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Metabolism of coprostanol-C^{14} and cholestanol-4-C^{14} in man*

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SUMMARY

Coprostanol-C^{14}, biosynthetically prepared, was administered orally to two patients, and cholestanol-4-C^{14} was administered to one of them 10 months later. At the time when radioactivity in the circulation was at a maximum, 3.6 and 3.5% of the administered labeled coprostanol was present in the plasma; the corresponding value after administration of cholestanol-C^{14} was 4.7%. The dynamic behavior of absorbed radioactive cholestanol was identical with that of orally ingested cholesterol-4-C^{14}, including the esterification process; on the other hand, virtually no coprostanol ester was present in the circulation. It is suggested that the conformation of the A/B rings or configuration at C-3 are related to the esterification mechanism of sterols. After 5 days, over 50% of both compounds had been excreted in the feces. The conversion of coprostanol-C^{14} to coprostanone-C^{14} has been demonstrated.

Since many sterols can be absorbed through the gastrointestinal tract (1–5), it seemed desirable to investigate the absorption of coprostanol[1] in man and to examine some aspects of its metabolism. It was found that coprostanol administered orally appeared in the circulation *only* as free sterol in contrast to the behavior of cholestanol, which underwent considerable esterification. Also coprostanone, isolated from feces, was shown to be derived in part from ingested coprostanol.

METHODS AND MATERIALS

Subjects. Two patients in good clinical condition and with plasma cholesterol levels in the normal range were studied. Coprostanol-C^{14} (72 mg) was dissolved in approximately 2.0 ml of sesame oil and administered by mouth to each subject. Cholestanol-4-C^{14} (1 mg) was administered in an identical way to one of the patients (patient 1M) 10 months after the coprostanol study. Details are reported in Table 1. Free and ester cholesterol were isolated from blood by digitonin precipitation (6). Stool collections were obtained for at least 1 week, and sterols were isolated by a procedure previously described (7). Urine was collected for 3 days and assayed for total radioactivity.

Radiochemical Purity of Cholestanol-4-C^{14}. This material[2] showed only one radioactive peak in the strip scanner graph supplied by the manufacturer. A tracer amount (5.38×10^5 disintegrations per minute [dpm]) was mixed with approximately 100 mg each of nonradioactive cholestanol and cholesterol. The mixture was treated with hydrogen peroxide–formic acid (8), and the reaction products were partitioned between 90% methanol and petroleum ether (9). Cholestane-3β,5α,6β-triol contained 9,000 dpm while the cholestanol contained 4.90×10^5 dpm. A second partition reduced the total radioactivity in the triol fraction to 5,000 dpm. Cholestane-3β,5α,6β-triol was treated with an acetic anhydride–pyridine mixture overnight at room temperature, and cholestane-3β,5α,6β-triol-3,6-diacetate was recrystallized from acetone–methanol; there was no detectable radioactivity in this fraction. Cholestanol (6,300 dpm/mg) was acetylated, and the mixture was chromatographed on alumina; cholestanol acetate was eluted with petroleum ether (6,700 dpm/mg, based on free sterol). Thus, cholestanol-4-C^{14} was free of labeled cholesterol within counting limitations.

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[1] Coprostanol = coprostan-3β-ol; cholestanol = cholestan-3β-ol.

[2] New England Nuclear Corporation.

TABLE 1. ABSORPTION AND EXCRETION OF RADIOACTIVE COPROSTANOL AND CHOLESTANOL

Subject	Age	Sex	Diagnosis	Compound Fed	Amount of Radioactivity Fed	Percentage of Administered Radioactivity Excreted*		Plasma Cholesterol Level		Maximum Concentration of Radioactivity in Plasma	Percentage of Administered Radioactivity in Plasma†
						Feces	Urine	Free	Ester		
					dpm	%	%	*mg/100 ml*		*dpm/ml*	%
1M	59	M	Multiple Sclerosis	Coprostanol-C^{14}	4.8×10^7	57	0.75	65	146	578	3.6
	60			Cholestanol-4-C^{14}	7.7×10^7	71	0	96	203	1202	4.7
2C	58	M	Alcoholic	Coprostanol-C^{14}	5.2×10^7	55	0	48	114	610	3.5

* Through fifth day after administration of radioactivity.
† Calculated from maximum dpm/ml in plasma and plasma volume.

