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METABOLISM OF CHOLESTANE-3β,5α,6β-TRIOL—I.
THE FATE OF CHOLESTANETRIOL IN THE RAT

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Abstract—The metabolic fate of cholestanetriol in the rat was studied. Twenty-four hr after the oral administration of 4-^{14}C-cholestanetriol, 84 per cent of the radioactivity recovered was present in the feces plus intestinal contents; 16 per cent of the recovered ^{14}C remained in the body. Ninety per cent of the radioactivity present in the body was equally distributed among the intestinal wall, carcass and blood. Of the remaining organs, only the liver contained a significant amount of radioactivity.

The ^{14}C present in the feces was equally distributed between neutral steroidal metabolites and bile acids. Two major neutral metabolites were found. The radioactivity present in the bile acids was separated into seven areas by thin-layer chromatography. Two major radioactive areas were found corresponding to: (1) a trihydroxy bile acid, and (2) a bile acid with a mobility intermediate between 7,12-diketolithocholic and hyodeoxycholic acids.

CHOLESTANE-3β,5α,6β-triol was recently found to be effective in preventing hypercholesterolemia in cholesterol-fed rabbits and chickens and in Triton-treated rats.[1,2] Imai *et al.*[3] reported that administration of cholestanetriol to rats resulted in increased fecal excretion of cholesterol with a concomitant increase in liver cholesterol biosynthesis. This paper reports the fate of cholestanetriol in the rat.

MATERIALS AND METHODS

4-^{14}C-cholestanetriol. Labeled cholestanetriol (sp. act., 0·23 μc/mg) was prepared by Dr. Milton Heller (Organic Chemical Research Section, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y.) from 4-^{14}C-cholesterol by the method of Fieser and Rajagopalan.[4] Ninety-eight per cent of the radioactivity in the preparation behaved as cholestanetriol on the basis of TLC in toluene:acetic acid:H_2O (5:5:1, v/v; top layer).

4-^{14}C-cholestanetriol emulsion. 4-^{14}C-cholestanetriol (125 mg) was placed in a Potter–Elvehjem homogenizer and dissolved in a minimal amount of ether. Tween 80* (0·45 ml) was added, and the ether was removed by gently shaking the homogenizer under hot running tap water. To the residue, 4·55 ml water was added, and the mixture was homogenized for 3 min. The resulting emulsion was stable for at least 1 day and contained 25 mg of 4-^{14}C-cholestanetriol/ml.

Animals. Three male CFE† rats (130 g) were placed on a diet of Purina laboratory

* Polyoxyethylene sorbitan monooleate, Atlas Powder Co., Wilmington, Del.

† Carworth Farms, New City, N.Y.

chow and water *ad libitum* for 5 days. During this period the animals gained normally and reached a weight of about 170 g. On the morning of the sixth day, 1·0 ml of the 4-^{14}C-cholestanetriol emulsion, containing 6·75 μc radioactivity (25 mg), was administered to each animal via stomach tube. Animals were placed in separate metabolism cages and allowed free access to food and water. Feces and urine were collected for 24 hr. Twenty-four hr after administering the 4-^{14}C-cholestanetriol, the animals were sacrificed by cranial fracture and decapitated. The thoracic cavity was exposed and an incision was made in the inferior vena cava. The animals were perfused with ice-cold 0·9% saline via an 18 gauge needle inserted into the thoracic aorta. After 5 min, the perfusion was stopped. The organs were removed, rinsed and extracted as described below.

Blood. Tail vein blood (0·2 ml) was withdrawn at the time of sacrifice and pipetted into about 4 ml acetone–alcohol (1:1, v/v) contained in a screw-top vial graduated to 5·0 ml; the tube was sealed and heated at 60° for 1 hr.[5] After cooling, the mixture was adjusted to 5·0 ml with solvent, the tube was centrifuged and 2·5 ml was counted. Total radioactivity was calculated on the basis that the blood volume is 8 per cent of the body weight.

Organs and Carcass. Immediately after removal and rinsing, the organs and carcass were placed in absolute ethanol and homogenized either in a Waring–Blendor or Virtis "45" homogenizer, depending on the size of the organ. After transferring the mixture to an Erlenmeyer flask, the appropriate volume of ether was added to give a solution of alcohol–ether (3:1, v/v) with a solvent–tissue ratio of at least 10:1. The mixture was brought to boiling on a steam bath and filtered through a sintered glass funnel. The solvent extraction was repeated twice. The filtrates were combined and brought to dryness, and the residue was dissolved in absolute ethanol.

