Bile acids. XXXV. Metabolism of 5α-cholestan-3β-ol in the Mongolian gerbil

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Abstract The principal bile acid of Mongolian gerbil bile is cholic acid, although small amounts of chenodeoxycholic and lesser amounts of deoxycholic acids are identified. Muricholic acids were not found in gerbil bile. The ratio of trihydroxy to dihydroxy bile acids in gerbil bile is approximately 11:1. After administration of [4-^{14}C]5α-cholestan-3β-ol to gerbils with bile fistulas, 4–7% of the administered ^{14}C was recovered in bile and 16% in urine on the first 6 days. Alkaline hydrolysis of the bile afforded the biliary acids which were separated by partition chromatography. The ^{14}C ratio of trihydroxy to dihydroxy bile acids was 11:1. Allocholic acid was identified as the major acidic biliary metabolite. From analysis of ^{14}C retained in selected tissues, the adrenal gland appears to be an important site for retention of cholestanol or its metabolites.

Supplementary key words allocholic acid · sterol metabolism · allocholanic acids · 5α-cholanoic acids

Recent reports have drawn attention to the Mongolian gerbil (*Meriones unguiculatus*), a rodent indigenous to the desert, as a suitable animal for a study of the relationship of dietary lipid and serum cholesterol in relation to atherosclerosis (1–4). The gerbil rapidly develops a hypercholesterolemia after 1 wk on a diet including 0.6% cholesterol (3) and maintains high serum cholesterol values up to 18 wk (2) or 180 days (4) in contrast to the rat, which shows little change in serum cholesterol on feeding the sterol. Roscoe and Fahrenbach (5) studied the metabolism of [4-^{14}C]cholesterol in the gerbil and reported an apparent lower turnover rate of cholesterol in comparison with the rat; 90% of the ^{14}C excreted after 14 days appeared in the feces, predominantly in the bile acid fraction, but the fecal bile acids were not identified.

Cholestanol (dihydrocholesterol) is a ubiquitous companion of cholesterol (6). Because of this universal association with cholesterol in normal and diseased states (7–10), the degradation of this saturated sterol to acidic metabolites excreted in bile has been a subject of investigation in this laboratory (11). We have previously established that allocholic acid is the major acidic biliary metabolite of cholestanol in the rat (11–13), and that allochenodeoxycholic acid (14) is the second major bile acid derived from this sterol. In view of an apparent difference in sterol metabolism in the gerbil compared with the rat and the reported interest in the Mongolian gerbil in relation to sterols (3), dietary lipids, and atherosclerosis (1), we report in this paper on the identification of the major biliary acids of gerbil bile and the nature of the principal acidic biliary metabolite of [4-^{14}C]5α-cholestan-3β-ol in this species. A preliminary report on some of these studies has appeared (15).

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; PLC, preparative-layer chromatography; TMSi, trimethylsilyl.

Systematic nomenclature of the compounds referred to in the text by their trivial names is as follows: cholestanol, 5α-cholestan-3β-ol; cholestanone, 5α-cholestan-3-one; cholesterol, cholest-5-en-3β-ol; coprostanol, 5β-cholestan-3β-ol; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; allochenodeoxycholic acid, 3α,7α-dihydroxy-5α-cholanoic acid; allocholic acid, 3α,7α,12α-trihydroxy-5α-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; α-muricholic acid, 3α,6β,7α-trihydroxy-5β-cholanoic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholanoic acid; Nembutal (Abbott), sodium 5-ethyl-5-(1-methylbutyl)-barbiturate; Tween 80, polyoxyethylene sorbitan monooleate.

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MATERIALS AND METHODS

Animals

Young adult male gerbils were purchased from Tumblebrook Farms, Inc., Brant Lake, N.Y., and were maintained on Purina Chow. Successful cannulation of the bile ducts was difficult because of the small size and nervous disposition of the animals. After fasting the animals for 12 hr, ether anesthesia was preceded by an intraperitoneal injection of Nembutal. The bile duct was ligated and a cannula was inserted into the gall bladder; it was externalized through the abdominal musculature and conducted subcutaneously up to a small vial taped to the animal's back. Bile was removed at regular intervals from the vial by syringe; the vial contained a small amount of 50% ethanol to suppress bacterial growth. The imperfections in this procedure were evidenced by the short duration of bile flow (never more than 7 days) despite ad lib. access to food, water, and a solution of 1.5% sodium chloride and 0.3% potassium bicarbonate.