Radiochemical Purity of Coprostanol-C^{14}. This substance was prepared by the reduction of cholesterol in beef brain homogenate by mixed fecal microorganisms in a procedure similar to that of Snog-Kjaer, Prange, and Dam (10). The substrate was labeled by the addition of 1 mg of cholesterol-4-C^{14} (1.70×10^8 dpm). Isolation of coprostanol-C^{14} by procedures that have been described (7) yielded 144 mg of coprostanol (660,000 dpm/mg). To 100 mg of nonradioactive coprostanol was added 0.109 mg of biosynthetically prepared coprostanol-C^{14}. This sterol (698 dpm/mg) was dissolved in a small amount of petroleum ether along with 200 mg of cholesterol. The mixture was chromatographed on alumina and separated by elution with mixtures of petroleum ether and benzene. Two fractions were obtained: coprostanol (700 dpm/mg) and cholesterol, which contained no measurable radioactivity. The coprostanol was oxidized with 2% chromic acid in 90% acetic acid for 2 hr. After extraction and chromatography of the product on alumina, 74 mg of coprostan-3-one was obtained (750 dpm/mg).

Contamination of Coprostanol-C^{14} with Coprostan-3-one-C^{14}. To 200 mg of nonradioactive coprostanone was added a trace of labeled coprostanol (mixture = 1,200 dpm/mg) and the mixture was chromatographed on alumina. Material from the coprostanone fraction (105 mg) was counted by the scintillation technique (3,400 dpm/g or approximately 4 dpm/mg). Therefore, maximum contamination of coprostanol with the ketone was 0.3%.

Fractionation of Radioactivity in Plasma Sterols After Administration of Coprostanol-C^{14}. Cholesterol digitonide, obtained from pooled plasma free cholesterol of patient 1M, was dissociated, and 71 mg of cholesterol was recovered. To this was added an equal amount of carrier coprostanol. The mixture was chromatographed on alumina, and 67 mg of coprostanol (140 dpm/mg) and 59 mg of cholesterol, devoid of radioactivity, were obtained. The coprostanol showed no change in specific activity after acetylation, perbenzoic acid treatment, and chromatography on alumina (9).

In patient 2C, combined supernatant fractions remaining after digitonin precipitation of cholesterol from the acetone-alcohol extracts of plasma (6) were concentrated and shaken with petroleum ether. The petroleum ether was evaporated, and the residue was chromatographed on alumina by a procedure that separates sterols from sterol esters (6). The ester fraction contained 21 mg (800 dpm); 5.5 mg (7,800 dpm) of "free sterol" was obtained.

Radiochemical Purity of Coprostanone-C^{14} Isolated from Feces. Coprostanone, isolated by the chromatography of the nonsaponifiable fraction (7) after oral administration of coprostanol-C^{14}, was diluted with nonradioactive material. Each sample was subjected to chromatography on alumina, and the eluted material was reacted with lithium aluminum hydride in ether. After extraction from the reaction mixture, the reduction product (coprostan-3α-ol) was chromatographed on alumina, the eluates containing this substance were combined, and the product was crystallized from acetone (m.p. 120°). Specific radioactivity was measured at each step, and the results are summarized in Table 2.

Radioactivity Measurements. Plasma, urine, and feces from both subjects and the sterol digitonides in the coprostanol-C^{14} study in patient 1M were directly plated and dried on stainless steel planchets. Sterols carried through the various purification steps were plated from dilute ethanolic solution and counted in the infinitely thin region.[3] Results are expressed as disintegrations per minute at infinite thickness by the use

[3] For which we thank Dr. H. Leon Bradlow.

