Metabolism of cholestane-3β,5α,6β-triol. II. Identification of two major neutral metabolites in the rat

HENRY G. ROSCOE and MARVIN J. FAHRENBACH

Department of Metabolic Chemotherapy, Experimental Therapeutics Research Section, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York 10965

ABSTRACT Rats were given a single oral dose of cholestane-3β,5α,6β-triol-4-^{14}C, and their feces were collected. The two major neutral metabolites were separated and isolated by use of solvent fractionation and chromatographic methods. The metabolites were identified as cholestane-3β,5α-diol-6-one and a mixture of long-chain fatty acid esters of cholestane-3β,5α,-6β-triol. Cholestane-3β,5α-diol-6-one was identified using thin-layer and gas–liquid chromatography, infrared spectroscopy, and the spectrum produced by reaction with 65% sulfuric acid. The mixed esters of cholestane-3β,5α,6β-triol were subjected to basic hydrolysis, and the steroid moiety was identified using the same techniques employed for cholestane-3β,5α-diol-6-one. The fatty acids were analyzed by gas–liquid chromatography of their methyl esters.

SUPPLEMENTARY KEY WORDS cholestane-3β,5α,diol-6-one · fatty acids · sterol esters

CHOLESTANE-3β,5α,6β-TRIOL and its derivatives have been reported to be active hypocholesterolemic agents in cholesterol-fed animals (1, 2). We have previously demonstrated in the rat that CT is excreted primarily in the feces as a mixture of unchanged CT and neutral and acidic metabolites (3). Thin-layer chromatography of the neutral lipid fraction obtained from feces of rats given a single oral dose of CT-4-^{14}C revealed the presence of four labeled compounds, viz., (*a*) unchanged CT, (*b*) compound 2,[1] slightly less polar than CT, (*c*) compound 3, less polar than compound 2, and (*d*) compound 4, which moved close to the solvent front on Silica Gel G plates developed in benzene–ethyl acetate 5:1 (v/v). Compounds 2 and 3 were the major radioactive neutral metabolites of CT. This report describes the isolation and identification of these metabolites.

Abbreviations: CT, cholestane-3β,5α,6β-triol.

[1] The word "compound" is used to denote the fact that 2, 3, and 4 moved as single entities on thin-layer chromatograms.

MATERIALS AND METHODS

Cholestane-3β,5α,6β-triol-4-^{14}C

CT-4-^{14}C (specific activity, 0.25 μCi/mg) was prepared by Dr. Milton Heller (Organic Chemical Research Section, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y.) from cholesterol-4-^{14}C by the method of Fieser and Rajagopalan (4). 98% of the radioactivity in the preparation behaved as CT by thin-layer chromatography in toluene–acetic acid–water 5:5:1 (v/v/v) (top layer). The radioactivity in the CT-4-^{14}C preparation also appeared as a single peak when subjected to reversed-phase partition chromatography using solvent system III (moving phase: isopropanol–water 1:1 [v/v]; stationary phase: chloroform–heptane 1:4 [v/v]) as described by Danielsson (5).

Cholestane-3β,5α-diol-6-one

The 6-one compound was prepared by selective oxidation of cholestane-3β,5α,6β-triol with *N*-bromosuccinimide in aqueous dioxane (4). Melting point: found, 234°C; reported, 232–233°C. The nuclear magnetic resonance spectrum of the product was consistent with cholestane-3β,5α-diol-6-one.

Routine Thin-Layer Chromatography

Chromatography was carried out on 20 × 20 cm plates coated with a 0.5-mm layer of Silica Gel G (E. Mar-

kag, Darmstadt, Germany) activated at 105°C for 1 hr. Plates were developed in either toluene–acetic acid–water 5:5:1 (v/v/v) (top layer), solvent A, or benzene–ethyl acetate 5:1 (v/v), solvent B.

After drying, the plates were placed in contact with No-Screen X-ray film (Eastman Kodak Co., Rochester, N.Y.) for 2–3 days to reveal the positions of radioactive compounds. Organic material was visualized by spraying the plate with 65% H_2SO_4 and heating it at 200°C.