Chromatography

Bile acids were separated by column partition chromatography (16). The fractions have been designated according to the percentage of benzene in hexane, e.g., fraction 80-1 represents the first fraction of the eluent containing 80% benzene in hexane.

Separation of neutral sterols was accomplished (14) with a column of silicic acid impregnated with silver nitrate, analogous to the procedure of Shefer, Milch, and Mosbach (17). In this manner [4-^{14}C]cholestanol (specific activity, 2.08×10^7 dpm/mg, New England Nuclear Corp.) was separated from contaminants (coprostanol and cholesterol). Previous experience has shown (14) that the maximum amount of [^{14}C]cholesterol that may be present after this procedure is 0.06%. An aliquot of the purified crystalline [4-^{14}C]cholestanol (1.08×10^6 dpm) was diluted with 106 mg of cholestanol, purified as above, to provide a sample with a calculated specific activity of 3.96×10^6 dpm/mmole; the specific activity was 4.00×10^6 dpm/mmole after crystallization from ethanol. The crystalline material was oxidized with chromium trioxide in acetic acid and the cholestanone was purified by chromatography on a silver nitrate–silicic acid column. The specific activity remained constant (4.08×10^6 dpm/mmole) after two crystallizations from ethanol and agreed with that of the stanol (14). The results established the radiopurity of the purified [4-^{14}C]cholestanol. TLC was carried out with solvent system S9 of Eneroth (18) (2,2,4-trimethylpentane–propan-2-ol–acetic acid 30:10:1) on glass plates (20 × 20 cm) with 0.25-mm layers of silica gel G as described (14).

GLC was carried out as reported (19) on an F & M model 402 gas chromatograph with a hydrogen flame detector and a U-shaped glass column (6 ft × 0.25 inch O.D.) packed with 3% QF-1 or 3% OV-17 on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories, State College, Pa.). TMSi ethers were prepared according to the procedure of Makita and Wells (20). Relative retention times were related to methyl deoxycholate as 1.00 or its bis-TMSi derivative as 1.00.

Injection solution

[4-^{14}C]Cholestanol was dissolved in 0.1 ml of warm ethanol, 80 mg of Tween 80 (Nutritional Biochemicals Corp., Cleveland, Ohio) was added, and the mixture was diluted with water to 1.00 ml for injection. Radioassay of solutions A and B showed 5.38×10^7 dpm/ml (2.58 mg/ml) and 3.71×10^7 dpm/ml (1.8 mg/ml), respectively.

Other methods

Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer, model 3314, equipped with external standardization. Bray's solution (21), 15 ml, was used for assays for specific activity, aliquots from partition chromatography, scrapings from TLC plates, and aqueous samples. Other materials soluble in toluene were assayed as previously reported (22). Melting point determinations were taken on a Fisher-Johns apparatus and are reported as read.

Bile acids

Cholic acid was a generous gift of the Wilson Laboratories, Chicago, Ill. Allocholic acid and its 3β-isomer were prepared from methyl cholate by allomerization with Raney nickel (23). Small amounts of the more common bile acids were at hand (19).

RESULTS

Bile acids of gerbil bile

Gerbil bile collected from four animals over a period of 13 animal-days was pooled and hydrolyzed with KOH according to the procedure of Mahowald et al. (24). The acid fraction (95 mg) was subjected to acetic acid partition chromatography (16) on 10 g of Celite (Fig. 1). Fraction 0-1, in which free fatty acids occur (16), was not investigated. In comparison with a similar profile from rat bile, an unusually large proportion (11:1) of mass appeared in regions associated with trihydroxy acids (fractions 80-1 to 100-4) compared with the regions associated with dihydroxy acids (fractions 40-1 to 40-3). No mass was present in the region associated with β-muricholic acid (fractions 60-3 and 60-4),

