Conversion of cholesterol injected into man to cholestanol via a 3-ketonic intermediate

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ABSTRACT Cholesterol-3-^{3}H,4-^{14}C was injected intravenously in man and its transformation to cholestanol was studied. From the ^{3}H:^{14}C ratios in cholestanol isolated from blood, evidence for the participation of a ketonic intermediate in the conversion was obtained. In a second subject given cholestanol-3-^{3}H,4-^{14}C the ^{3}H:^{14}C ratios in blood sterols remained unchanged for as long as 1 wk after the injection, which showed that cholestanol did not lose tritium by interconversion with cholestanone.

KEY WORDS cholesterol–cholestanol conversion · ketonic intermediate · man · cholesterol-3-^{3}H,4-^{14}C metabolism

THAT CHOLESTANOL is formed from cholesterol in animal tissues has been reported by several investigators (1–3); recently, Werbin, Chaikoff, and Phillips showed that the transformation takes place in the intact guinea pig (4). Although the steps of the conversion have not been unequivocally proven, it is probable that the sequence cholesterol → cholestenone → cholestanone → cholestanol represents the metabolic pathway. Several groups have reported that cholestenone is converted to cholestanol in experimental animals (2, 5–7) and in vitro studies with tissue preparations have demonstrated the conversion of cholesterol to cholestenone (8), cholestenone to cholestanone (9), and cholestanone to cholestanol (5, 10).

This sequence very probably occurs in man and the conversion of cholestenone-4-^{14}C to cholestanol-4-^{14}C has been established (11). Investigations of the cholesterol–cholestanol pathway in humans by radiochemical techniques, where peripheral blood is the only conveniently available source from which to separate and isolate the saturated sterol, are hampered by several factors: (*a*) low levels of cholestanol, usually less than 1% of the circulating sterol (12, 13); (*b*) small conversion of precursor (3); (*c*) difficulty in separation of product and precursor; and (*d*) the presence of labeled contaminant, inseparable from or identical with cholestanol, in tracer cholesterol. With regard to (*d*), the preparation of cholesterol with a specific activity sufficiently high for studies in man yet completely free from impurities would require facilities beyond the scope of most laboratories. Therefore to investigate the cholesterol–cholestanol transformation, it is necessary to know precisely the quantity of radioactive impurity in the administered cholesterol. With these considerations in mind we have studied the conversion of cholesterol to cholestanol in humans and the results establish that the transformation occurs in vivo by a mechanism in which the 3β-hydroxy group is oxidized. A preliminary account of this study has been published (14).

MATERIALS

Subjects

A 19 yr old diabetic male (plasma cholesterol 206 mg/100 ml, 28% free) on an 1800 cal/day diet containing about 0.15 g of plant sterols, was given cholesterol-3-^{3}H,4-^{14}C. The tracer (2 mg, SA = 14.5×10^{7} cpm/mg of ^{3}H, 3.95×10^{7} cpm/mg of ^{14}C; ^{3}H:^{14}C ratio = 3.67) was dissolved in 2 ml of ethanol, mixed with 35 ml of 5% dextrose solution, and immediately administered by rapid intravenous injection. To a 42 yr old diabetic woman (plasma cholesterol 237 mg/100 ml, 33% free) on

Abbreviations: SA, specific activity; MCPBA, *m*-chloroperbenzoic acid; TLC, thin-layer chromatography; GLC, gas–liquid chromatography. The following trivial names have been used: cholestanol, 5α-cholestan-3β-ol; coprostanol, 5β-cholestan-3β-ol; cholestenone, Δ^{4}-cholesten-3-one; cholestanone, 5α-cholestan-3-one.

a 2400 cal/day diet containing approximately 0.20 g of plant sterols, we administered in the same way 1 mg of cholestanol-3-^{3}H,4-^{14}C (SA = 16.5 × 10^{7} cpm/mg of ^{3}H, 4.05 × 10^{7} cpm/mg of ^{14}C; ^{3}H:^{14}C ratio = 4.08). Blood was drawn into heparinized tubes at specified times after the start of the experiment and separated into plasma and cells. In the study of doubly labeled cholesterol, free cholesterol and cholesterol esters were isolated from plasma by a chromatographic procedure (15). In both studies, red cell cholesterol was obtained from the nonsaponifiable fraction of an acetone–ethanol extract of cells (13). Sterols were obtained from stools as previously described (16, 17).

