Transformation of 5α-cholest-7-en-3β-ol to cholesterol and cholestanol in cerebrotendinous xanthomatosis

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Abstract The metabolism of Δ^7-cholestenol, cholesterol, and cholestanol was examined in a patient with cerebrotendinous xanthomatosis after intravenous pulse-labeling with a mixture of DL-[2-^{14}C]mevalonate and stereospecific 3S,4S,3R,4R-[4-^{3}H]-mevalonate. Silver nitrate and reversed-phase thin-layer chromatography were used to purify the sterols isolated from the feces, and their identities were confirmed by gas–liquid chromatography–mass spectrometry. The specific activities were determined and plotted as a function of time. Isotope ratio measurements and specific activity decay curves showed that sterol synthesis proceeded in the following sequence: mevalonate, squalene, lanosterol, Δ^7-cholestenol, cholesterol, cholestanol. Labeled cholesterol precursors might be advantageously used to measure changes in cholesterol synthesis because they appear to equilibrate rapidly and have very short turnover times.

Supplementary key words β-sitosterol · gas–liquid chromatography · mass spectrometry · silver nitrate thin-layer chromatography · reversed-phase thin-layer chromatography · two-pool model · ^{3}H/^{14}C isotope ratio · specific activity decay curves

Lathosterol[1] (Δ^7-cholestenol), a suspected intermediate in the cholesterol biosynthetic pathway, is probably derived from lanosterol by reduction of the double bond at carbons 24,25 before the double bond at carbons 8,9 shifts to carbons 5,6 in ring B (1–7). Substantial quantities of Δ^7-cholestenol, cholestanol, and lanosterol are secreted in the bile (8, 9) of patients with cerebrotendinous xanthomatosis. This rare inherited disease is characterized by increased storage of cholesterol and cholestanol in all body tissues but especially in brain, lung, and Achilles tendon xanthomas (8, 9). Recently, these massive tissue sterol deposits have been related to hyperactive cholesterol and cholestanol synthesis (10). Although cholestanol has been conclusively demonstrated to originate from cholesterol (9) and not directly from lanosterol, which is 5α-H saturated, the role of Δ^7-cholestenol in cholesterol and cholestanol biosynthesis has not been convincingly established. The present investigation demonstrates that Δ^7-cholestenol is synthesized from mevalonate and is transformed into cholesterol, and confirms that cholestanol is formed only from cholesterol.

METHODS

Patient

E.D.S. is a 46-yr-old white woman with cerebrotendinous xanthomatosis (8, 9). She was hospitalized on the metabolic ward at the Rockefeller University Hospital and was fed a formula diet, prepared according to Ahrens (11), that contained 45% glucose, 15% protein, and 40% fat as cottonseed oil. Less than 45 mg/day of cholesterol, and no cholestanol, was present in the diet.

Experimental procedure

As described in a previous study (9) the patient was pulse-labeled with a mixture of 400 μCi of DL-[2-^{14}C]mevalonate (13.4 mCi/mmole) and 2000 μCi of stereospecifically labeled 3S,4S,3R,4R-[4-^{3}H]mevalonate (116 mCi/mmole) obtained from Amersham/Searle Corp., Des Plaines, Ill. Prior to administration, the two mevalonate preparations were mixed and converted to the sodium salt by incubating with $NaHCO_3$ at 37°C. The isotopic mixture was then dispersed in 150 ml of physiological saline and immediately infused intravenously.[2] Stools were col-

[1] The following are the systematic names of sterols referred to by trivial names: cholesterol, cholest-5-en-3β-ol; cholestanol, 5α-cholestan-3β-ol; lanosterol, lanosta-8,24-dien-3β-ol; Δ^7-cholestenol (lathosterol), 5α-cholest-7-en-3β-ol; β-sitosterol, cholest-5-en-24-methyl-3β-ol; campesterol, cholest-5-en-24-ethyl-3β-ol; desmosterol, cholest-5,24-dien-3β-ol; dihydrolanosterol, lanosta-8-en-3β-ol.

Abbreviations: $AgNO_3$-TLC, silver nitrate thin-layer chromatography; RP-TLC, reversed-phase thin-layer chromatography; GLC, gas–liquid chromatography; CTX, cerebrotendinous xanthomatosis; TMS, trimethylsilyl.

