Biosynthesis of 5α-Cholestan-3β-ol in Cerebrotendinous Xanthomatosis

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ABSTRACT Cerebrotendinous Xanthomatosis is a rare, inherited disease characterized by an extraordinary accumulation of cholestanol in all tissues, xanthomatous deposits in the brain, lungs, and Achilles tendons, premature atherosclerosis, and low plasma cholesterol concentrations. In two patients with the disease, the biosynthesis of cholestanol was examined by different techniques. After cholesterol-4-^{14}C was injected intravenously into one patient, cholestanol and cholesterol isolated from the bile on 3 different days over the ensuing week contained significant radioactivity. The specific radioactivity–time curves for cholesterol-^{14}C and cholestanol-^{14}C suggested a precursor product relationship and provided additional evidence for the transformation of cholesterol into cholestanol. The second patient received intravenously a mixture of mevalonate-2-^{14}C and stereospecifically labeled mevalonate-3R,4R-^{3}H. Again cholesterol and cholestanol were isolated from the bile, and the ^{3}H/^{14}C ratio in both sterols was almost the same. This experiment again demonstrated that the biosynthetic path of cholestanol proceeded through cholesterol and not directly from earlier 5α-H–saturated precursors. These two independent lines of evidence indicate that the extraordinary deposition of cholestanol in Cerebrotendinous Xanthomatosis arises from cholesterol presumably through the accentuation of the normal biosynthetic pathway.

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INTRODUCTION

Cholestanol,[1] the 5α-dihydro derivative of cholesterol is detectable in small amounts in tissues of normal laboratory animals and man. The impressive accumulation of this sterol in man was first shown by Menkes, Schimshock, and Swanson (1) who reported finding large amounts in the brain of a patient with a rare disorder called Cerebrotendinous Xanthomatosis (CTX).[2] The fully developed syndrome is characterized by progressive neurologic dysfunctions, premature atherosclerosis, cataracts, and xanthomatosis of brain, lungs, and tendons despite normal concentrations of plasma cholesterol. In 1969, Salen, Ahrens, and Naarden (2) found elevated concentrations of cholestanol in plasma, brain, tendon, and lung xanthomata, as well as in 12 other tissues in a patient with CTX indicating that the cholestenol deposition in CTX is not restricted to the brain.

The present report is concerned with observations on cholestanol biosynthesis in two patients with this disease studied on a metabolic ward. Two series of experiments have led us to the conclusion that cholestanol is derived from cholesterol. Experiments in these patients support the observations of Rosenfeld, Zumoff, and Hellman (3) in normal man as well as the preliminary findings of Mueckenhausen, Derby, and Moser (4) in a CTX patient that cholestanol arises from cholesterol and indi-

[1] The following systematic names are given to sterols referred to by trivial names: cholesterol, cholest-5-en-3β-ol; cholestanol, 5α-cholestan-3β-ol; lanosterol, lanosta-8,24-dien-3β-ol; dihydrolanosterol, lanosta-8-en-3β-ol; Δ^7-cholestenol, 5α-cholest-7-en-3β-ol.

[2] *Abbreviations used in this paper:* $AgNO_3$-TLC, argentation thin-layer chromatography; CTX, Cerebrotendinous Xanthomatosis; GLC, gas-liquid chromatography; TLC, thin-layer chromatography.

cate that the increased cholestanol in CTX was produced via the normal pathway.

METHODS

Patients. Patient 1 (E. D. S.) is a 46 yr old white woman who manifests moderately severe neurologic dysfunction (corticospinal tract paresis), bilateral Achilles tendon xanthomata, coronary insufficiency, and low levels of plasma cholesterol. Patient 2 (J. C.) is a 32 yr old black man who manifests bilateral Achilles tendon xanthomata, mild pulmonary insufficiency, and low concentrations of plasma cholesterol. Detailed clinical descriptions and metabolic studies will be presented elsewhere.