Feces plus intestinal contents. Intestinal contents, obtained by washing out the gastrointestinal tract with 0·9% saline, were added to the 24-hr collection of feces and the combined sample was dried for 2 days at 56° *in vacuo*. The dried residue was homogenized in a Waring–Blendor with 200 ml absolute ethanol for 15 min. The mixture was transferred to a flask, heated on a steam bath for 15 min, and filtered through a sintered glass funnel. After extracting the residue twice more, with 100-ml vol. of absolute ethanol, the ethanol extracts were combined, 50 ml water was added and the total volume was adjusted to 500 ml with absolute ethanol.

Urine. Urine was centrifuged and an aliquot was pipetted directly into DAM phosphor[6] for counting.

^{14}C-counting. Aliquots of the lipid extracts, prepared as described above, were pipetted into DAM phosphor and counted on a Packard Tri-Carb scintillation spectrophotometer. Counts per min (cpm) were converted into disintegrations per min (dpm) by use of an internal standard.

Neutral and acidic fecal compounds. The 90% ethanolic extract of feces, prepared as described above, was fractionated into neutral and acidic components with Dowex-1.[7] This method was checked by the solvent fractionation method of Roscoe and Fahrenbach,[8] modified as follows: to a 50-ml aliquot of the alcoholic fecal extract was added 2 drops of saturated K_2CO_3, and the solution was brought to dryness. The residue was extracted at room temperature with three 10-ml vol. of *n*-hexane and the extracts were combined. After adjusting the volume to 50 ml, an aliquot was counted (hexane-soluble lipids). The residue remaining after the hexane extraction

was suspended in 5 ml water and acidified with 1 N HCl by using Congo red paper. The acidified aqueous solution was extracted with four 15-ml vol. of chloroform; the extracts were combined and brought to dryness. Twenty ml diethyl ether was added to solubilize the residue, and the bile acids were extracted with three 10-ml vol. of 5% aqueous KOH followed by three 10-ml washings with water. The ether solution was adjusted to 50 ml and an aliquot was counted (ether-soluble neutral lipids). The KOH extracts were combined together with the first water wash, and the solution was acidified with 1 N HCl to Congo red paper. Bile acids were extracted with three 100-ml vol. of chloroform. After combination of these extracts, the volume was reduced to 25 ml and an aliquot was counted (bile acids).

Thin-layer chromatography (TLC). Lipid fractions were examined by chromatography on Silica Gel G, (E. Markag, Darmstadt, Germany) 0·5 mm thick, spread on 20 × 20 cm plates and activated at 105° for 1 hr. Position of radioactive material was located by placing a piece of NO-screen X-ray film (Eastman Kodak, Rochester, N.Y.) in contact with the plate and exposing the film for 2–5 days. The amount of radioactivity in each metabolite was determined by scraping the radioactive area off the plate, extracting the silica gel with ethanol and counting an aliquot of the extract.

RESULTS AND DISCUSSION

Distribution of radioactivity in the rat. The distribution of ^{14}C found in the rat 24 hr after the gastric intubation of 25 mg labeled cholestanetriol is presented in Table 1. Most of the label was present in the feces plus intestinal contents, whereas the urine

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN THE RAT AFTER ADMINISTRATION OF 4-^{14}C-CHOLESTANETRIOL

Compartment	Radioactivity found	
	^{14}C-(μc)	Per cent of recovered ^{14}C
Body	0·96 (0·79–1·17)*	16·1 (13·2–19·6)
Feces + intestinal contents	5·01 (4·60–5·46)	83·8 (76·9–91·3)
Urine	0·01 (0·01–0·01)	0·1 (0·1–0·1)
Total	5·98	100·0

* Range.

contained no significant amount of radioactivity. Sixteen per cent of the ^{14}C administered was found in the body 24 hr after administration of the 4-^{14}C-cholestanetriol. The total recovery of ^{14}C was 88·5 per cent.