[2] A mixture of DL-[2-^{14}C]mevalonate and 3S,4S,3R,4R-[4-^{3}H]mevalonate was injected intravenously. Since only L-[2-^{14}C]mevalonate and 3R,4R-[4-^{3}H]mevalonate are metabolically active, only half of the quantities referred to in the text represent the amount of biologically active material injected. The radioactivity in the metabolically inactive mevalonate was recovered in the urine as mevalonic acid during the next day.

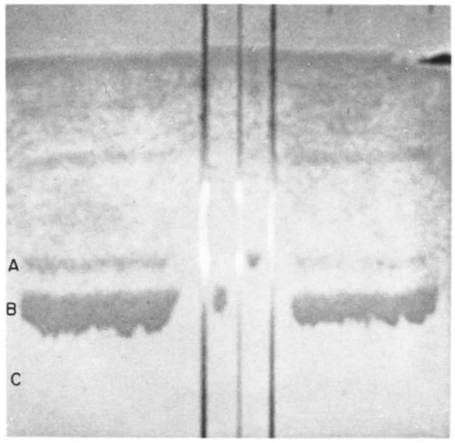

Fig. 1. Reversed-phase separation of cholestanol and Δ^7-cholestenol. Keiselguhr plus paraffin oil, developed in acetone–water 4:1 at 4°C. *A*, Δ^7-cholestenol; *B*, cholestanol; *C*, region of high ^{3}H activity.

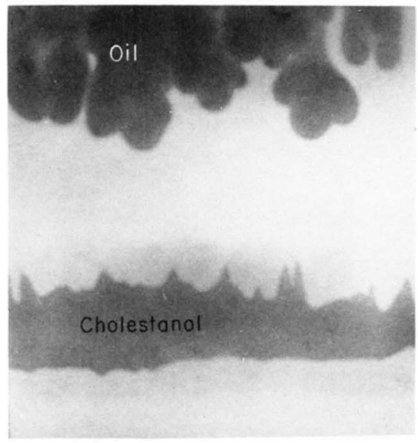

Fig. 2. Separation of paraffin oil from cholestanol in reversed-phase system. Keiselguhr, developed twice in *n*-heptane at room temperature.

lected daily for the next 12 days, homogenized, and stored at −15°C. The neutral sterol fraction was extracted and purified by $AgNO_3$ and reversed-phase TLC. Specific activities of the purified sterols were measured and plotted versus time. The decay curves for Δ^7-cholestenol, cholesterol, and cholestanol were analyzed for precursor–product relationships.

Sterol analysis

The stool samples were divided into 5–7-g portions and saponified by refluxing at 110°C for 1 hr in 1 N ethanolic NaOH (12). After adding 10 ml of water, the neutral sterols were extracted with hexane (boiling range 60–80°C).

Thin-layer chromatography

The neutral sterol fraction and reference standards of cholesterol and cholestanol were plated (13) as broad bands on 20 × 20 cm glass plates coated with a 0.5-mm layer of silica gel H containing $AgNO_3$, 10% (w/w). The plates were developed in chloroform–acetone 97:3 at 4°C. Sterol bands were made visible by spraying with a half-saturated aqueous solution of rhodamine G (Allied Chemical Corp., Morristown, N.J.) and were viewed under UV illumination.

Bands corresponding to the reference standards were eluted with ethyl ether. GLC and GLC–mass spectrometry indicated that the cholesterol band also contained the plant sterols campesterol and β-sitosterol, and the cholestanol band contained both cholestanol and Δ^7-cholestenol. The latter two sterols were separated by reversed-phase thin-layer chromatography using a modification of the system described by de Souza and Nes (14). Glass plates 20 × 20 cm were coated with 0.5-mm layers of kieselguhr G (Brinkmann Instruments, Inc., Westbury, N.Y.). After activation for 1 hr at 110°C, the plates were impregnated with paraffin oil (U.S.P.) by permitting a 5% solution of the oil in petroleum ether to migrate to the top of the plate. The plates were used as soon as the petroleum ether had evaporated. A maximum of 400–500 μg of sterol could be applied to the plates as a narrow band with a 100-μl Hamilton syringe. The plates were developed in acetone–water 4:1 at 4°C, and the sterol bands were visualized by spraying lightly with a half-saturated aqueous solution of rhodamine G. A typical chromatogram is illustrated in Fig. 1. Table 1 lists the R_F and R_S (S = cholesterol) values obtained for five different neutral sterols. The kieselguhr that contained the sterol bands was eluted first with ethyl ether, then with hexane, and finally with a 1:1 mixture of the two. This sequence of solvents was used to obtain maximal recovery of the sterols. However, paraffin oil was also eluted with this solvent system.