Determination of Radioactivity

Radioactivity was determined by liquid scintillation counting as described previously (3).

Animals

1 ml of a 10% Tween 80[2] emulsion (3) containing 25 mg of CT-4-^{14}C (6.25 μCi) was administered orally to each of three male CFE rats (Carworth Farms, Inc., New City, N.Y.) (avg wt, 170 g). Animals were placed in separate metabolism cages and feces were collected for 24 hr, during which time the animals were allowed free access to food and water.

Reagents

All organic solvents were reagent grade and were used without further purification.

Isolation of Cholestane-3β,5α,6β-triol Metabolites, Compounds 2 and 3, from Feces

Preliminary Separation. Alcoholic extracts of the feces plus intestinal contents were prepared as described previously (3), and combined (total volume = 1200 ml). 2 g of solid K_2CO_3 was added, and the solvent was removed in vacuo over steam. After extracting the dry residue with five 100-ml volumes of hot *n*-hexane, the extracts were pooled and filtered through a sintered glass funnel. The combined hexane extract, which contained labeled compound 3 plus a small amount of compound 2, was washed with water and dried over Na_2SO_4. The water washes from the hexane extract were added back to the flask containing the hexane-extracted residue. The aqueous mixture was acidified with 2 N HCl (Congo red) and extracted with three 100-ml volumes of chloroform. After pooling the extracts, the chloroform was removed over steam in vacuo. The residue was dissolved in 90 ml of diethyl ether, and the bile acids were removed by extraction with four 30-ml volumes of 5% aqueous KOH. The KOH extracts were combined and extracted with three 100-ml volumes of diethyl ether. The ether extracts were combined and added to the original ether solution. The combined ether extract was washed with three 50-ml volumes of water and dried over Na_2SO_4. Thin-layer chromatography revealed that the ether extract contained labeled CT and compound 2 only.

Isolation of Compound 2. The ethereal solution containing CT-4-^{14}C and compound 2 was contaminated with a green pigment. To remove this pigment, the solution was brought to dryness in vacuo at room temperature, and the residue was dissolved in 20 ml of benzene–methanol 1:1 (v/v). 1 g of Darco G-60, prepared as described by Roscoe and Fahrenbach (6), was added and the mixture was stirred and filtered. After bringing the filtrate to dryness under nitrogen at room temperature, 35.6 mg of a yellowish, amorphous powder containing 3.40 μCi of radioactivity was obtained. The powder was dissolved in 10 ml of isopropanol.

An aliquot of the isopropanol solution which contained 26.8 mg (2.38 μCi) of the charcoal-treated material was concentrated under nitrogen, and the sample was applied as a strip on a Silica Gel G plate. Following development in solvent A, the chromatogram was dried and the radioactive areas were located by radioautography as described above. Two well-separated major radioactive bands were obtained corresponding to CT (R_F = 0.19) and compound 2 (R_F = 0.38). The areas containing each labeled band were scraped from the plate and extracted with three 10-ml volumes of isopropanol. After removing the solvent under nitrogen at room temperature, 5.6 mg (0.51 μCi) of compound 2 and 7.5 mg (1.11 μCi) of CT were recovered.

In preliminary experiments, infrared spectroscopy indicated that compound 2 and CT, extracted from the thin-layer chromatogram, were contaminated with a material tentatively identified as calcium acetate. In an attempt to remove this contaminant, compound 2 was subjected to reversed-phase partition chromatography using solvent system III according to Danielsson (5). Compound 2 was dissolved in 2 ml of moving phase and placed on a column, 16 cm × 1 cm I.D., containing 4.5 g of siliconized Hyflo Super-Cel. The column was eluted with approximately 200 ml of moving phase, and 5-ml fractions were obtained (average flow rate: 5 ml/15 min). Appearance of radioactivity was followed by counting 0.05-ml aliquots from each tube. The fecal CT fraction was also chromatographed on a separate column under identical conditions for comparison. Each compound displayed a single radioactive peak and the order of elution was consistent with that expected from thin-layer chromatography on Silica Gel G. CT was eluted between 40 and 60 ml of moving phase, whereas compound 2 appeared between 100 and 150 ml of eluate, suggesting that compound 2 is less polar than the triol. The tubes under each radioactive peak were combined and brought to dryness under nitrogen. 1.8 mg (0.44 μCi) of compound 2 and 4.3 mg (1.10 μCi) of CT were