Sterol Tracers

Radiochemical Purity of Cholesterol-3-^{3}H,4-^{14}C. About 0.06% of the radioactive dose (New England Nuclear Corp., Boston, Mass.) was mixed with 200.7 mg of cholesterol, SA of mixture = 890 cpm/mg of ^{3}H, 250 cpm/mg of ^{14}C; ^{3}H:^{14}C ratio = 3.66. Sequential purification steps, as outlined in Table 1, demonstrated that the isotope ratio was essentially unchanged after four procedures in which the hydroxyl group at C-3 was not altered, but that oxidation to the 3-ketone removed over 99% of the tritium.

Contamination of Administered Cholesterol-3-^{3}H,4-^{14}C with Saturated Radioactive Sterols. The same labeled cholesterol administered to the patient was added to 107 mg of unlabeled cholestanol; SA of the sterol mixture = 122,000 cpm/mg of ^{3}H, 33,600 cpm/mg of ^{14}C; ^{3}H:^{14}C = 3.64. After performic acid oxidation (18), the product was partitioned between petroleum ether and 90% methanol (19). Saturated sterol from the petroleum ether phase was dissolved in 5 ml of chloroform and mixed with a solution of 150 mg of MCPBA (FMC Corporation, New York, N.Y.) in 5 ml of chloroform. After 45 min at room temperature, the solution was diluted with 60 ml of ether and washed with 5 ml of cold 5% potassium hydroxide solution, then with water until the washings were neutral. The organic phase was concentrated and the residue was chromatographed on 15 g of alumina. The column was developed with petroleum ether–benzene mixtures; cholestanol was eluted with 100% benzene. Cholestanol so obtained was once more subjected to the performic acid-partition procedure and crystallized from acetone and acetone–methanol to constant specific activity. These several procedures were carried out to insure complete removal of cholesterol from saturated sterols. This sequence is summarized in Table 2; the ^{3}H:^{14}C ratio of saturated contaminant was 1.21 and the quantities represented 0.15% of the ^{3}H and 0.44% of the ^{14}C in the administered cholesterol.

Radiochemical Purity of Administered Tracer. Since the two isotopes were incorporated into the cholesterol mole-

TABLE 1 RADIOCHEMICAL PURITY OF ADMINISTERED CHOLESTEROL-3-^{3}H,4-^{14}C

Step	Procedure	SA ^{3}H	SA ^{14}C	^{3}H:^{14}C
		cpm/mg		
1	Tracer diluted with nonradioactive cholesterol*	890	250	3.66
2	Digitonin separation: β-fraction	930	250	3.72
3	β-Fraction from step 2: oxidized to cholestanetriol; 86.9 mg of product mixed with 100.5 mg of nonradioactive cholestanetriol; diluted material converted to cholestane-3β,-5α-diol-6-one by *N*-bromosuccinimide oxidation (18)	870†	220†	3.96
4	Cholestane-3β,5α-diol-6-one crystallized from methanol–acetone; mp 224–226 °C	860	220	3.90
5	Product from step 4 refluxed in 5% KOH in 80% ethanol	840	230	3.65
6	Cholestane-3β,5α-diol-6-one from step 5 oxidized to cholestane-5α-ol-3,6-dione with chromic oxide–sulfuric acid (21)	15	260	0.058
7	Cholestane-5α-ol-3,6-dione after chromatography on alumina	<5	230	<0.022

* 0.064% of the administered tracer was mixed with 200.7 mg of cholesterol.

† SA before dilution; factor = 0.42.