TABLE 2. RADIOCHEMICAL PURITY OF COPROSTANONE ISOLATED FROM FECES AFTER ORAL ADMINISTRATION OF COPROSTANOL-C^{14}

Sample	Specific Radioactivity: Patient 1M	Patient 2C
	dpm/mg	*dpm/mg*
72 mg of isolated coprostanone + 32 mg carrier	1,892	—
25 mg of isolated coprostanone + 87 mg carrier	—	836
Coprostanone after chromatography on alumina	1,550	710
Coprostan-3α-ol from reduction	1,580	434
Coprostan-3α-ol after recrystallization	1,520	428*

* The total radioactivity in the nonsaponifiable fraction of feces in patient 2C was 1.05×10^7 dpm; the corresponding value for patient 1M was 8.52×10^6 dpm. In 2C, if it is assumed that the radioactivity is present only in coprostanol, which could be contaminated by 0.3% coprostanone (see text), then $(1.05 \times 10^7 \text{ dpm}) \times (0.003) = 3.15 \times 10^4$ dpm would be the maximum amount of radioactivity that could be found in coprostanone. From radiochemically pure material isolated at the final step of purification, 112 mg × 428 dpm/mg = 4.80×10^4 dpm was actually found.

$$\frac{\text{Coprostanone radioactivity (isolated)}}{\text{Coprostanone radioactivity (possible contaminant)}} = \frac{4.80 \times 10^4}{3.15 \times 10^4} = 1.5$$

of experimentally determined conversion factors. All other radioactivity measurements were made with a Packard TriCarb liquid scintillation spectrometer.

RESULTS

Figure 1 shows the dynamic behavior of labeled plasma sterols in subject 1M after the ingestion of cholestanol-4-C^{14}. For comparison, plasma sterol radioactivity curves after oral ingestion of Δ^4-cholestene 3-one-4-C^{14} to the same patient about two years earlier are also included (9). The specific activity curves of plasma sterol are similar regardless of the labeled steroid administered.

Figure 2 shows the specific activity of plasma sterols as a function of time in the two subjects to whom coprostanol-C^{14} had been administered. The points are connected by straight lines because no information is available on the kinetic behavior of coprostanol in the circulation, and, in any case, the data in patient 2C would not permit construction of a smooth curve. Another complicating feature is that only trace amounts of radioactive coprostanol were present in the plasma sterol, and the methods previously validated for the measurement of cholesterol digitonide (6) may not be completely applicable for coprostanol. However, extraction and chromatography of the combined supernatant solutions after precipitation of plasma free sterols showed that over 90% of the radioactivity in the unprecipitated material was associated with free sterol. At the peak of the plasma radioactivity, patient 1M

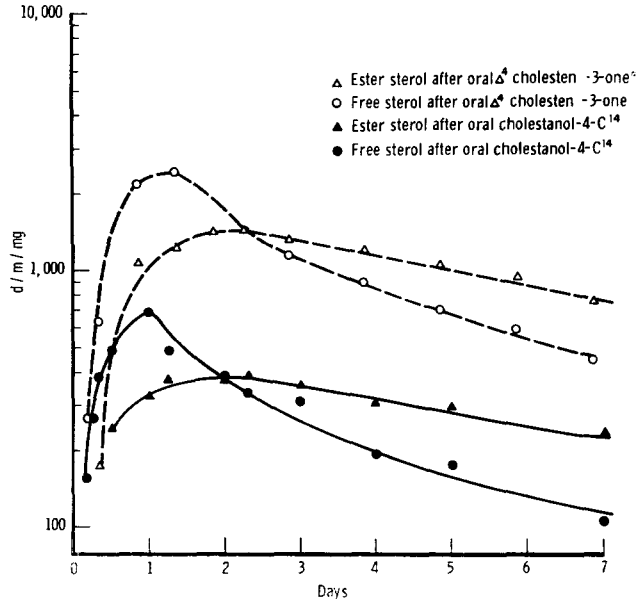

FIG. 1. Specific radioactivity of plasma free and esterified sterols as a function of time after oral administration of cholestanol-4-C^{14} (solid lines) and of Δ^4-cholesten-3-one-4-C^{14} (dotted lines) to subject 1M. *From a previous study (9).