Tissue distribution of 4-^{14}C-cholestanetriol. When the distribution of radioactivity present in the body was studied, it was found that there was a pooling of the label in the intestinal wall and carcass, each containing about 30 per cent of the recovered radioactivity from the body (Table 2). An additional 30 per cent of the body radioactivity was present in the blood. Thus about 90 per cent of the radioactivity recovered from the body was equally distributed among the intestinal wall, carcass and blood. Of the remaining organs, only the liver contained a significant amount of radioactivity.

Distribution of ^{14}C in feces plus intestinal contents. The distribution of radioactivity

in the feces plus intestinal contents is shown in Table 3. Excellent agreement between the two methods of analysis was found.

The radioactivity was distributed between the neutral sterols and bile acids with an average ^{14}C neutral sterol/^{14}C bile acid ratio of 2·47. Since it was reasonable to

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN THE RAT BODY AFTER ADMINISTRATION OF 4-^{14}C-CHOLESTANETRIOL

Tissue or organ	Radioactivity found	
	^{14}C (μc)	Per cent of recovered ^{14}C*
Intestine	0·312 (0·275–0·351)†	32·45 (29·97–35·03)
Carcass	0·283 (0·224–0·385)	29·43 (25·78–32·88)
Blood	0·271 (0·193–0·330)	28·19 (24·59–31·26)
Liver	0·059 (0·055–0·065)	6·20 (5·55–7·39)
Adrenals	0·001 (0·000–0·001)	0·07 (0·03–0·14)
Testes	0·001 (0·001–0·002)	0·10 (0·09–0·17)
Spleen	0·003 (0·003–0·003)	0·32 (0·26–0·38)
Brain	0·003 (0·003–0·004)	0·35 (0·34–0·43)
Epididymal fat pads	0·003 (0·003–0·003)	0·34 (0·34–0·34)
Lung	0·010 (0·009–0·010)	1·02 (0·85–1·27)
Heart	0·002 (0·002–0·002)	0·17 (0·17–0·17)
Kidneys	0·014 (0·012–0·016)	1·40 (1·37–1·53)

* Per cent of ^{14}C recovered from the rat body.
† Range.

TABLE 3. DISTRIBUTION OF ^{14}C IN THE FECES + INTESTINAL CONTENTS AFTER ORAL ADMINISTRATION OF 4-^{14}C-CHOLESTANETRIOL

Animal	Radioactivity found in			
	Neutral sterols*	Bile acids*	Neutral sterols†	Bile acids†
	(μc)	(μc)	(μc)	(μc)
1	3·38	1·77	2·89	1·89
2	3·54	1·08	3·23	1·14
3	4·21	1·10	3·69	1·56
Av.	3·70	1·31	3·25	1·53

* Dowex-1 method.
† Modified solvent extraction, TLC method.

assume that part of the neutral sterol radioactivity was due to unabsorbed cholestanetriol, the distribution of ^{14}C in the neutral lipid fraction of feces was studied.

Fecal neutral sterols. The hexane-soluble neutral lipid fraction obtained by the modified solvent fractionation method was subjected to TLC (Fig. 1). Four radioactive spots were found. The first spot represents cholestanetriol and the second