[2] Polyoxyethylene sorbitan monooleate, Atlas Powder Co., Wilmington, Del.

recovered. Both compounds were of satisfactory purity as judged by their specific activities: compound 2, 0.24 μCi/mg; fecal CT, 0.25 μCi/mg; and CT originally administered to the rats, 0.25 μCi/mg.

Isolation of Compound 3. The hexane extract, obtained from the preliminary separation described above, was brought to dryness in vacuo and the residue, which weighed 268 mg (1.75 μCi), was dissolved in 10 ml of benzene. 40 g of silicic acid, 100 mesh (Mallinckrodt Chemical Works, New York, N.Y.), previously activated at 105°C overnight, was mixed with 20 g of Celite 503. The mixture was slurried in benzene and poured into a glass column, 2.5 cm I.D., and allowed to pack under gravity to a height of 27.3 cm. Following the addition of the benzene solution which contained compound 3, the sample was rinsed into the column with an additional 5 ml of benzene, and the column was eluted with 250 ml of benzene followed by 600 ml of benzene–ethyl acetate 5:1 (v/v). 10-ml fractions were collected every 2.5 minutes and aliquots from each tube were assayed for radioactivity.

Chromatography of the crude hexane-soluble material on silicic acid resulted in the appearance of five radioactive peaks (Fig. 1). Each radioactive fraction was examined by routine thin-layer chromatography in solvent B. Fraction III, which contained 67% (1.17 μCi) of the recovered ^{14}C, chromatographed as a single radioactive spot corresponding to compound 3. This fraction was grossly contaminated with nonradioactive material, much of which was tentatively identified as free sterol by gas–liquid chromatography. Small-scale experimentation showed that compound 3 obtained from column chromatography did not form an insoluble digitonide. Thus, to remove contaminating sterols, column fraction III was brought to dryness under nitrogen at room temperature, and the residue (41.6 mg, 1.17 μCi) was dissolved in 5.0 ml of isopropanol. 20 ml of 1% digitonin in 50% aqueous ethanol was added, and the mixture was shaken, then allowed to stand for 1.5 hr at 37°C. The mixture was filtered, and the precipitate was washed with acetone–alcohol 1:1 (v/v) and diethyl ether. The washes were combined with the filtrate, and the solvent was removed under nitrogen at room temperature. The residue was extracted with seven 50-ml volumes of boiling ether, and the ether extracts were combined, washed with water, and dried over Na_2SO_4.

The ether solution containing compound 3 was brought to dryness under nitrogen at room temperature. The residue, which weighed 15.5 mg (0.72 μCi), was dissolved in 0.21 ml of isopropanol and chromatographed by thin-layer chromatography in solvent B to remove contaminating pigments. After locating compound 3 by radioautography, the area containing compound 3 was scraped from the plate, placed in a test tube, and extracted with three 10-ml volumes of isopropanol. The isopropanol extracts were combined and brought to dryness. The residue of compound 3 weighed 3.1 mg and contained 0.50 μCi of radioactivity.