The patients were hospitalized on the metabolic ward at the Rockefeller University Hospital. Patient 1 was fed a formula diet composed of glucose, 45% protein, 15%, and fat as cottonseed oil, 40% that was prepared according to methods developed by Ahrens (5). The diet contained less than 45 mg/day of cholesterol and was free of cholestanol. Patient 2 ate regular food during the study.

Experimental design.[3] Patient 1 was given intravenously a single injection of a mixture of 200 μCi of mevalonate-2-^{14}C (13.4 mCi/mmole) plus 1000 μCi of stereospecifically labeled mevalonate-3R,4R-4-^{3}H (116 mCi/mmole) (6); the two preparations of mevalonate were obtained from Amersham-Searle Corp., Des Plaines, Ill. To ascertain the labeling ratios in sterol precursors produced from the mevalonate mixture used in this patient, samples containing 1 μCi of ^{14}C and 5 μCi of ^{3}H were incubated anaerobically with rat liver homogenates to produce squalene, and aerobically to produce lanosterol, dihydrolanosterol, and cholesterol (7). The sterols were purified by $AgNO_3$-TLC until constant ^{3}H/^{14}C ratios were obtained. Squalene was purified by column chromatography on alumina; the specimen was shown by gas-liquid radiochromatography to contain no radioactive substance other than squalene. The sterol mixture produced aerobically was subjected to the same succession of TLC steps described below for the isolation of individual pure sterols from the patients. The ^{3}H/^{14}C ratios obtained for these sterols served as standards.

After intravenous infusion of the radioactive mevalonate mixture, specimens of duodenal fluid rich with bile were obtained daily for the next 4 days. Cholecystokinin (obtained from Professor Erik Jorpes, Karolinska Institute, Stockholm, Sweden) was administered intravenously to facilitate the collection of gallbladder bile. The sterols were isolated from the bile and repeatedly purified by TLC (see below). Isotope ratios (^{3}H/^{14}C) were first expressed as ^{3}H specific activity/^{14}C specific activity; then these ratios were related to that of the squalene produced by in vitro incubation of the same mevalonate mixture with the rat liver homogenate.

Patient 2 received intravenously a pulse label of 30 μCi of cholesterol-4-^{14}C (55.2 mCi/mmole), (obtained from New England Nuclear Corp., Boston. Mass.) Before injection, the radioactive cholesterol was purified by $AgNO_3$-TLC, and 1.3% of the radioactivity was removed as a saturated contaminant. It was then dissolved in 1 ml of ethanol and administered intravenously dispersed in 150 ml of saline. During the ensuing week, three specimens of duodenal bile were obtained (days 1, 2, and 5). The neutral sterol fractions were isolated and repeatedly purified by $AgNO_3$-TLC and ^{14}C radioactivity was sought in cholestanol.

Radioactivity assay. Measured portions of the purified sterols and of the sterol precursor standards were dissolved in toluene phosphor (4.2% Liquifluor, New England Nuclear Corp.) and assayed for radioactivity in liquid scintillation spectrometers with appropriate corrections for cross-over and quenching (Packard Tri-Carb, model 3003, Packard Instrument Co., Inc., Downers Grove, Ill., and Beckman model LS-250, Beckman Instruments, Fullerton, Calif. (8). The efficiency for counting ^{3}H and ^{14}C was 51% and 71%, respectively.

Sterol analysis. (9) Specimens of intestinal bile were saponified at 70°C for 1 hr in 1 N ethanolic NaOH; the neutral sterols were then extracted with hexane. The solvent was evaporated, and the sterols were applied to thin-layer plates coated with $AgNO_3$-Silica Gel H (10% w/w); the plates were developed in chloroform: acetone, 97:3 (10). After spraying lightly with a half-saturated solution of Rhodamine 6G (Allied Chemical Corp., Morristown, N. J.), the sterols were identified under ultraviolet light and were individually eluted with ethyl ether in a vacuum aspirator. A portion of each sterol fraction was assayed for radioactivity and the remainder quantitated as the trimethylsilyl (TMS) ether derivatives by GLC on 6-ft columns packed with 3% QF-1 on Gas-Chrom Q (Applied Science Laboratories, Inc., State College, Pa.).