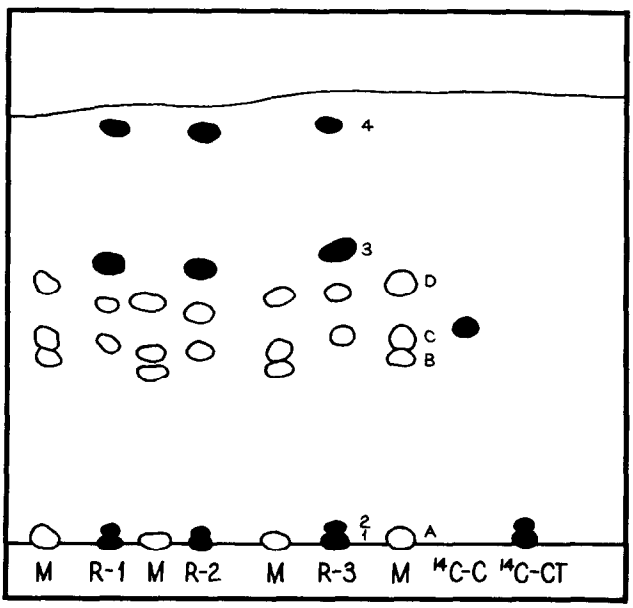

FIG. 1. TLC of the fecal hexane-soluble neutral lipid fraction from rats given 4-^{14}C-cholestanetriol M = mixture of sterols: cholestanetriol = A; Δ^7-cholestene-3β-ol = B; cholesterol = C; and coprostanol = D. ^{14}C-C = 4-^{14}C-cholesterol; ^{14}C-CT = 4-^{14}C-cholestanetriol. R-1, R-2 and R-3 = neutral lipid fractions from rats 1, 2 and 3. Open circles were visualized by spraying with 50% H_2SO_4 and heating at 200°. Closed circles are radioactive spots visualized by radioautography. Solvent system: benzene–ethyl acetate (5:1, v/v).

represents a contaminant found in the original cholestanetriol preparation. The third spot moved farther than coprostanol and a fourth spot moved just behind the solvent front.

The ether-soluble neutral lipid fraction was also subjected to thin-layer chromatography and radioautography. Two radioactive spots were found corresponding (1l) to cholestanetriol and (2) to the impurity present in the original cholestanetriol preparation.

The quantitative distributions of radioactivity in the hexane-soluble and ether-soluble neutral lipid fractions were determined (Table 4). Eighty-one per cent of the total radioactivity found in the neutral lipid fraction was present in cholestanetriol plus spot 2. Spots 3 and 4 contained 17 and 2 per cent of the total neutral lipid radioactivity respectively.

It was observed upon radioautography that spot 2, present in the ether-soluble neutral lipid fraction, contained relatively more radioactivity than did the impurity present in the original labeled cholestanetriol. In the solvent system used, i.e. benzene:

ethyl acetate (5:1, v/v), cholestanetriol and spot 2 were not clearly separated. Therefore, the ether-soluble neutral lipid fractions were pooled and rerun on TLC plates in a more polar system (toluene:HOAc:H_2O, 5:5:1, v/v; top layer). After development, the radioactive spots were located and recovered from the silica gel as described above.

TABLE 4. DISTRIBUTION OF RADIOACTIVITY IN THE LIPID NEUTRAL FRACTIONS OF RAT FECES + INTESTINAL CONTENTS 24 HR AFTER ORAL ADMINISTRATION OF 4-^{14}C-CHOLESTANETRIOL

	Radioactivity found in				
	Cholestanetriol (spot 1) + spot 2			Spot 3	Spot 4
Animal	Ether-soluble (μc)	*n*-Hexane-soluble (μc)	Total (μc)	*n*-Hexane-soluble (μc)	(μc)
1	2·12	0·52	2·64	0·22	0·04
2	1·83	0·92	2·75	0·64	0·07
3	2·11	0·72	2·83	0·84	0·10
Av.	2·02	0·72	2·74	0·57	0·07

In this system cholestanetriol and spot 2 were cleanly separated from each other with R_f values of 0·40 and 0·49 respectively. The radioactivity in each spot was then determined by liquid scintillation counting. Twenty-seven per cent (0·77 μc) of the radioactivity found in the ether-soluble neutral lipid fraction was present in spot 2. In contrast to this, only 2 per cent of the radioactivity present in the original cholestanetriol was found in spot 2, suggesting that this spot represents a real metabolite of cholestanetriol. A summary of the distribution of ^{14}C in feces is presented in Table 5.

TABLE 5. SUMMARY OF THE DISTRIBUTION OF RADIOACTIVITY IN NEUTRAL LIPIDS OF RAT FECES + INTESTINAL CONTENTS 24 HR AFTER ORAL ADMINISTRATION OF 4-^{14}C-CHOLESTANETRIOL

Fraction	Solubility	Component	^{14}C (μc)
Spot 1	Ether	Cholestanetriol	1·97
Spot 2	Ether	?	0·77
Spot 3	*n*-Hexane	?	0·57
Spot 4	*n*-Hexane	?	0·07
Total			3·38

Of the 3·38 μc of ^{14}C present in the neutral lipids, 1·41 μc (spots 2 + 3 + 4) was actually representative of the neutral metabolites of cholestanetriol. Thus the amount of radioactivity present in the neutral metabolites was essentially equal to that found in the bile acids (see Table 3). These results are in good agreement with those of Kikuchi *et al.*,[9] who reported metabolites less polar than cholestanetriol to be present in the chyle and intestinal contents from thoracic duct-cannulated rats after the oral administration of ^{14}C-cholestanetriol.