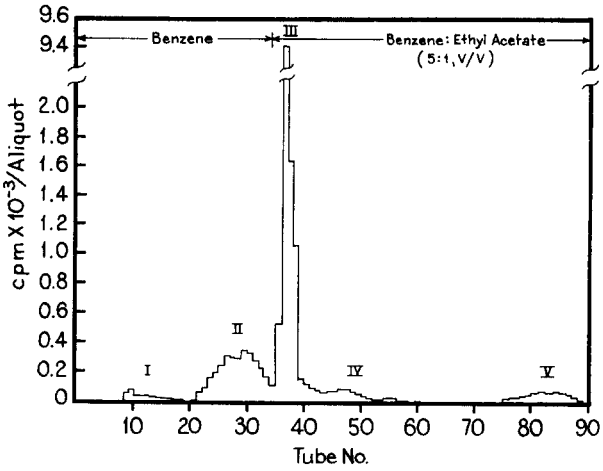

FIG. 1. Silicic acid chromatography of hexane-soluble material from rat feces. The column contained 40 g of silicic acid and 20 g of Celite 503. Material was eluted with 250 ml of benzene followed by 600 ml of benzene–ethyl acetate 5:1 (v/v).

Identification of Compounds 2 and 3

Saponification. Approximately 900 μg of either compound 2 or 3 was dissolved in chloroform and pipetted into a Teflon-stoppered Pyrex culture tube. After removing the solvent under nitrogen at room temperature, 0.2 ml of 30% aqueous KOH and 1.0 ml of ethanol were added to the residue. The tube was flushed with nitrogen, capped, and heated on a steam bath for 40 min. After cooling, 1 ml of water was added, and the sample was extracted with three 5-ml volumes of diethyl ether. The ether extracts were combined, washed with water, and dried over Na_2SO_4. After drying, the Na_2SO_4 was filtered off, and the ether was removed under nitrogen over steam. The residue was dissolved in 10 ml of isopropanol. The aqueous solution remaining after the ether extraction was acidified with 10% HCl (Congo red) and extracted with three 5-ml volumes of fresh diethyl ether. The ether extracts were combined, washed with water, and dried over Na_2SO_4. The Na_2SO_4 was removed by filtration, and the ether phase containing fatty acids was brought to dryness under nitrogen at room temperature. The residue of fatty acids was dissolved in 10 ml of isopropanol.

Gas–Liquid Chromatography. All gas–liquid chromatography was performed using a Biomedical Gas Chromatograph, Model 400, equipped with a flame ionization detector (F & M Scientific Corp., Avondale, Pa.). Steroids were chromatographed in either their free form or after trimethylsilylation according to Makita and

Wells (7). Gas-Chrom P, 100–120 mesh (Applied Science Laboratories Inc., State College, Pa.), was prepared and coated as described by Sjövall, Meloni, and Turner (8), except that the Gas-Chrom P was siliconized by exposing it overnight to an atmosphere of dimethyldichlorosilane (Analytical Engineering Laboratories, Hamden, Conn.) under reduced pressure. The column was prepared by packing one arm of a 6-ft glass U-tube with 0.5% SE-52 (Analytical Engineering Laboratories) on Gas-Chrom P and the other arm with 1.0% QF-1 (Analytical Engineering Laboratories) on Gas-Chrom P. The column conditions used were: column temperature, 210°C–245°C; flash heater, 300°C; and flow rate, 40–70 ml/min.

Fatty acids were converted to their methyl esters (9) and chromatographed on a 6-ft glass column containing Gas-Chrom Q (Applied Science Laboratories Inc.) coated with 5% diethylene glycol succinate. Column temperature, 148°C and 190°C; flash heater, 200°C; and flow rate, 40 ml/min.

Absorption Spectrum in Sulfuric Acid. The absorption spectrum after reaction with 65% sulfuric acid was measured essentially as described by Mosbach, Kalinsky, Halpern, and Kendall (10). An aliquot containing 75–125 μg of the sample in ethanol was pipetted into a glass-stoppered test tube and the alcohol was removed under nitrogen at room temperature. 3 ml of 65% H_2SO_4 was added, and the tube was heated at 60°C for 15 min. After cooling, the absorption spectrum was determined in a Beckman DU Quartz Spectrophotometer using silica cells with a 1-cm light path.

Infrared Spectroscopy. Infrared spectra were determined in a Perkin–Elmer Double Beam Spectrophotometer, Model 21.