In order to remove the oil, it was necessary to rechromatograph the sterol–paraffin oil mixture on 20 × 20 cm glass plates coated with activated, oil-free, kieselguhr G. When the plates were developed twice in *n*-heptane, the oil migrated with the solvent front leaving the sterol band behind. A typical chromatogram illustrating the separation of the oil from the neutral sterols is shown in Fig. 2. The minimum amount of sterol that could be visualized was 30–40 μg. The average recovery of the plant sterol β-sitosterol after reversed-phase TLC and oil removal was 75%.

TABLE 1. R_S (S = cholesterol) and R_F values in the reversed-phase system[a]

Sterol	R_S	R_F
Cholesterol	1.00	0.41
Campesterol	0.90	0.37
β-Sitosterol	0.82	0.34
Cholestanol	0.88	0.36
Δ^7-Cholestenol	1.12	0.46

[a] Paraffin oil-impregnated kieselguhr G developed in acetone–water 4:1 at 2–5°C.

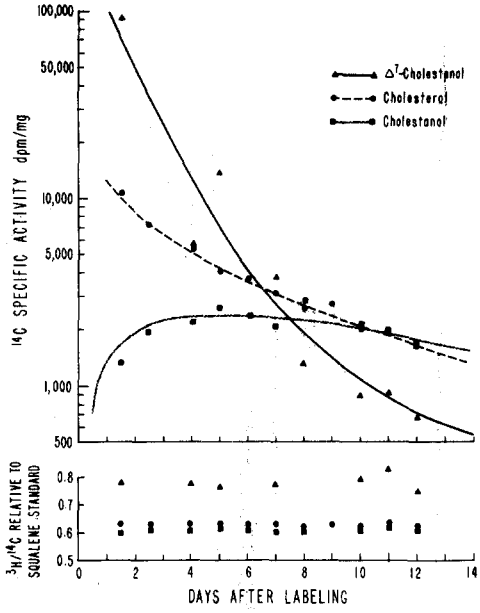

Fig. 3. ^{14}C specific activity and ^{3}H/^{14}C isotope ratio vs. time, in days, after injection of radioactive mevalonate, for cholesterol, cholestanol, and Δ^7-cholestenol isolated from the feces.

The purified sterols were then dissolved in ethyl acetate containing a known quantity of 5α-cholestane, and aliquots were taken for radioactive assay and GLC analysis.

Radioactive assay and GLC

Measured portions of the sterol solution were dissolved in toluene phosphor (4.2% Liquifluor, New England Nuclear Corp., Boston, Mass.) and assayed for radioactivity in a Beckman liquid scintillation system (model LS-250, Beckman Instruments, Fullerton, Calif.). Corrections were made for background, crossover, and quench by the channels-ratio method. The ^{3}H counting efficiency was set at approximately 45% and ^{14}C counting efficiency at about 70%.

Mass measurements were made on a Packard gas chromatograph, model 7300 (Packard Instruments, Downers Grove, Ill.), equipped with a flame ionization detector and an electronic integrator (model CRS-104, Infotronics Corp., Houston, Texas). Trimethylsilyl ether derivatives were formed by dissolving the sterol in 50–100 μl of Sil-Prep (Applied Science Laboratories, Inc., State College, Pa.). Between 1 and 5 μg of the sterols was injected onto 6 ft × ¼ inch OD glass columns packed with 3% QF-1 on Gas-Chrom Q, 80–100 mesh (Applied Science Laboratories). The column temperature was 240°C, the flash heater and detector were kept at 260°C, and the nitrogen carrier gas flow was maintained at 40 cm^3/min. The mass of the sterol was determined by comparing the area under the GLC peak produced by the sterol TMS ether derivatives with the area produced by a known quantity of 5α-cholestane (12, 15). The initial identification of the sterols was made by comparing the retention times (relative to 5α-cholestane) with the retention times of known standards (Applied Science Laboratories).