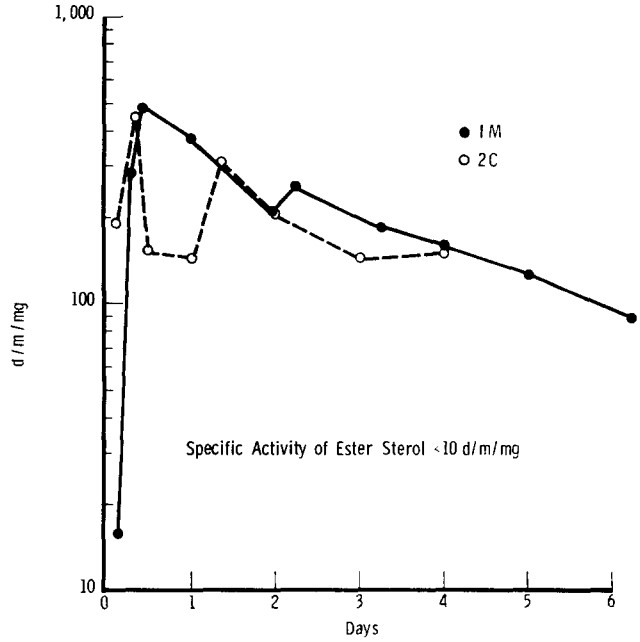

FIG. 2. Specific radioactivity of plasma free sterols after oral ingestion of biosynthetically labeled coprostanol-C^{14}. The specific radioactivity of ester sterols was at all times less than 10 dpm/mg.

had 3.6% of the administered coprostanol-C^{14} in the plasma, and patient 2C had 3.5% (Table 1). In patient 1M, 4.7% of the ingested cholestanol-4-C^{14} was present in the plasma at the peak of plasma radioactivity. The excretion of radioactivity in the urine amounted to less than 0.75% in all three experiments. After administration of coprostanol-C^{14}, the excretion of radioactivity in the feces amounted to 57 and 55% of the dose, respectively, 5 days after the start of the experiments. Patient 1M had excreted 71% of the ingested cholestanol-4-C^{14} in the feces after 5 days.

After the administration of labeled coprostanol, coprostanone isolated from the feces of both patients contained significant amounts of radioactivity. The only source of label was coprostanol-C^{14}, which, as discussed above, could be contaminated by a maximum of 0.3% of coprostanone. It can be calculated that the coprostanone isolated from subject 2C contained 1.5 times the maximum amount of C^{14} it would have contained had the radioactivity all represented labeled coprostanone contaminating the administered radioactive coprostanol. For patient 1M, the isolated and purified coprostanone had over six times the radioactivity that would have been expected from such an impurity. It should be emphasized that these ratios are minimum values, since the 0.3% contamination of coprostanol-C^{14} by coprostanone-C^{14} represents a *maximum figure*. It seems certain, therefore, that under the condition of these studies some transformation to coprostanone occurred.

DISCUSSION

Earlier work showed that absorbed Δ^4-cholestenone-4-C^{14} was converted to cholestanol-C^{14}. The similarity of the curves in Fig. 1 confirms the observation that the reduction of cholestenone to cholestanol in vivo must be rapid, and, regardless of the precursor fed, the same material must have become available for esterification. The relative role of the intestinal mucosa and the liver in the esterification of the 3β-hydroxy group is not known. It is certain that large amounts of cholesterol are esterified during absorption (11), presumably in the mucosa (12). The delayed appearance of the labeled ester in the plasma, however, makes it probable that the liver must also be involved either in combination of the ester with lipoprotein, or in hydrolysis and re-esterification reactions, or in both.