RESULTS

Identification of Compound 2

Examination of compound 2 by thin-layer chromatography revealed that this compound was almost as polar as CT itself (3). When examined by gas–liquid chromatography, compound 2 appeared with a retention time greater than CT and exhibited thermal decomposition. These properties are suggestive of a polyhydroxylated sterol in which one or more of the hydroxyl groups has been converted into a ketone, analogous to the behavior of the bile acids (11, 12). The fact that compound 2 moved close to CT on thin-layer chromatography suggested that only one hydroxyl group had been oxidized.

Infrared Spectroscopy. Compound 2 displayed a strong carbonyl absorption at 5.85 μ, characteristic of a 6-membered cyclic ketone, and distinct from an ester carbonyl which absorbs from 5.68 to 5.81 μ (13). Since the movement on thin-layer chromatography suggested that compound 2 may be a diolone, two possibilities exist: namely, that compound 2 is cholestane-3β,5α,-diol-6-one or cholestane-3-one-5α,6β-diol. Therefore, cholestane-3,5-diol-6-one was prepared as described under METHODS. Cholestane-3-one-5α,6β-diol was not available, and a description of its synthesis could not be found. The infrared spectrum of the authentic cholestane-3,5-diol-6-one compared quite well with that of compound 2 (Fig. 2).

Thin-Layer Chromatography. Compound 2 and cholestane-3,5-diol-6-one were compared by thin-layer chromatography in solvent A. Both compounds had the same mobility, and when chromatographed together, the radioactivity due to compound 2 was superimposable on cholestane-3,5-diol-6-one.

Sulfuric Acid Spectrum. Further evidence for the identity of compound 2 was supplied by the spectrum produced by reaction with 65% sulfuric acid at 60°C for 15 min. The spectra produced by compound 2 and cholestane-3,5-diol-6-one were identical, each having two maxima, i.e., 320 and 390 nm (Fig. 3).

Gas–Liquid Chromatography. Attempts to examine compound 2 by gas–liquid chromatography resulted in thermal decomposition of the material. It was found that if compound 2 was first converted into its trimethylsilyl ether, no thermal decomposition occurred, and a sharp peak was obtained. Therefore, aliquots of compound 2 and cholestane-3,5-diol-6-one were converted into their trimethylsilyl ethers and chromatographed. Compound 2 chromatographed with the same retention time as did cholestane-3,5-diol-6-one, and when chromatographed together, a single sharp peak was obtained.

Identification of Compound 3

The fact that compound 3 had a higher mobility than coprostanol when examined by thin-layer chromatography (3), together with the observation that compound 3 did not form an insoluble digitonide, suggested that the 3β-hydroxyl had been modified. Evidence indicating that compound 3 was an ester came from the observation that no peaks were observed when the isolated material was subjected to gas–liquid chromatography under conditions which are known to separate a number of free steroids with different degrees of polarity from cholestane to cholestanetriol. Infrared spectroscopy of compound 3 revealed a strong carbonyl absorption at 5.8 μ coupled with a strong absorption in the 7.75 to 9.0 μ range, suggesting that this metabolite is an ester rather than a ketone (13). Final proof that compound 3 was an ester came from saponification studies.

Nonsaponifiable Material. The mobility of the nonsaponifiable material obtained from compound 3 was compared to that of the original metabolite on thin-layer chromatography combined with radioautography in solvent B. Compound 3 moved 6.9 cm from the origin