Mass spectrometry

The identity and purity of the sterols were confirmed by GLC–mass spectrometry using a Varian model 111 "Gnome" (Varian/MAT, Palo Alto, Calif.). About 5–10 μg of the sterol TMS ether derivative was injected onto 6 ft × ⅛ inch ID glass columns packed with 3% QF-1 as described above. The columns were maintained at 240°C with a helium carrier flow of 15 cm^3/min. The inlet and molecular separator temperatures were set 10–15°C above the column temperature. The ionization current was approximately 270 μA with an electron energy of 80 eV. The source temperature was about 300°C, and the source pressure was approximately 3 × 10^{-6} Torr (mmHg). The same mass of the TMS ether derivative of a reference compound was also injected and the two mass spectra compared line by line.

RESULTS

Separation of the sterols

The ^{3}H/^{14}C isotope ratios and the specific activities (^{14}C) of Δ^7-cholestenol, cholesterol, and cholestanol that were isolated from the stools over a 12-day period are presented in Fig. 3. The ^{3}H/^{14}C isotope ratios shown in Fig. 3 and Table 2 were calculated by dividing the measured ^{3}H/^{14}C specific activity ratio of each sterol by the

TABLE 2. ^{3}H/^{14}C isotope ratios of cholesterol, cholestanol, and Δ^7-cholestenol relative to squalene standard

Sterol	$AgNO_3$-TLC[a]	RP-TLC[a]	Mean ± SD (n) (entire experiment)	Bile (Ref. 9)	Theoretical (Fig. 4)
Cholesterol	0.62		0.63 ± 0.01 (16)	0.64	0.60
Cholestanol	1.14[b]	0.60	0.61 ± 0.01 (11)	0.65[c]	0.60[d]
Δ^7-Cholestenol		0.78	0.78 ± 0.02 (7)		0.80

[a] Pooled feces from days 1 and 2.
[b] After $AgNO_3$-TLC this band also contained Δ^7-cholestenol.
[c] After peroxyformic acid oxidation.
[d] Assuming cholestanol is synthesized entirely from cholesterol.

$^3H/^{14}C$ specific activity ratio of a squalene standard prepared from a portion of the injected mevalonate mixture.[3] This procedure removes the dependence of the $^3H/^{14}C$ specific activity ratio on the ^3H and ^{14}C activities of the injected mevalonate (9). Therefore, because the ratio of the number of ^3H atoms to the number of ^{14}C atoms in squalene synthesized from the biologically active labeled mevalonate is assumed to be 6/6, or 1.0 (9, 16, 17), the $^3H/^{14}C$ ratios in Fig. 3 and Table 2 represent the ratio of the average number of ^3H atoms to ^{14}C atoms in the sterol molecule.

It was necessary to separate the Δ^7-cholestenol from cholestanol by RP-TLC because these sterols migrate together on $AgNO_3$-TLC (13). In a previous report (9), the cholestanol was purified by oxidation with peroxyformic acid (18). This procedure destroyed the Δ^7-cholestenol and consequently no information about its metabolism was obtained. Furthermore, the results of that study suggested that the cholestanol–Δ^7-cholestenol band contained other radioactive impurities because the $^3H/^{14}C$ ratio after $AgNO_3$-TLC was greater than 1.0. The $^3H/^{14}C$ ratios of the cholestanol band after $AgNO_3$-TLC in the current study were also greater than 1.0 (as illustrated by the data for days 1 and 2 presented in Table 2). However, after RP-TLC the mean $^3H/^{14}C$ ratio for the entire 12 days (Table 2) was 0.78 ± 0.02 for Δ^7-cholestenol and 0.61 ± 0.01 for cholestanol. No combination of these two sterols alone could yield a $^3H/^{14}C$ ratio greater than the value of 0.78, obtained for pure Δ^7-cholestenol. Therefore, before RP-TLC the Δ^7-cholestenol–cholestanol band must have contained at least one other radioactive impurity whose $^3H/^{14}C$ ratio was greater than 1.0. Although it was not possible to characterize this compound(s), an area of excessive ^3H radioactivity was detected beneath the cholestanol band on the RP-TLC plates (Fig. 1).