TABLE 2 PRESENCE OF SATURATED CONTAMINANT IN ADMINISTERED CHOLESTEROL-3-^{3}H,4-^{14}C*

Step	Procedure	SA ^{3}H	SA ^{14}C	^{3}H:^{14}C
		cpm/mg		
1	Tracer diluted with 107 mg of nonradioactive cholestanol	122,000	33,600	3.64
2	Performic acid oxidation: petroleum ether-soluble fraction treated with MCPBA and chromatographed: cholestanol	1300	560	2.32
3	Repeat of step 2: cholestanol	530	277	1.90
4	Crystallized cholestanol from acetone	200	148	1.35
5	Crystallized from methanol–acetone	160	133	1.20
6	Repeat of step 5	180	149	1.21

* ^{3}H in saturated contaminant in dose, 180: 122,000 = 0.15%; ^{14}C in saturated contaminant in dose, 149: 33,600 = 0.44%.

cule by different procedures, it is to be expected that the amounts of ^{3}H- and ^{14}C-labeled contaminants would differ. Consequently (Table 2), the ^{3}H:^{14}C ratio of saturated sterols in the dose, 1.21, was quite different from that in the radioactive cholesterol, 3.67. It is assumed that the saturated substance was cholestanol since analysis by reverse isotope dilution techniques showed that the radioactivity could not be dissociated from this sterol.

Preparation and Radiochemical Purity of Cholestanol-3-^{3}H,4-^{14}C. Cholestanol-4-^{14}C (New England Nuclear Corp., Boston, Mass.) was used as received. Dilution of 1×10^{6} cpm of this material with 100 mg of cholesterol and 100 mg of cholestanol showed that more than 99% of the radioactivity remained with cholestanol after oxidation and removal of the cholesterol (18, 19). Cholestanol-3-^{3}H was obtained by reduction of cholestanone with sodium borohydride-^{3}H in methanol and was separated from 5α-cholestan-3α-ol-3-^{3}H by TLC in a 1:1 cyclohexane–ethyl acetate system. The ^{3}H- and ^{14}C-labeled cholestanol samples were mixed. A trace quantity of the doubly labeled compound, 2.32×10^{6} cpm of ^{3}H, was diluted with 126 mg of unlabeled cholestanol. This was mixed with 25.1 mg of 5α-cholestane-3α-ol and the β-fraction was precipitated with digitonin solution. Dissociation of the digitonide with dimethyl sulfoxide (20) afforded cholestanol with 92% of the original radioactivity, and two recrystallizations from acetone–methanol caused no change in the specific activity. Oxidation with chromic oxide–sulfuric acid in acetone (21) yielded cholestanone with a loss of over 99% of the tritium. Therefore cholestanol-3-^{3}H,4-^{14}C used in the study could contain no more than 8% of the 3α-epimer and had over 99% of the ^{3}H at C-3.

Study with Cholesterol-3-^{3}H,4-^{14}C

Purification of Isolated Sterols: Plasma. Free cholesterol and cholesterol esters were isolated from three pooled plasma samples obtained during the 10 days following administration. Cholesterol esters were saponified and the sterol obtained was counted before being combined with the corresponding free cholesterol to form a total sterol fraction. Each pool was then processed separately. The sterol was treated with formic acid–hydrogen peroxide (18) and saturated sterols were removed by petroleum ether–90% methanol partition. Cholestane-3β,5α,6β-triol from the methanol layer was acetylated by reaction with pyridine–acetic anhydride at room temperature overnight and the cholestanetriol-3,6-diacetate was crystallized from methanol until its specific activity was constant. Cholestanol in the petroleum ether phase was determined by GLC (13) so that the dilution factor could be calculated after the addition of non-radioactive cholestanol. After dilution, 10 mg of cholesterol was added and the fraction was treated with MCPBA in chloroform and chromatographed on alumina to remove the oxidation products. Cholestanol, eluted in the petroleum ether–benzene fraction, was treated a second time with MCPBA and chromatographed. It was crystallized once from methanol, acetylated, and chromatographed on alumina. Cholestanol acetate was finally crystallized from acetone–methanol until constant isotope ratio and specific activities were achieved.

Red Cells. Two red cell pools were made from the blood collected during the 10 day period and the lipid fractions were saponified. Total sterols were treated in the same way as plasma sterols.

Feces. Coprostanol and cholesterol were separated by chromatography of the nonsaponifiable fraction of stools from the 2nd and 3rd day after administration of the tracer (22). Coprostanol was acetylated and the product was crystallized from acetone–methanol. Cholesterol, containing small quantities of cholestanol, was converted to cholestanetriol and the saturated sterol was removed by solvent partition. Each fraction was purified to constant specific activity as described above.