Although earlier investigators had considered coprostanol to be unabsorbed in the animal body (13, 14), it was not surprising to find that the present procedures unequivocally revealed its presence after ingestion of coprostanol-C^{14} since the radioactive isotope method is more sensitive than balance techniques and since coprostanol is ordinarily not present in the portion of the intestine in which active absorption occurs.

The appearance of coprostanol in the plasma only in the free form is further evidence that certain spatial requirements in the A and B rings must be met for in vivo esterification of sterols. Thus cholesterol, desmosterol, and cholestanol, all of which exist in the plasma largely as esters (9, 15, 16), have similar conformations in the A and B rings,[4] and the 3β-hydroxyl of each is equatorial. On the other hand, coprostanol (A/B *cis;* 3β-hydroxyl axial) and Δ^5-cholesten-3α-ol (3α-hydroxy axial) (2) are absorbed without esterification.

Isolation of coprostanone-C^{14} after the administration of coprostanol-C^{14} provides additional evidence for a coprostanol-coprostanone oxidation system in the intestine. The transformation is reversible since Anchel and Schoenheimer demonstrated in their classical studies that coprostanone can be converted to coprostanol in vivo (17).

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REFERENCES

1. Harold, F. M., D. D. Chapman, and I. L. Chaikoff. *J. Biol. Chem.* **224:** 609, 1957.
2. Swell, L., E. Stutzman, M. D. Law, and C. R. Treadwell. *Arch. Biochem. Biophys.* **97:** 411, 1962.
3. Dunham, L. W., R. E. Fortner, R. D. Moore, H. W. Culp, and C. N. Rice. *Arch. Biochem. Biophys.* **82:** 50, 1959.
4. Swell, L., E. C. Trout, Jr., H. Field, Jr., and C. R. Treadwell. *Proc. Soc. Exptl. Biol. Med.* **100:** 140, 1959.
5. Setty, C. S. N., and A. C. Ivy. *Am. J. Physiol.* **199:** 1008, 1960.
6. Rosenfeld, R. S., L. Hellman, W. J. Considine, and T. F. Gallagher. *J. Biol. Chem.* **208:** 73, 1954.
7. Rosenfeld, R. S., D. K. Fukushima, L. Hellman, and T. F. Gallagher. *J. Biol. Chem.* **211:** 301, 1954.
8. Fieser, L. F., and S. Rajagopalan. *J. Am. Chem. Soc.* **71:** 3938, 1949.
9. Rosenfeld, R. S., B. Zumoff, and L. Hellman. *Arch. Biochem. Biophys.* **96:** 84, 1962.
10. Snog-Kjaer, A., I. Prange, and H. Dam. *J. Gen. Microbiol.* **14:** 256, 1956.
11. Hellman, L., E. L. Frazell, and R. S. Rosenfeld. *J. Clin. Invest.* **39:** 1288, 1960.
12. Daskalakis, E. G., and I. L. Chaikoff. *Arch. Biochem. Biophys.* **58:** 373, 1955.
13. Bürger, M., and W. Winterseel. *Z. Physiol. Chem.* **202:** 237, 1931.
14. Breusch, F. L. *J. Biol. Chem.* **124:** 151, 1938.
15. Hellman, L., R. S. Rosenfeld, and T. F. Gallagher. *J. Clin. Invest.* **33:** 142, 1954.
16. Goodman, DeW. S., J. Avigan, and H. Wilson. *J. Clin. Invest.* **41:** 962, 1962.
17. Anchel, M., and R. Schoenheimer. *J. Biol. Chem.* **125:** 23, 1938.

[4] The A/B trans juncture and the Δ^5 structure in the steroids have a similar conformation.