In order to eliminate any unsaturated impurities in the isolated cholestanol, peroxyformic acid oxidation was carried out on this fraction according to the method of Mosbach, Blum, Arroyo, and Milch (11).[4] The sterol fraction was exposed to a mixture of hydrogen peroxide and formic acid which results in the production of sterol formates. After hydrolysis, unsaturated compounds are converted into more polar derivatives, while cholestanol formate is hydrolyzed to cholestanol and can be separated from the bulk of the more polar sterols by solvent partition from 50% aqueous ethanol with hexane. To ensure that only cholestanol remained. the hexane extract was rechromatographed on thin-layer plates coated with $AgNO_3$-Silica Gel H. A sample was assayed for radioactivity and the remainder quantitated by GLC as described previously.

RESULTS

Neutral sterol composition of bile. The neutral sterols isolated by $AgNO_3$-TLC from specimens of duodenal bile of patients 1 and 2 were identified and quantitated by GLC. The results are displayed in Table I. Substantial quantities of cholestanol, lanosterol, dihydrolanosterol, and Δ^7-cholestenol were secreted in the bile. The concentrations of these sterols in normal bile is negligible, and therefore, we believe this characteristic biliary neutral sterol pattern in CTX is helpful in proving the diagnosis (12). Although the absolute quantities of the individual biliary sterols may have little significance, the appearance of the cholesterol precursors with cholestanol in the bile suggests that CTX is associated with a defect in the regulation of sterol biosynthesis.

[3] This experiment was suggested by Dr. George Popják of University of California at Los Angeles School of Medicine, Los Angeles, Calif.

[4] This procedure was kindly performed by Dr. Erwin H. Mosbach.

TABLE I

Sterol Composition in Duodenal Drainage

Days after pulse labeling	Cholesterol		Cholestanol		Δ^7-Cholestenol*		Lanosterol		Dihydro-lanosterol‡	
	mg	%	*mg*	%	*mg*	%	*mg*	%	*mg*	%
Patient 1										
1	30.1	93.8	1.58	4.9	0.12	0.4	0.25	0.8	0.04	0.1
3	29.1	89.6	2.27	7.0	0.23	0.7	0.72	2.2	0.15	0.5
5	34.9	85.8	4.00	9.9	0.30	0.7	1.13	2.8	0.34	0.8
Patient 2										
1	7.5	93.3	0.41	5.0	0.06	0.7	0.05	0.7	0.02	0.3
2	9.7	92.7	0.56	5.4	0.13	1.2	0.03	0.3	0.05	0.4
5	18.3	89.9	1.36	6.7	0.33	1.6	0.26	1.3	0.10	0.5

* Δ^7-Cholestenol could not be separated from cholestanol by $AgNO_3$-TLC, but was easily distinguished and quantitated by GLC.

‡ Contained traces of 14-nor-dihydrolanosterol.

The conversion of cholesterol-4-^{14}C into cholestanol-4-^{14}C. To assess the transformation of cholesterol directly into cholestanol, we administered intravenously to patient 2 approximately 30 μCi of purified cholesterol-4-^{14}C as a pulse label. Neutral sterols were isolated from bile and ^{14}C radioactivity measured in cholestanol and the sterol precursors lanosterol and dihydrolanosterol, and their specific activities were determined (Fig. 1).

The presence of label in cholestanol proves that cholesterol was converted into cholestanol. After isolation of the cholestanol by $AgNO_3$-TLC, peroxyformic acid oxidation was performed to eliminate traces of cholesterol-^{14}C which might have migrated with cholestanol on TLC. Although it is unlikely that any cholesterol-^{14}C was transformed into its precursor Δ^7-cholestenol-^{14}C, had any been present, the oxidation procedure also would remove this sterol.