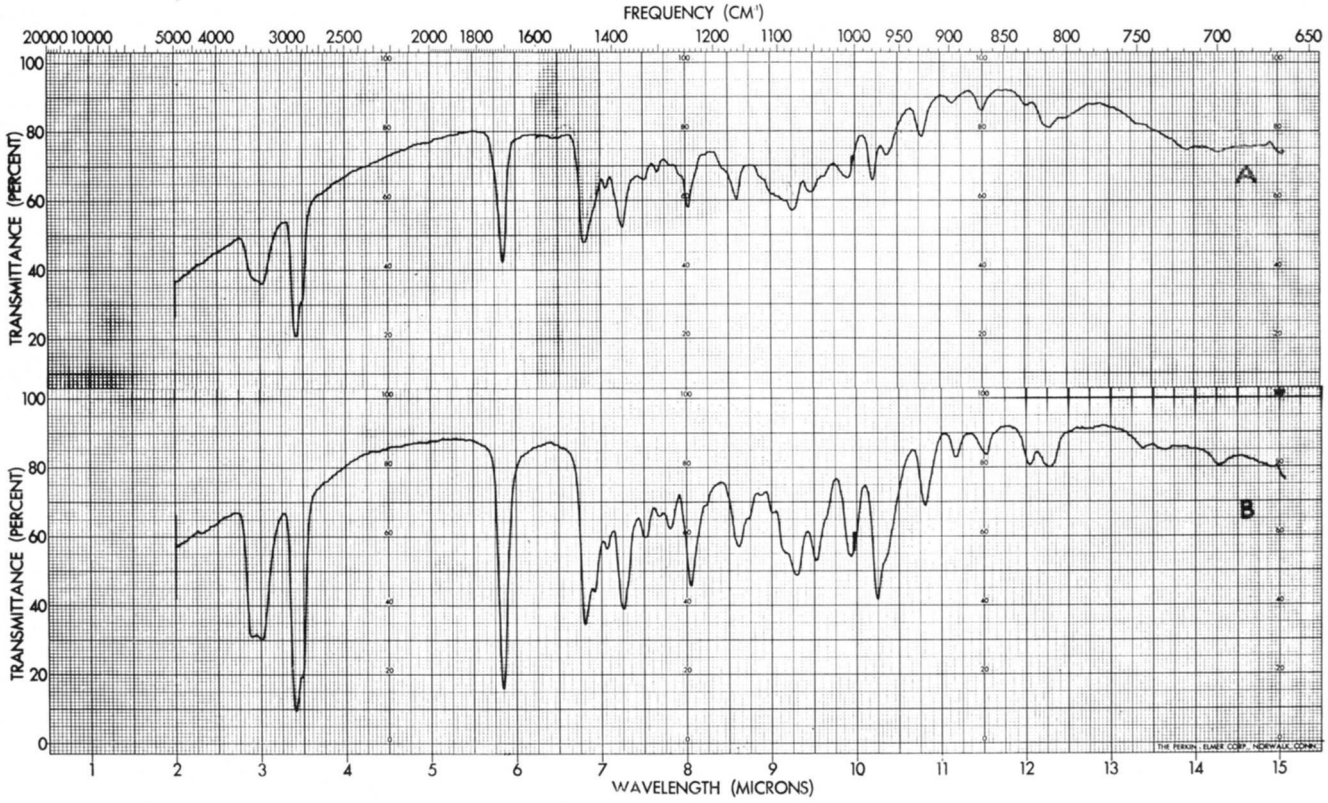

FIG. 2. Infrared spectra of compound 2 (*A*) and cholestane-3,5-diol-6-one (*B*). Samples were pelleted in potassium bromide for spectral determinations.

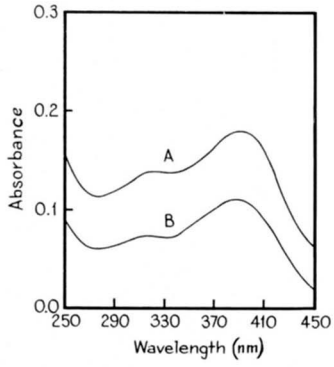

FIG. 3. Comparison of spectra of cholestane-3β,5α-diol-6-one and compound 2. Compounds were heated with 3.0 ml of 65% H_2SO_4 at 60°C for 15 min. Spectra were determined on a Beckman Quartz Spectrophotometer, model DU, using silica cells with a 1-cm light path. *A*, 200 μg cholestane-3β,5α-diol-6-one; *B*, 125 μg of compound 2.

while the nonsaponifiable material remained at the point of application. Chromatography of the nonsaponifiable material in the more polar solvent system A resulted in a single radioactive band which moved with the same mobility as cholestane-3β,5α,6β-triol.