The demonstration of a $^3H/^{14}C$ ratio of 0.78 ± 0.02 for Δ^7-cholestenol is consistent with the expected number and location of the $^3H/^{14}C$ atoms in this compound as illustrated in Fig. 4. The $^3H/^{14}C$ ratio of the lanosterol is 5/6, or 0.83 (6, 17). Presumably, during the transformation of lanosterol to Δ^7-cholestenol, one ^{14}C atom is lost with the methyl group at carbon 4, and one ^3H atom located at carbon 3 is also lost (19). Therefore, the predicted $^3H/^{14}C$ ratio of Δ^7-cholestenol should be 4/5, or 0.80.

Δ^7-Cholestenol

Analysis of the Δ^7-[^{14}C]cholestenol specific activity–time curves (Fig. 3) revealed that the specific activity was considerably higher and the curve decayed much more

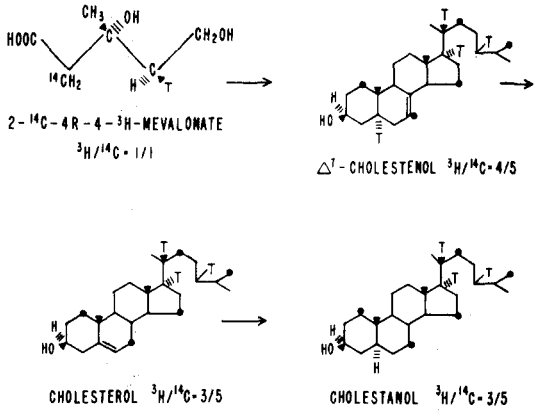

Fig. 4. Stereospecific labeling of Δ^7-cholestenol, cholesterol, and cholestanol after administration of [2-^{14}C-4R,4-^{3}H]mevalonate. T, ^3H; ●, ^{14}C; $^3H/^{14}C$, number of atoms of ^3H/number of atoms of ^{14}C.

rapidly than the specific activity–time curves for [^{14}C]cholesterol and [^{14}C]cholestanol. These observations are consistent with rapid conversion of Δ^7-cholestenol into cholesterol.

The turnover of Δ^7-cholestenol is so rapid that after 12 days its specific activity decreased to about 0.7% of its initial value. A decay in specific activity of this magnitude is found in pulse-labeled cholesterol experiments 40–60 wk after isotope injection (20, 21).

Fecal sterols were used for these analyses because (*a*) there are relatively large amounts of Δ^7-cholestenol excreted in the feces of CTX subjects, and (*b*) the Δ^7-cholestenol in the feces is lost from the pool and thus fecal sampling would not remove material that could possibly alter pool size or kinetic turnover. Apparently the liver preferentially secretes Δ^7-cholestenol into the bile because the concentration of this sterol relative to cholesterol in bile (8, 9) is considerably higher than it is in plasma, where Δ^7-cholestenol is barely detectable.

β-Sitosterol

One disadvantage of studying fecal sterols was that the cholesterol fraction isolated by $AgNO_3$-TLC contained large quantities of the plant sterols campesterol and β-sitosterol. These sterols were inherent in the dietary fat, and they appear in quantity in the feces because they are poorly absorbed from the intestine (22, 23). To rule out the possibility that a portion of these plant sterols might have arisen from the mevalonate mixture by de novo synthesis (24), an aliquot of the cholesterol–plant sterol mixture that was isolated by $AgNO_3$-TLC was further purified by RP-TLC. After four successive RP-TLC rechromatographs of the β-sitosterol band, the plant sterol was essentially free from cholesterol. Since no significant radioactivity was detected in the purified β-sitosterol, it was concluded that the plant sterol was not formed in the body. This finding is consistent with several previous observations which also showed that the β-sitosterol that cir-

[3] The squalene standard was from an aliquot of the same radioactive mevalonate mixture administered to the patient and was prepared by anaerobic incubation of the radioactive mevalonate mixture with a rat liver homogenate. This standard was used in a previous experiment on this subject (9) and was prepared by Drs. George Popják and Alan Polito.