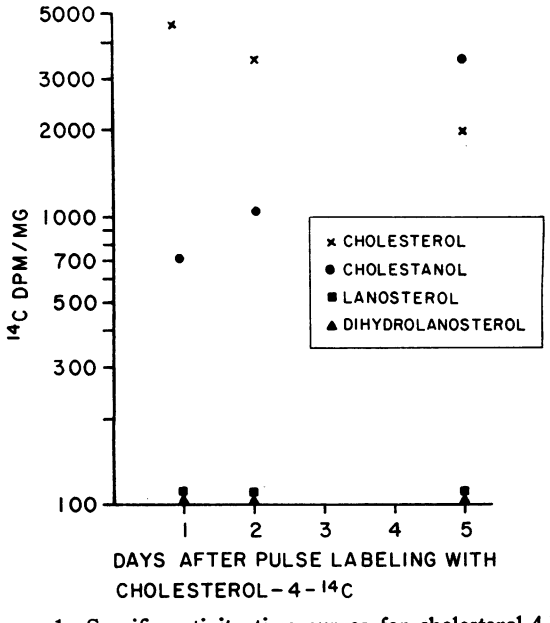

FIGURE 1 Specific activity–time curves for cholesterol-4-^{14}C and cholestanol-4-^{14}C isolated from bile after patient 2 received 30 μCi of cholesterol-4-^{14}C intravenously. The specific activities of cholesterol-4-^{14}C decreased while the specific activities of cholestanol-^{14}C increased and exceeded cholesterol-^{14}C by the 5th day.

The demonstration of increasing radiospecific activity of cholestanol-4-^{14}C over the 5 day study period with the specific activity of cholestanol ultimately exceeding that of cholesterol-4-^{14}C is consistent with a precursor product relationship between cholesterol and cholestanol. This is additional evidence for the transformation of cholesterol into cholestanol. Had cholestanol arisen from the reversal of the cholesterol synthetic pathway, label would have been expected in the cholesterol precursors lanosterol and dihydrolanosterol. Since significant counts were not found in these compounds, this possibility is considered excluded.

The biosynthesis of cholestanol from stereospecifically labeled mevalonate.[8] Patient 1 was given a mixture of stereospecifically labeled mevalonate-^3H and -^{14}C as a single intravenous pulse label. The choice of this mixture of radioactive mevalonates was based on the experiments of Cornforth, Popják, and colleagues (6, 13). These workers proved by degradative studies the precise locations of the ^{14}C and ^3H atoms in cholesterol, lanosterol, and squalene that were synthesized by liver homogenates from the mevalonate mixture (13). Because of the stereospecific nature of the enzymatic reactions involved in the biosynthesis of these sterols, the radioactive atoms are found in fixed locations (Fig. 2).

Table II shows the isotope ratios established for the squalene, lanosterol, dihydrolanosterol, and cholesterol standards formed after in vitro incubation with a rat liver homogenate, using a portion of the mixture administered to the patient. The actual isotopic ratios in

TABLE II

Isotope Ratios ($^3H/^{14}C$) of Sterols Isolated after Incubation of Mevalonate-2-^{14}C and Mevalonate-3R, 4R-^3H with Rat Liver Homogenates

Standards	^3H	^{14}C	$^3H/^{14}C$ Actual	$^3H/^{14}C$ Relative to squalene	$^3H/^{14}C$ Predicted
	dpm				
Squalene	29,700	6,200	4.79	1.00	—
Lanosterol*	9,800	2,400	4.08	0.85	0.83
Dihydrolanosterol*	15,900	3,800	4.18	0.87	0.83
Cholesterol*	62,500	20,100	3.11	0.65	0.60

* Purification facilitated by the addition of chemically pure, nonradioactive sterol before $AgNO_3$-TLC.

the sterol standards have no intrinsic meaning because they are determined solely by the proportion of the two labeled mevalonates in the injected mixture. However, when each sterol ratio was expressed relative to squalene ([actual isotopic ratio/squalene isotopic ratio] = ratio relative to squalene) and then compared with the theoretical ratios (Fig. 2) excellent agreements were obtained.