Study with Cholestanol-3-^{3}H,4-^{14}C

Purification of Blood Sterols. Total sterols, obtained from the nonsaponifiable fraction of plasma or red cell lipids, were oxidized with performic acid (18,19) and partitioned into cholestanetriol and saturated sterol fractions. The latter fractions contained about 2% cholestanol as judged by GLC (13); each was mixed with 50 mg of nonradioactive cholestanol. Reaction with MCPBA and chromatography were applied as in the previous study. Cholestanol fractions from the column were combined and crystallized to constant specific activity.

Feces. Chromatography of the petroleum ether-soluble lipids on alumina (16) afforded sterol ester fractions and more polar fractions containing free sterols. Coprostanol was partially separated from cholesterol. TLC of the individual sterol ester fractions in cyclohexane–benzene 2:1 (17) showed that they were mainly coprostanol esters, although toward the end of the elution peak, small amounts of cholesterol esters appeared. The ester fractions were combined, mixed with nonradioactive cholestanol, and chromatographed on an alumina column. Sterol esters, from which all traces of radioactive free sterols had been removed, were refluxed in 10% KOH in 70% ethanol for 7 hr. The nonsaponifiable fraction, in which only coprostanol and cholesterol were detectable in the ratio 9:1 by GLC, was mixed with 53 mg of nonradioactive cholestanol and the sterols were treated with MCPBA and chromatographed. By this procedure, coprostanol, cholestanol, and 5,6-oxidocholesterol were separated. Cholestanol was crystallized from acetone–methanol.

In the initial chromatographic separation of fecal lipids, fractions immediately following the main portion

of free coprostanol were mixtures. These were combined and analyzed by GLC on a well-conditioned column of 3% QF-1 (methyl fluoroalkyl silicone) on 100–120 mesh Gas-Chrom P at 250°C; coprostanol, cholesterol, and cholestanol were present in a ratio of 3.4:3:1. Cholesterol was removed by means of performic acid and the saturated sterols were diluted with an equal weight of nonradioactive cholestanol and chromatographed on alumina to separate coprostanol and cholestanol.

Radioactive Measurements. All samples were simultaneously assayed for ^{3}H and ^{14}C in a Packard Tri-Carb liquid scintillation counter according to methods which have been described in detail by Bradlow, Fukushima, Zumoff, Hellman, and Gallagher (23).

RESULTS

Study with Cholesterol-3-^{3}H,4-^{14}C

Isolated Sterols. Table 3 presents a summary of the ^{3}H:^{14}C ratios of the sterols in three plasma pools and two red cell pools. Total sterols and cholesterol, ultimately purified as cholestanetriol-3,6-diacetate, show isotope ratios almost identical with that of the administered dose (3.67), as would be expected from mixing with circulating sterol, which is over 99% cholesterol. On the other hand, cholestanol from each pool has ^{3}H:^{14}C ratios of 0.45–0.62, less than 20% of the ratio in cholesterol. The radiochemical data for the stepwise purification of cholesterol and cholestanol from the pooled plasma collected between 0.5 and 2.0 days after the start of the study are shown in Table 4. Data from the remaining four pools (Table 3) were very similar and differed only in specific activities, since each pool was obtained from blood withdrawn at different times after administration of the radioactive tracer. These results show that cholestanol was derived in vivo from cholesterol by a pathway which involved a ketonic intermediate, since the ^{3}H:^{14}C ratio in plasma cholestanol was considerably less than that in cholesterol and, more important, *about 50% lower than the ^{3}H:^{14}C ratio in the saturated sterol that contaminated the administered dose* (*Table 2*). Loss of tritium in cholestanol by interchange with cholestanone, the only other reasonable explanation for a smaller ^{3}H:^{14}C ratio, did not occur (see below).

TABLE 3 ^{3}H:^{14}C Ratio of Purified Blood Sterols after Injection of Cholesterol-3-^{3}H,4-^{14}C

Pooled Samples: Days after Tracer	^{3}H:^{14}C Ratios					
	Plasma			Cells		
	Total Sterols	Cholesterol	Cholestanol	Total Sterols	Cholesterol	Cholestanol
0.5–2	3.62	3.69	0.62	3.62	3.70	0.62
3–7	3.64	3.73	0.53	3.48*	3.62	0.47
9–11	3.64	3.71	0.45			

* Cell sterols from 3–11 days were pooled.