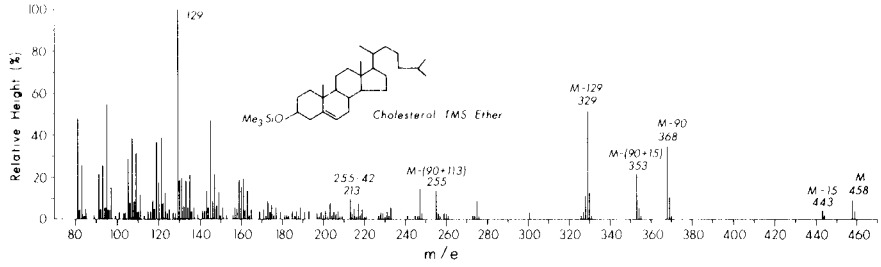

Fig. 5. Mass spectrum of the TMS ether derivative of cholesterol isolated from feces.

culates in the body originates only from dietary plant sterol (22, 23).

Cholesterol and cholestanol

The ^{14}C specific activity curves for cholesterol and cholestanol (Fig. 3) demonstrate a typical precursor–product relationship (25). This, combined with their $^{3}H/^{14}C$ ratios, confirms the conclusions of a previous study (9), that cholesterol is the precursor of cholestanol and that cholestanol is not synthesized directly from any of the cholesterol precursors.

Mass spectrometry

The GLC–mass spectra of the TMS ether derivatives of cholesterol, cholestanol, and Δ^7-cholestenol isolated from the feces were virtually identical with the mass spectra of reference standards of these compounds. No extraneous lines of any significant intensity were detected in the mass spectra of these fecal sterols.

The spectrum of cholesterol TMS ether (Fig. 5) exhibited a base peak at *m/e* 129 $[(CH_3)_3SiO(CH)_2CH_2]^+$ and prominent lines at *m/e* 458 (molecular ion), 443 (M−15), 368 (M−90), 353 (M−90−15), 329 (M−129), 255 (M−90−C_8H_{17} side chain), 247, and 213. The appearance of the spectrum was almost identical with that obtained by Diekman and Djerassi (26) except for slight differences in relative intensities of the *m/e* 255, 247, and 213 peaks. This is to be expected because the above authors have shown that the spectrum of this compound is very sensitive to the conditions and instruments used.

The TMS ether derivative of cholestanol (Fig. 6) exhibited a base peak at *m/e* 215 and major peaks at *m/e* 460 (molecular ion), 445 (M−15), 403, 370 (M−90), 355 (M−90−15), 306, 305, 230, 216, 215, and 201 as reported by Eneroth and Nyström (27). The peak at *m/e* 129, typical of 3-trimethylsilyloxy-Δ^5 compounds, was quite small, indicating that the purified cholestanol contained almost no cholesterol. The absence of a peak at *m/e* 458 (the molecular ion peak of cholesterol TMS ether) confirmed this point.

The spectrum of the TMS ether derivative of Δ^7-cholestenol (Fig. 7) showed a base peak at *m/e* 255 and other peaks at *m/e* 458 (molecular ion), 443 (M−15), 401, 368 (M−90), 353 (M−90−15), 255 (M−90−C_8H_{17} side chain), 229, and 213, which are typical of those reported for this compound (28, 29).

DISCUSSION

The data presented above show that Δ^7-cholestenol is an intermediate in cholesterol biosynthesis in patients with CTX and that it can be isolated from a mixture of closely related sterols by the RP-TLC procedure described above.