In Tables III and IV are shown the ($^3H/^{14}C$) ratios for the sterols isolated from bile specimens of patient 1 at various intervals after isotope injection. The isotope ratios in cholesterol and lanosterol as expected were constant and were nearly identical with values obtained for these sterols produced by the in vitro procedure (Table II) and predicted by previous experiments in lower species (Fig. 2). However, the ratios of $^3H/^{14}C$ in the cholestanol isolated by $AgNO_3$-TLC was considerably higher than cholesterol,[5] and also varied considerably from day to day. Since we found that Δ^7-cholestenol was in this fraction, it was necessary to remove this sterol and any other unsaturated contaminants by peroxyformic acid oxidation before the true $^3H/^{14}C$ ratio for cholestanol could be established. Table IV displays the results for cholestanol after oxidation and shows that the $^3H/^{14}C$ ratio of the pure cholestanol was now virtually identical with that of cholesterol. The close correspondence of these values provides strong evidence that cholestanol was derived directly from cholesterol.

DISCUSSION

The results of these experiments extend to patients with CTX the previous findings of Rosenfeld et al. (3) that cholesterol was converted to cholestanol in a normal man. Similar findings have been reported in rats, guinea pigs, and rabbits by Shefer, Milch, and Mosbach (14), and Werbin, Chaikoff, and Imada (15), and in one patient with CTX by Mueckenhausen et al. (4). These latter investigators gave cholesterol-4-^{14}C to a man with CTX, and when he died 3 yr later isolated radioactive cholestanol from the myelin of his brain indicating that cholesterol served as the precursor.

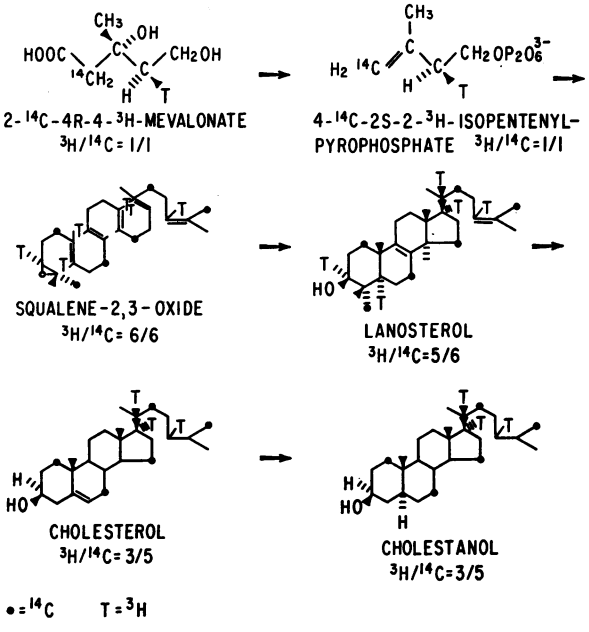

FIGURE 2 Stereospecific labeling of squalene, lanosterol, and cholesterol based on previous studies (6, 13) in lower species, after administration of mevalonate-2-^{14}C-4R,4-^3H, and postulated distribution of radioactive atoms in 5α-cholestanol. Experimental finding of a 3/5 ratio of $^3H/^{14}C$ in 5α-cholestanol relative to squalene indicates that cholesterol ($^3H/^{14}C = 3/5$) served as the direct precursor.

[5] The $^3H/^{14}C$ ratio of 1.40 obtained for the cholestanol-Δ^7-cholestenol fraction isolated on day 1 suggests the presence of additional contaminants. It is exceedingly unlikely that the squalene biosynthesized from the mevalonate mixture would give rise to any sterol with a $^3H/^{14}C$ ratio greater than 1.0.