TABLE 4 Stepwise Purification of Sterols in Plasma 0.5–2 Days after Cholesterol-3-^{3}H,4-^{14}C Injection

Step	Procedure	SA		^{3}H:^{14}C
		^{3}H	^{14}C	
		cpm/mg		
Cholesterol				
1	Total sterol	9,330	2,580	3.62
2	Cholestane-3β,5α,6β-triol	8,450	1,900	4.45
3	Triol-3,6-diacetate	10,300	2,780	3.70
4	Diacetate from step 3: twice crystallized	10,350	2,800	3.69
Cholestanol				
1	Saturated fraction (5.9% cholestanol)	—	—	—
2	Carrier cholestanol (MCPBA and chromatography)	2,960*	2,830*	1.05
3	Cholestanol from step 2 crystallized from methanol	1,430	2,240	0.64
4	Cholestanol from step 3 acetylated and chromatographed	1,250	2,020	0.62
5	Cholestanol acetate from step 4 crystallized from acetone–methanol	1,350	2,200	0.62

* These and following values calculated from the dilution factor.

Although it would be interesting to supplement these observations by direct demonstration of the presence of more ^{14}C in blood and tissue cholestanol than was originally present as an impurity in the administered tracer, this was impossible under the conditions of the experiment. More than 99.6% of the ^{14}C in the injected tracer was in cholesterol, but the small amount of ^{14}C in the saturated contaminant which could not be practically removed was more than sufficient to account for the ^{14}C in cholestanol of blood and excreted sterols. Cholestanol in plasma, red cells, and feces contained (Table 5) 139,000 cpm of ^{14}C, corresponding to 40% of the ^{14}C in the saturated contaminant of the dose, but certainly not originating solely from it, during the first 2 days.[1] It is probable that cholestanol-^{14}C in other sterol pools, for example liver, lung, intestine, kidney, adrenal, vascular system, and muscle, would more than exceed the remaining 60% of the radioactivity; however, studies in man preclude direct sampling of these sites.

[1] In plasma, the SA of cholestanol-^{14}C was 2200 cpm/mg (2nd day). Plasma sterol was 2.0 mg/ml, from which a pool of 6000 mg can be calculated, if the plasma volume is assumed to be 3 liters. Since plasma sterol contained 0.6% cholestanol, or 36 mg, the total ^{14}C radioactivity in plasma cholestanol could be obtained (Table 5). A similar calculation was made for radioactivity in red cells.

TABLE 5 CHOLESTANOL-^{14}C IN BLOOD AND FECES
0.5–2 Days after Cholesterol-3-^{3}H,4-^{14}C Injection

	Cholestanol-^{14}C*	% of Saturated Contaminant in Injected Tracer†
	cpm	
Plasma	79,000	23.0
Red cells	33,000	9.5
Feces	27,000	7.8

* Product of the specific activity and milligrams of cholestanol. The latter value was calculated from the percentage of cholestanol in total sterols of each pooled sample.

† 3.48×10^5 cpm present as saturated impurity in the dose.

Table 6 presents the specific activities and isotope ratios of fecal sterols. Since the diet contained some plant sterols, the specific activity values of cholesterol and coprostanol are lower than they would be for C_{27} sterols alone. The methods employed in the purification of these substances would not separate C_{27} sterols from their phytosterol analogues; however, this in no way affects the validity of the measurements. The cholestanol that was chemically separated from cholesterol and carried through the several purification steps had an isotope ratio of 1.14. The 5β-epimer, coprostanol, the principal fecal sterol, had a ^{3}H:^{14}C ratio of 1.66.

Study with Cholestanol 3-^{3}H,4-^{14}C

Radiochemical Purity of Tracer. Although only 92% of the tritium was associated with cholestanol, the remaining 8% was present in epimeric cholestan-3α-ol and in no way affected the validity of the study since the isotope in both epimers was bound only to C-3.

