69 + 129 + 15).

Quantitative aspects of sterols from different sources

Table 1 shows the average composition of the sterols in vernix caseosa, amniotic fluid, meconium, placenta and maternal serum from three different deliveries. Cholesterol forms almost all (99 %) of the placental and serum sterols, in contrast to vernix caseosa, amniotic fluid, and meconium, in which cholestanol and cholesterol precursors comprise from 10 to 20 % of the total sterols. The proportion of cholestanol in the material from the three latter sources was 2—3-fold that in the serum and placenta. The difference is even more marked for Δ^7-cholestenol, which is one of the main components of the non-cholesterol sterol fraction in vernix caseosa, amniotic fluid and meconium. Only traces of this sterol were found in the placenta and serum. Other C—27 sterols were detected in small amounts in all five samples, their proportion being highest in meconium. The content of C_{30}, C_{29}, and C_{28} sterols was negligible in the serum and placenta (below 0.1 % of total) as compared with the other three sources (about 2 %). Since the GLC patterns of Fractions I and II were qualitatively the same in all samples, detailed quantitative analysis was only carried out for meconium. The figures at

Table 1. Relative amounts (ppm) of sterols from vernix caseosa, amniotic fluid, meconium, placenta, and maternal serum.

Compound	Vernix caseosa	Amniotic fluid	Meconium	Placenta	Maternal serum
Cholesterol	788.73	903.96	850.27	989.76	990.36
Cholestanol	17.14	14.18	23.78	7.12	6.39
Δ^7-Cholestenol	170.18	57.45	95.80	0.33	0.33
7-Dehydrocholesterol	1.48	3.62	3.48	1.46	1.95
Δ^*-Cholestenol [a]	0.49	0.73	2.04	0.36	0.23
Desmosterol	0.84	0.95	5.68	0.47	0.40
Methyl sterols [b]	21.14	19.11	18.95	0.50	0.34
Total	1000.00	1000.00	1000.00	1000.00	1000.00

[a] An unlocalized double bond in the nucleus.
[b] Sum of Fractions I and II; contains lanostenols, dimethyl cholestenols, and methostenols.

the top of each peak in Figs. 2—4 show that the lanosterols were major components as compared to the methostenols, of which the delta-8 isomer dominated. As already presented, traces of β-sitosterol and β-sitostanol were found in meconium (see also Figs. 2—4). Although no mass spectrometric analyses were carried out in every case, small peaks corresponding to these sterols were found in all other samples. Quantitatively, the amounts were negligible even in serum.

Bile acids in meconium

Bile acids were analyzed quantitatively in two different specimens of meconium. The amounts were surprisingly low, 16.4 and 18.6 μg/g of meconium, respectively. No attempt was made to identify individual bile acids.

DISCUSSION

Since during intrauterine life the fetus drinks amniotic fluid into which skin cells and surface lipids have been released, the similarity of the sterol patterns found in amniotic fluid, vernix caseosa, and meconium is understandable. The fate of the swallowed amniotic sterols is not known, however. Sterols can only be absorbed if bile acids are excreted into the intestine to form the micellar phase which is necessary for sterol absorption.[10-12] Although bile acids are present in the duodenal contents of infants at birth [13-14] this does not necessarily indicate that the critical micellar concentration is reached in the duodenal and jejunal contents of fetuses after drinking amniotic fluid. A markedly low bile acid concentration (about 20 μg/g of meconium in contrast to 4 mg/g of meconium for cholesterol) suggests that the intestinal bile acid concentration may be low during fetal life. Thus, a considerable proportion of the sterols swallowed may be passed into the meconium. Furthermore, the intestinal mucosa, being a tissue of ectodermal origin, may synthesize sterols that resemble those of the skin.

Comparison between the sterol patterns in meconium and in amniotic fluid showed that the proportion of non-cholesterol sterols, mainly cholestanol and Δ^7-cholestenol, was significantly higher in the former than in the latter. This may be due to better intestinal absorption of cholesterol as compared to other sterols or to secretion of non-cholesterol sterols by the intestinal wall, whilst it is also possible that the composition of amniotic fluid at term differs from that during the last trimester of pregnancy. Calculations showed that 1 g of meconium corresponds to approximately 100 ml of amniotic fluid on the basis of cholesterol, and 350 ml and 300 ml on the basis of lathosterol and cholestanol, respectively.

The pattern of meconium sterols differs markedly from that found in the feces of adult human subjects,[3,8] mainly because only traces of plant sterols are present and because of the absence of bacterial conversion products of sterols. However, the proportion of cholesterol precursors in meconium markedly exceeds that found in the feces of adult human subjects on a sterol-free diet.[3] Thus, meconium resembles rat feces, which also contain large amounts of cholesterol precursors [9] even on a sterol-free diet.[1] If the rat is prevented from fur-licking, the amount of these sterols in the feces decreases, suggesting that, as in the case of meconium, they may originate in part from the skin.[1]

The results of detailed TLC, GLC, and mass spectrometric analysis of meconium sterols in the present study showed the presence of 1) methyl sterols, 2) precursor sterols with 27 carbons, 3) cholestanol, and 4) small amounts of plant sterols. At least four different lanosterols were found, *viz.* lanosterol, Δ^8-lanostenol, Δ^7-lanostenol, and a lanostenol that, according to the mass spectrum, had a double bond in the 5—6 position. This type of compound, Δ^5-sterol with methyl groups still attached at carbon 4, has not previously been found in any other mammalian tissue.[15] The finding suggests that the mammalian organism is also able to convert Δ^5-lanostenol to cholesterol. No corresponding di- or monomethyl derivatives were detected, suggesting that there were no rate-limiting steps in the demethylation or that this pathway was of minor importance. As compared to lanosterols, the amounts of di- and monomethyl derivatives were relatively small in meconium, suggesting that removal of the methyl group at C-14 had been a rate-limiting step. Mono- and dimethyl sterols are reported to predominate among the methyl sterols found in rat skin, while the amounts of lanosterols are negligible.[16]

Recent studies have shown rather convincingly that cholestanol is not formed by bacteria from cholesterol in the intestinal lumen.[17,18] The presence of this stanol in specimens from the fetus which, since the latter is living under sterile conditions, can not be a bacterial product, suggests that the stanol is synthesized by the fetal organism itself. However, the presence of traces of plant sterols in meconium, amniotic fluid, vernix caseosa, placenta, and maternal serum indicates that sterols, including cholestanol, can be transported from mother to child. Bloch [19] demonstrated the *in vivo* conversion of deuterated cholesterol to pregnanediol in pregnancy, which suggests, as recently pointed out by Ryan *et al.*,[20] that the maternal blood is the source of placental cholesterol. Yet the relatively high proportion of cholestanol

in fetal sterols seems to point to local synthesis rather than enrichment of transported stanol.

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