Another aliquot of the nonsaponifiable fraction obtained from compound 3 was examined by gas–liquid chromatography. Compound 3 itself did not come off the gas chromatographic column. However, after saponification, compound 3 was converted into a compound which had a retention time identical to that of authentic CT.

Additional evidence that compound 3 was converted into CT was supplied by an examination of the spectrum produced by reaction with 65% sulfuric acid. Authentic CT and the nonsaponifiable material from compound 3 gave identical spectra after reaction with 65% sulfuric acid (Fig. 4).

Fatty Acids. Fatty acids obtained from saponification of compound 3 were methylated and examined by gas–liquid chromatography. When run at 148°C, an excellent separation of the fatty acid methyl esters was obtained for saturated and unsaturated fatty acids of chain lengths C_{12} to C_{18}. For fatty acid methyl esters of chain length greater than C_{18}, a temperature of 190°C was employed. When examined at 148°C, the predominant fatty acids found were palmitic, stearic, oleic, and linoleic. Examination at 190°C revealed the presence of three other major peaks. The first peak, 1, had a retention time between those for 22:0 and 24:0, and peaks 2 and 3 had retention times greater than that for 24:0. Peaks 1 and 2 were tentatively identified as fatty acid methyl esters of 22:4 and 22:6, respectively (14). Peak 3 was not identified.

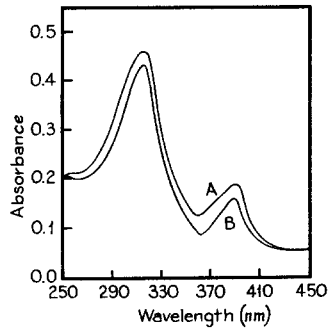

FIG. 4. Comparison of spectra of CT and nonsaponifiable matter from compound 3. For conditions, see Fig. 3. *A*, 80 μg CT; *B*, 75 μg nonsaponifiable matter from compound 3.

Since compound 3 was isolated from feces, the possibility existed that some of the fatty acid moieties were less than 12 carbons in length. To examine this possibility, compound 3 was reacted directly with hydroxylamine, and the hydroxamic acids formed were separated by paper chromatography (15). Using this method, no fatty acids containing 1 to 10 carbons were detected.

DISCUSSION

From the data presented above, compound 2 was found to be identical to cholestane-3β,5α-diol-6-one, whereas compound 3 was identified as a mixture of CT esters containing predominately long-chain fatty acids. Although we were unable to ascertain at which position the fatty acids are bonded, the fact that these esters were not precipitable with digitonin suggests that the 3β-hydroxyl group was esterified.

Compound 2 and the steroidal moiety of compound 3 were identified as the 6-one and CT, respectively, by comparison of infrared (compound 2) and ultraviolet spectra and behavior on chromatography with that of the authentic compounds. Due to lack of material, it was not possible to perform additional experiments such as (*a*) mixed melting point determinations and (*b*) crystallization to constant specific activity after the addition of authentic material. Although a more rigorous proof for the structures of the steroidal moieties would be desirable, the evidence presented above is strongly suggestive of the proposed structures.

Recently, Kikuchi, Imai, Ziro, Matsuo, and Noguchi (16) described the appearance of a major neutral steroidal metabolite in rat intestine after the oral administration of 25 mg of CT-4-^{14}C to animals with thoracic duct fistulas. Although the metabolite was not identified, its chromatographic properties were similar to those of cholestane-3β,5α-diol-6-one (compound 2). However, these authors found no metabolites corresponding to esterified CT, suggesting that the liver may be the site of their synthesis and that the intact enterohepatic circulation may be required for their appearance in the intestine.

The quantitative importance of the neutral metabolites of CT appears to vary with the route of administration. Thus, during the 24 hr following a single oral dose of 25 mg (6.25 μCi) of CT-4-^{14}C to intact rats, equal amounts of neutral and bile acid metabolites (1.41 μCi and 1.42 μCi, respectively) were recovered from feces (3). On the other hand, when intact rats were injected intravenously with labeled CT, only 2.6% of the fecal radioactivity was present in the neutral steroid fraction, whereas greater than 90% of the label appeared as bile acids (16). However, the fact that CT is active when given orally (1) suggests that the neutral steroidal metabolites may be quantitatively important catabolic products of this steroid in normal usage.