Isolated Sterols: Blood. The ^{3}H:^{14}C ratio in the injected doubly labeled cholestanol was 4.08. After extensive purification of the cholestanol from plasma and red cells obtained at intervals of from 12 hr to 7 days after the start of the study, constant specific activity was achieved and there was virtually no change in the isotope ratio. These data are summarized in Table 7. Higher ratios (up to 4.30) obtained in some of the purified cholestanol samples might result from an enrichment of cholestanol by cholestan-3α-ol-3-^{3}H during chromatographic purification of the MCPBA oxidation product. Oxidation of the combined cholestanol-3-^{3}H,4-^{14}C from plasma and red cells removed over 99% of the tritium, which showed that it was present at C-3. This and the constant isotope ratio in circulating sterol, even 1 wk after the injection of the tracer, show that no significant conversion of cholestanol and cholestanone occurred in this patient.

From the maximum concentration of radioactivity in plasma and red cells (cpm/ml) and the total circulating volume of each (approximately 3 and 2 liters respectively), the total counts per minute in the circulation at peak radioactivity can be calculated. Maximum blood radioactivity occurred at 24–48 hr and amounted to 15% of the administered dose. This value is lower than the maximum counts per minute in the circulation after intravenous cholesterol (28% of the dose). Whether this represents a significant difference cannot be concluded from the two studies.

TABLE 6 PURIFICATION OF STEROLS IN FECES
2–3 Days after Cholesterol-3-^{3}H,4-^{14}C Injection

Step	Procedure	SA ^{3}H	SA ^{14}C	^{3}H:^{14}C
		cpm/mg		
Cholesterol				
1	Cholesterol fractions (109 mg) from chromatography of fecal nonsaponifiable fraction	4450	1280	3.46
2	Cholestane-3β,5α,6β-triol from oxidation of step 1	2940	720	4.08
3	Cholestane-3β,5α,6β-triol-3,6-diacetate from acetylation of triol	5150	1260	4.09
4	Cholestanetriol-3,6-diacetate, twice recrystallized from acetone–methanol	5280	1300	4.06
Cholestanol				
1	Saturated sterol fraction (6 mg) separated by solvent partition after performic acid oxidation of cholesterol (above); 33.7% cholestanol by GLC	—	—	—
2	Carrier cholestanol added. MCPBA reaction and chromatography: cholestanol	6910	4860	1.42
3	Cholestanol from step 2 crystallized from methanol–acetone	6150	4590	1.34
4	Cholestanol: second recrystallization	5080	4150	1.23
5	Cholestanol: third recrystallization	4780	4190	1.14
Coprostanol				
1	Coprostanol fractions (334 mg) from chromatography of fecal nonsaponifiable fraction	1230	630	1.95
2	Acetylation and chromatography: coprostanoyl acetate	1220	710	1.72
3	Coprostanol acetate, recrystallized from acetone	1160	700	1.66

Feces. Cholestanol isolated by reverse isotope dilution from the free sterol fraction, after purification to constant specific activity, had a ^{3}H:^{14}C ratio of 3.50 (Table 8), about 12% lower than the ratio in the administered tracer. Coprostanol and cholesterol were almost devoid of ^{14}C. Free coprostanol obtained from the initial chromatography of the fecal lipids, and not further purified, had a specific activity of 49 cpm/mg of ^{14}C. After removal of cholesterol from the cholestanol-containing fraction by

TABLE 7 RADIOCHEMICAL PURITY OF CHOLESTANOL-^{3}H,^{14}C AND ISOTOPE RATIO IN BLOOD AFTER ADMINISTRATION OF CHOLESTANOL-3-^{3}H,4-^{14}C