Fecal bile acids. Since the fecal bile acids represent the other major class of cholestanetriol metabolites found in the feces, it was of interest to examine the distribution of radioactivity within this class of compounds.

The bile acid fraction obtained by solvent fractionation from the feces of rats fed 4-^{14}C-cholestanetriol for 24 hr, was subjected to TLC (see Fig. 2). Radioautography revealed that the major radioactive bile acid chromatographed with cholic acid (spot 1). The position of this spot indicates that the bile acid is a trihydroxy bile acid.

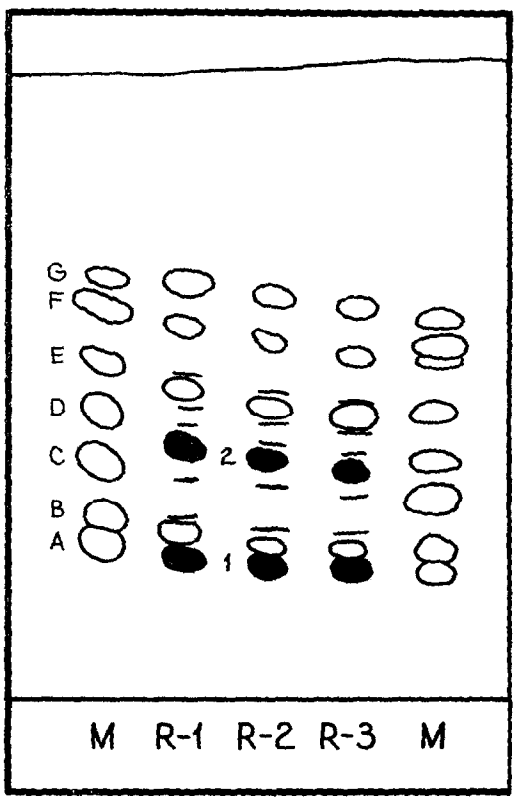

FIG. 2. TLC of the fecal bile acid fraction from rats given 4-^{14}C-cholestanetriol. M = mixture of bile acids: cholic = A; hyocholic = B; 7,12-diketolithocholic = C; hyodeoxycholic = D; deoxycholic = E; dehydrodeoxycholic = F; and lithocholic = G. R-1, R-2 and R-3 = bile acid fractions from rats 1, 2 and 3. Open circles were visualized by spraying with 50% H_2SO_4 and heating at 200°. Closed circles are radioactive spots visualized by radioautography. Solvent system: toluene–HoAc–H_2O (5:5:1, v/v; top layer).

A second major radioactive spot was found which chromatographed between 7,12-diketolithocholic and hyodeoxycholic acids (spot 2). In addition, there were five other minor radioactive spots present, indicating some bacterial degradation of the bile acids formed from cholestanetriol.

For the determination of ^{14}C distribution, another aliquot of the bile acid fraction was chromatographed under the same conditions as described for Fig. 2. Radioactive areas corresponding to the two major radioactive bile acids and the five minor radioactive bile acids were removed from the plate, extracted and counted. The results are shown in Table 6. As can be seen, most of the radioactivity was located in the trihydroxy bile acid spot and in the spot moving between 7,12-diketolithocholic and

hyodeoxycholic acids (65·7 per cent); the remaining 34·2 per cent was distributed among the five minor bile acids.

Thus, it is apparent that cholestanetriol is readily converted into bile acids as

TABLE 6. DISTRIBUTION OF RADIOACTIVITY IN FECAL BILE ACIDS AFTER ADMINISTRATION OF 4-^{14}C-CHOLESTRANETRIOL

Fraction	Radioactivity	
	(μc)	(%)
Spot 1	0·56	42·5
Spot 2	0·31	23·2
other (5 minor)	0·45	34·2

well as neutral metabolites by the rat. These findings are in agreement with those of Kikuchi *et al.*,[9] who have reported the rapid conversion of cholestanetriol into bile acids in bile duct-cannulated rats.

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