This system permits the recovery of pure cholestanol without resorting to oxidation with peroxyformic acid. Thus, pure Δ^7-cholestenol and other compounds can be recovered. Since cholesterol subsequently is transformed into cholestanol, Δ^7-cholestenol is also a distant precursor of cholestanol. The identification of Δ^7-cholestenol is based on its GLC retention time and confirmed by mass spectrometry. The $^{3}H/^{14}C$ ratios served as an additional method of structural confirmation (Fig. 4 and Table 2);

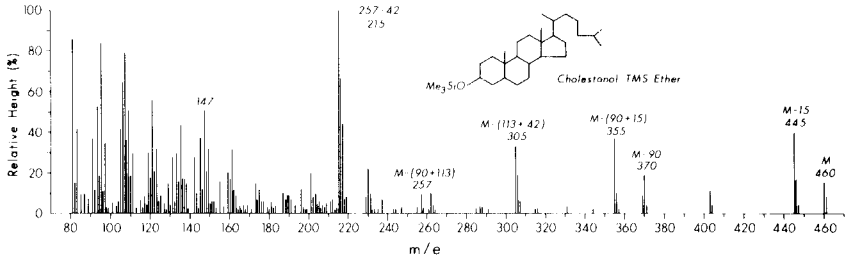

Fig. 6. Mass spectrum of the TMS ether derivative of cholestanol isolated from feces.

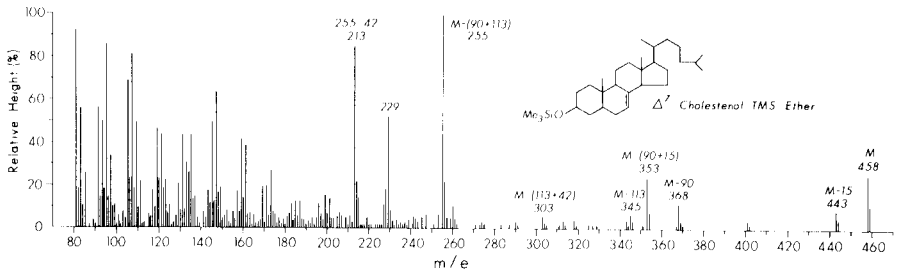

Fig. 7. Mass spectrum of the TMS ether derivative of Δ^7-cholestenol isolated from feces.

the experimental ratio of 0.78 ± 0.02 for Δ^7-cholestenol is in excellent agreement with the theoretical value of 0.80. It has been demonstrated (1, 2) that lanosterol is an obligate precursor in the synthesis of cholesterol by man. Both lanosterol and its Δ^{24} saturated product dihydrolanosterol have been isolated from the bile of this and other CTX subjects (8, 9). However, the question of the precise pathway from lanosterol to cholesterol is still unresolved. Clayton (2) has summarized the conflicting experimental results that suggest that synthesis proceeds either through desmosterol (the final step: reduction of the Δ^{24} double bond) or via Δ^7-cholestenol (early reduction of the Δ^{24} double bond).

The $^3H/^{14}C$ ratio and specific activity decay data presented here indicate that, in patients with CTX, cholesterol synthesis undoubtedly proceeds via Δ^7-cholestenol. The data do not, however, rule out the possibility that the alternate desmosterol pathway might also be operable.

The rapid turnover of this cholesterol precursor suggests that Δ^7-cholestenol, or another cholesterol precursor, might be useful in measuring cholesterol synthesis under a series of different experimental conditions, provided the compound was an obligatory precursor of cholesterol or it could be shown that its specific activity decay curve could be correlated to the production of cholesterol.

Since the turnover rate of the cholesterol precursors are extremely rapid, these measurements could be repeated several times during the course of an experiment. This would seem to be more convenient than measuring cholesterol production by either the kinetic or the sterol balance method. For instance, patients could be relabeled with Δ^7-cholestenol at intervals of 3 wk because the specific activity remaining from the first injection would be negligible. A simple calculation, using the observed Δ^7-[^{14}C]cholestenol specific activity curve (Fig. 3), indicates that for a series of measurements made over the next 3 wk the residual radioactivity from the first dose would at any time be less than 3–4% of the newly measured specific activities. An additional advantage is that the precursor pool turns over quite fast and would consequently reach equilibrium rapidly, in contrast to cholesterol, for which a prolonged interval is required. Thus, kinetic measurements for the precursor could be repeated quite often, over a reasonable period of time, because a steady state would be reached rapidly. However, a major disadvantage of this technique is that it is necessary to isolate a precursor that may be absent, or present only in vary small concentrations, in most body tissues (plasma). However, if the precursor can be isolated from bile or feces, relatively large quantities would be available.

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