The authors would like to thank Mr. George Morton, Department of Structure and Analysis in the Organic Chemical Research Section, for interpreting the infrared and nuclear magnetic resonance spectra.

Manuscript received 3 June 1970; accepted 14 September 1970.

REFERENCES

1. Aramaki, Y., T. Kobayashi, Y. Imai, S. Kikuchi, T. Matsukawa, and K. Kanazawa. 1967. Biological studies of cholestane-3β,5α,6β-triol and its derivatives. *J. Atheroscler. Res.* **7:** 653–669.
2. Armstrong, M. L., W. E. Connor, and J. C. Hoak. 1968. Serum and tissue lipids in Rhesus monkeys fed cholesterol and a trihydroxy analogue. *Circulation.* **38:** VI–1.
3. Roscoe, H. G., R. Goldstein, and M. J. Fahrenbach. 1968. Metabolism of cholestane-3β,5α,6β-triol. I. The fate of cholestanetriol in the rat. *Biochem. Pharmacol.* **17:** 1189–1196.
4. Fieser, L. F., and S. Rajagopalan. 1949. Selective oxidation with *N*-bromosuccinimide. II. Cholestane-3β,5α,6β-triol. *J. Amer. Chem. Soc.* **71:** 3938–3941.
5. Danielsson, H. 1958. Reversed phase partition chromatography of some C_{27}-steroids. Bile acids and steroids 38. *Biochim. Biophys. Acta.* **27:** 401–405.
6. Roscoe, H. G., and M. J. Fahrenbach. 1963. Removal of fecal pigments and its application to the determination of fecal bile acids in the rat. *Anal. Biochem.* **6:** 520–529.
7. Makita, M., and W. W. Wells. 1963. Quantitative analysis of fecal bile acids by gas–liquid chromatography. *Anal. Biochem.* **5:** 523–530.
8. Sjövall, J., C. R. Meloni, and D. A. Turner. 1961. A study of the separation of substituted cholanic acids by gas–liquid chromatography. *J. Lipid Res.* **2:** 317–320.
9. Schlenk, H., and J. L. Gellerman. 1960. Esterification of fatty acids with diazomethane on a small scale. *Anal. Chem.* **32:** 1412–1414.
10. Mosbach, E. H., H. J. Kalinsky, E. Halpern, and F. E. Kendall. 1954. Determination of deoxycholic and cholic acids in bile. *Arch. Biochem. Biophys.* **51:** 402–410.
11. Eneroth, P. 1963. Thin-layer chromatography of bile acids. *J. Lipid Res.* **4:** 11–16.

12. Sjövall, J. 1962. Qualitative analysis of bile acids by gas chromatography. *Acta Chem. Scand.* **16:** 1761–1764.
13. Randall, H. M., R. G. Fowler, N. Fuson, and J. R. Dangl. 1949. Infrared Determination of Organic Structures. D. Van Nostrand Co., New York. 4–6.
14. Harlan, W. R., Jr., and S. J. Wakil. 1963. Synthesis of fatty acids in animal tissues. I. Incorporation of C^{14}-acetyl coenzyme A into a variety of long chain fatty acids by subcellular particles. *J. Biol. Chem.* **238:** 3216–3223.
15. Thompson, A. R. 1951. Separation of saturated monohydroxamic acids by partition chromatography on paper. *Aust. J. Sci. Res., Series B.* **4:** 180–186.
16. Kikuchi, S., Y. Imai, S. Ziro, T. Matsuo, and S. Noguchi. 1968. Biologic studies of cholestane-3β,5α,6β-triol and its derivatives. III. The metabolic fate and metabolites of cholestane-3β,5α,6β-triol in animals. *J. Pharmacol. Exp. Ther.* **159:** 399–408.