TABLE III

Isotope Ratios ($^3H/^{14}C$) and Specific Activities of Sterols Isolated from the Bile of Patient 1

Sterols	Days after pulse labeling	Mass	^3H	^{14}C	$^3H/^{14}C$ Actual	$^3H/^{14}C$ Relative to squalene	Specific activities ^3H	Specific activities ^{14}C
		mg	*dpm*				*dpm/mg*	
Cholesterol	1	30.10	1,380,700	449,600	3.07	0.64	45,800	14,900
	3	29.10	479,200	156,800	3.06	0.64	16,400	5,380
	5	34.90	427,600	139,300	3.07	0.64	12,300	4,000
Cholestanol-Δ^7-cholestenol[5]	1	1.70	106,300	15,900	6.69	1.40	62,400	9,340
	3	2.50	18,100	3,960	4.57	0.95	7,200	1,570
	5	4.50	25,700	6,190	4.15	0.87	5,940	1,430
Lanosterol	1	0.25	6,100	1,390	4.39	0.92	24,600	5,610
	3	0.72	1,230	280	4.39	0.92	1,710	390
	5	1.13	1,190	270	4.41	0.92	1,050	240
Dihydrolanosterol	1	0.04	1,760	440	4.00	0.84	48,100	11,900
	3	0.15	850	250	3.40	0.71	5,700	1,660
	5	0.34	1,770	540	3.28	0.68	5,220	1,600

Our conclusion is based upon two independent lines of evidence. (*a*) The direct conversion of cholesterol-4-^{14}C into cholestanol was established in patient 2. The demonstration of ^{14}C radioactivity in cholestanol and the apparent attainment of the precursor product relationship for the specific activity–time curves of cholesterol and cholestanol after intravenous pulse labeling with cholesterol-4-^{14}C (Fig. 1) support this conclusion. The higher specific activity of cholestanol-^{14}C on day 5 is also evidence that only a single pathway through cholesterol exists for the formation of cholestanol. If synthesis of cholestanol also occurred by another route bypassing cholesterol, then the specific activity of cholestanol would probably always be lower than that of cholesterol reflecting the dilution of cholestanol-^{14}C with the extra nonradioactive cholestanol. (*b*) The nearly identical $^3H/^{14}C$ ratios of cholestanol and cholesterol isolated from the bile of patient 1 after pulse labeling with a mixture of mevalonate-2-^{14}C, 3R,4R-^3H, proved that cholestanol had been formed from cholesterol. The correspondence of isotope ratios ($^3H/^{14}C$) in both sterols eliminated lanosterol and Δ^7-cholestenol as direct precursors of cholestanol. Both lanosterol and Δ^7-cholestenol contain extra tritium atoms at the 5α carbon position (Fig. 2). Therefore, had they been directly transformed into cholestanol, the $^3H/^{14}C$ ratio would have exceeded that in cholesterol. Thus, it appears that cholestanol biosynthesis although accentuated in CTX is similar to that of normal man and lower animals.

The importance of the peroxyformic acid oxidation step was emphasized because this technique removes unsaturated contaminants including Δ^7-cholestenol and cholesterol from cholestanol. However, the ratio of 1.40 on day 1 (relative to squalene) which was observed in the cholestanol-Δ^7-cholestenol fraction could not be explained solely by the presence of Δ^7-cholestenol. The predicted $^3H/^{14}C$ ratio for this sterol relative to squalene is 0.80. Consequently, the $^3H/^{14}C$ ratio of 1.40 must be caused by an additional contaminant synthesized from

TABLE IV

Isotope Ratios ($^3H/^{14}C$) in Cholestanol after Peroxyformic Acid Oxidation

	Days after pulse labeling	Mass	^3H	^{14}C	$^3H/^{14}C$ Actual	$^3H/^{14}C$ Relative to squalene	Specific activities ^3H	Specific activities ^{14}C
		mg	*dpm*				*dpm/mg*	
Cholestanol	1	0.20	1600	490	3.26	0.68	8000	2500
	3	0.12	600	200	3.00	0.63	5200	1700
	5	0.49	3700	1200	3.08	0.64	7600	2500

mevalonate. The structure of this material is not known, but must be preferentially formed from stereospecifically labeled mevalonate-3R,4R-^{3}H in order to show a higher ^{3}H/^{14}C ratio than squalene. Fortunately, after oxidation only cholestanol remained.

The isotope and mass measurements were made on biliary rather than plasma sterols because bile contained appreciable quantities of cholesterol precursors not detectable in plasma and also 5 times more cholestanol than plasma. Thus, after the rigorous purification of cholestanol sufficient sterol remained for conclusive identification and confident isotope measurement.