Step	Procedure	SA Plasma ^{3}H	SA Plasma ^{14}C	SA RBC ^{3}H	SA RBC ^{14}C	^{3}H:^{14}C Ratios 12 hr Plasma	12 hr RBC	12–48 hr Plasma	12–48 hr RBC	60–88 hr Plasma	60–88 hr RBC	144–168 hr Plasma	144–168 hr RBC
		cpm/mg at 12 hr											
1	Nonsaponifiable fraction	1610	410	2740	670	3.93	4.10	4.01	3.95	3.98	3.86	3.97	3.90
2	Saturated sterol fraction from performic acid oxidation: cholestanol carrier added	780	190	840	210	4.10	4.00	3.96	3.91	4.02	3.92	4.00	4.02
3	Saturated sterols from step 2: MCPBA oxidation and chromatography: cholestanol	820	190	900	210	4.30	4.28	4.17	4.08	4.08	4.10	4.09	4.08
4	Cholestanol crystallized from acetone–methanol	820	190	880	210	4.30	4.18	4.18	4.11	4.05	4.10	4.18	4.02
5	Cholestanone from oxidation of cholestanol* in step 4							^{3}H:^{14}C = 0.016					

RBC, red blood cells.
* Cholestanol from plasma and red cells were combined for the oxidation step.

TABLE 8 RADIOCHEMICAL PURITY OF CHOLESTANOL-^{3}H,^{14}C IN FREE AND ESTERIFIED STEROLS OF FECAL LIPID*

Step	Procedure	SA ^{3}H	SA ^{14}C	^{3}H:^{14}C
		cpm/mg		
Sterol esters: 8.1 × 10⁴ cpm of ^{14}C				
1	Sterol esters after cholestanol washout	—	—	4.58
2	Product from step 1 saponified, carrier cholestanol added, MCPBA oxidation and chromatography: cholestanol	3,640†	920†	3.94
3	Cholestanol from step 2 crystallized	3,740	940	3.98
Free sterols: 3.26 × 10⁶ cpm of ^{14}C				
1	From chromatography of fecal lipids	—	—	3.50
2	Sterols from step 1 oxidized with performic acid: saturated sterols	—	—	3.42
3	Sterols from step 2 diluted with cholestanol: mixture chromatographed: cholestanol fraction	10,800	3,100	3.48
4	Cholestanol from step 3 crystallized	10,900	3,130	3.50

* This fraction contained 3.36 × 10⁶ cpm of ^{14}C.
† The values in this table represent specific activities of the diluted material.

the performic acid procedure, over 99% of the radioactivity remained in the saturated sterols and 90% of this was accounted for in purified cholestanol.

DISCUSSION

The fact that the ^{3}H:^{14}C ratio in cholestanol (0.55 ± 0.08) isolated from plasma and red cells was strikingly different from the ^{3}H:^{14}C ratio in the saturated impurity (1.2) which accompanied the injected cholesterol-3-^{3}H,4-^{14}C provides conclusive evidence for the formation of cholestanol from cholesterol. This difference was well outside of the experimental counting error and cannot be related to the process of purification of cholestanol, which was essentially the same for the tracer and for plasma or red cell sterol. Furthermore, the decrease in ratio could not have been due to loss of ^{3}H from preformed cholestanol by interchange with cholestanone in vivo, since the cholestanol-3-^{3}H,4-^{14}C study demonstrated a constant isotope ratio (4.1) throughout. This is in contrast to the 3-hydroxy ⇄ 3-ketone interconversion in vivo in the C_{19} series (H. L. Bradlow, personal communication). Thus, new cholestanol was formed in vivo from administered doubly labeled cholesterol by a process where ^{14}C was retained but ^{3}H was lost; i.e.,

cholesterol → cholestenone → cholestanol.

Although the transformation of cholesterol to cholestenone in vivo has not been unequivocally demonstrated, cholestenone is rapidly converted to cholestanol in animals (2, 6, 7) and man (11), and its intermediate role is very probable. That some cholesterol was also transformed to cholestanol by reduction of the double bond, without a 3-ketonic intermediate, cannot be excluded. However, such a reaction would have resulted in an *increase* in the ^{3}H:^{14}C ratio of cholestanol, since the ^{3}H:^{14}C ratio of injected cholesterol was 3 times that of the saturated sterol present in the tracer dose. Thus saturation of the double bond with no participation of a ketonic intermediate, if it occurred at all, must have been a minor pathway.

These conclusions would be confirmed if it could be shown that the amount of cholestanol-^{14}C in the body of the subject who received cholesterol-^{3}H,^{14}C were greater than the amount injected with the cholesterol. Werbin et al. (4) were able to do this in guinea pigs after injection of cholesterol-4β-^{3}H,4-^{14}C (containing 0.1% saturated

^{14}C-sterol) by isolation of sterol from the adrenals, liver, and intestine. Clearcut demonstration of conversion in man would require either (*a*) a knowledge of the exchangeable cholestanol pool, so that the amount of cholestanol in the circulation might be extrapolated to a total body content, or (*b*) perhaps measurement of ^{14}C in fecal cholestanol and 5α-bile acids for an extended period of time until accumulated radioactivity exceeded that which was introduced in the dose. Exchangeable cholestanol cannot be calculated since there is no information concerning its rate of interchange in sterols of blood and tissues and few data are available regarding the amount of cholestanol in these various sterol pools; nevertheless, the biologically similar behavior of cholesterol and cholestanol (24) makes it a reasonable first approximation to assume that their distributions are similar. After 1–2 days, the exchangeable cholesterol pool is approximately 5 times the size of the blood cholesterol pool.[2,3] A comparable value was found by Bieberdorf and Wilson in rabbits, where 30% of the total body cholesterol had equilibrated with serum cholesterol 1 day after the injection of cholesterol-4-^{14}C (27). Therefore, if 45 g is considered to be the size of the miscible sterol pool in man (5–6 times the amount used in calculating the data of Table 5) it can be calculated[4] that the amount of cholestanol originally injected was increased by a factor of 1.7. This is close to the "increase factor" of 1.9 which is obtained by dividing the ^{3}H:^{14}C ratio of the cholestanol that contaminated the injected cholesterol, 1.2, by that of blood cholestanol, 0.62.

With regard to measurements of fecal radioactivity for long periods of time: low radioactivity might make the estimation difficult, a knowledge of the contribution of fecal bacteria to cholestanol formation would be required, and there are difficulties in the quantification of 5α-cholanic acid derivatives in stool.

The isotope ratios of the fecal sterols merit some comment. In the study that employed doubly labeled cholesterol, plasma and biliary cholesterol were the major sources of labeled fecal sterols. The ^{3}H:^{14}C ratio of 1.66 in coprostanol (Table 6) must be the resultant of three processes that can be carried out by intestinal microorganisms: (*a*) saturation of the cholesterol double bond (retention of ^{3}H) (22); (*b*) production of coprostanol via a ketonic intermediate (loss of ^{3}H) (28, 29); (*c*) interconversion of coprostanol and coprostanone (loss of ^{3}H) (24, 29). It is impossible to assess the relative contribution of each process. The isotope ratio in fecal cholestanol (1.14), although nearly identical with that of the contaminant in the injected material, probably does not result from preferential secretion of this saturated sterol into the lumen. It is likely that transformations in the intestine similar to those occurring in the formation of the 5β-epimer were taking place; that fecal sterols contain a higher percentage of cholestanol than any other sterol fractions examined (13) supports this contention.

In the cholestanol-3-^{3}H,4-^{14}C study (Table 8), the lower isotope ratio (3.50) of fecal free cholestanol as compared with that in plasma sterol must be a consequence of the loss of ^{3}H due to interconversion with cholestanone carried out by fecal microorganisms. Although cholesterol and coprostanol were not rigorously purified in this study, there was comparatively little radioactivity in these sterols and it is probable that this was due to trace contamination by cholestanol-^{14}C. It should be noted that in feces, over 90% of the cholestanol was free. This is in contrast to the larger amount of coprostanol esters found (16) and supports the observation that in stool, only coprostanol is readily esterified.[4]

It is of considerable interest that conversion of cholesterol in vivo to its ring A reduced products, cholestanol (5α-H) and bile acids (5β-H), requires a 3-ketonic intermediate (30–32). Reduction of cholesterol without a 3-ketonic intermediate is a bacterial reaction apparently not carried out by mammalian tissue. The details of these pathways and the biological significance of the intermediate products remain subjects for future investigation.

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[2] Unpublished experimental results from this laboratory.

[3] The exchangeable cholesterol pool undergoes rapid expansion during the first few days and then increases gradually (25). Chobanian, Burrows, and Hollander (26) found that 59–100 days after administration of tracer cholesterol-^{14}C, exchangeable cholesterol amounted to 125–349 g.

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