The biosynthetic pathway of cholesterol to cholestanol has been considered by Rosenfeld et al. (3) who showed the almost complete elimination of the 3α-^{3}H in cholestanol formed from cholesterol-3α-^{3}H, 4-^{14}C given intravenously to a 23 yr old man. These workers, therefore, concluded that the first step in the formation of cholestanol involved the oxidation of the 3β-hydroxyl group of cholesterol to a ketonic intermediate (Fig. 3). Although this key intermediate has not been conclusively identified, there is evidence which indicates it to be Δ^4-cholesten-3-one. The earliest study dealing with this subject was the report of Anker and Bloch (16) who showed that uniformly deuterated Δ^4-cholesten-3-one was converted into saturated sterols but not cholesterol. Similar results were later presented by Harold, Abraham, and Chaikoff (17) who also demonstrated that Δ^4-cholesten-3-one-4-^{14}C was transformed into cholestanol and bile acids in the rat. Although the bile acids were quantitatively the most important metabolites produced, they were not further identified. These observations were subsequently substantiated by Shefer, Hauser, and Mosbach (18) who conclusively showed that cholestanol was formed in the rabbit and guinea pig from injected Δ^4-cholesten-3-one. Nevertheless, despite the evidence in favor of a ketonic intermediate, the oxidation of cholesterol into Δ^4-cholestenone has never been proven.

A number of interesting questions are suggested by these studies. For example, (*a*) what are the factors which regulate cholestanol production in man? The demonstration that small amounts of cholestanol accompany cholesterol in virtually every tissue indicates that cholestanol is produced normally in all people; while the extraordinary deposits of cholestanol found in the tissues of CTX patients (2) is suggestive of accentuated synthesis. In a preliminary communication we have reported that cholestanol production in CTX is 5 times greater than normal (19). Thus, the cholestanolosis in CTX is associated with increased production probably related to the derepression of its biosynthetic pathway. (*b*) In what organ is cholestanol made?

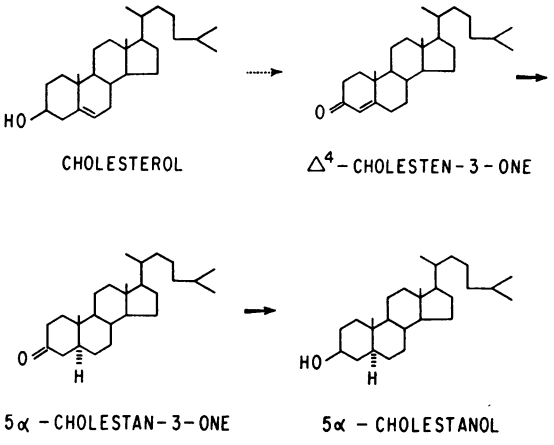

FIGURE 3 The biosynthesis of cholestanol from cholesterol requires at least three reactions. The conversion of Δ^4-cholesten-3-one into 5α-cholestan-3-one and then into cholestanol have been amply authenticated (18). However, the transformation of cholesterol into Δ^4-cholesten-3-one requires substantiation and is therefore indicated by the broken arrow.

Are the large amounts of cholestanol found in the brain and xanthomata of CTX individuals produced locally by these tissues, or is cholestanol produced elsewhere and transported to those sites? (*c*) What compound serves as the feedback inhibitor of cholestanol synthesis? The failure of elevated plasma and tissues levels of cholestanol in CTX to inhibit synthesis militates against relatively low levels of cholestanol acting as its own inhibitor and may mean that synthesis is not under negative feedback control. (*d*) Finally, what role does cholestanol play in the development of the devastating symptomology in CTX? Is this sterol directly toxic to nervous tissues? Can increased cholestanol concentrations in plasma of CTX subjects promote the development of xanthomas and atherosclerosis at low plasma cholesterol concentrations? The answers to these key questions and others suggested by this intriguing disorder will fill many fundamental gaps in our knowledge about sterol metabolism.

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