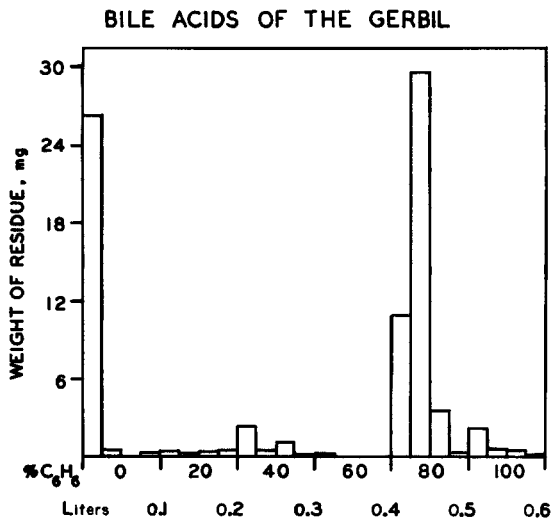

FIG. 1. Partition chromatography of the free bile acids derived from pooled bile obtained from four gerbils with bile fistulas. The chromatographic procedure is described in the text.

a metabolite of chenodeoxycholic acid in the rat (24) and in mouse (25).

Crystallization of material from fractions 80-1 and 80-2 provided cholic acid, mp 201°C (reported, 198°C [26] and 199–200°C [27]); the infrared spectrum was identical with that of authentic cholic acid. Portions of material from fractions 80-1, 80-2, and 80-3 were methylated with diazomethane, converted to the TMSi ethers, and analyzed by GLC. A single component appeared in each preparation, relative retention time 0.90, corresponding to the TMSi ether of methyl cholate (19).

Residue from fractions 40-1 and 40-2 was methylated, silylated, and analyzed by GLC on 3% QF-1. Fraction 40-1 contained four components with the following relative retention times: 0.95 (unidentified), 5%; 1.00 (deoxycholate), 12%; 1.10 (chenodeoxycholate), 79%; and 1.44 (unidentified), 5%. Fraction 40-2 contained three constituents in the ratio 3:1:3 with the following retention times: 1.00 (deoxycholate); 1.12 (chenodeoxycholate); and 1.27 (unidentified). Fraction 40-3 was analyzed only as the methyl ester as follows: 1.37 (unidentified), 5%; 1.56 (unidentified), 6%; and 3.49 (possibly 7-oxo-3α,12α-dihydroxy-5β-cholanate), 89%. The residue remaining in fraction 40-4 after several manipulations was not analyzed because of the paucity of material. Analysis of material in fractions 40-1 to 40-4 by TLC showed that fraction 40-1 contained chenodeoxycholic acid (R_F 0.46) as the major component and deoxycholic acid (R_F 0.52) as the minor component. The principal material in fractions 40-2, 40-3, and 40-4 had an R_F intermediate between chenodeoxycholic and deoxycholic acids and produced a reddish-brown color after spraying with the phosphomolybdic acid reagent instead of the usual blue-green.

Analysis by GLC on 3% OV-17 of material from fractions 60-1 to 60-4, 80-4, and 100-1 to 100-4 after methylation and silylation showed the presence of a number of unidentified constituents in low concentrations. A relative retention time of 1.60 corresponding to 3-oxo-12α-hydroxy- or 3-oxo-7α,12α-dihydroxy-5β-cholanate was observed as a small, well-defined peak in fractions 60-3 and 60-4, but the identification was not completed.

Although α-muricholic acid is frequently eluted with cholic acid in partition chromatography, the TMSi derivatives of the methyl esters are readily resolved (19); thus neither α- nor β-muricholic acids were detected as constituents of these samples of gerbil bile. Since only cholic acid was found in the trihydroxy acid fractions and since only 2.0 mg of the dihydroxy acid fractions can be chenodeoxycholic acid, the ratio of these two acids was 23:1.

Metabolic studies

[4-^{14}C]5α-Cholestan-3β-ol was administered intraperitoneally to gerbils with bile fistulas that functioned for 22 hr postoperatively. Gerbil A received 1.5 mg (3.2×10^7 dpm from solution A) and gerbil B received 0.45 mg (9.28×10^6 dpm from solution B). Bile flow continued normally for the next 12–36 hr but then decreased, indicating obstruction of the cannulas. After 5 days, 4.4% of the radioactivity administered to gerbil A was recovered from bile and 16% from urine; 7.1% of the ^{14}C was recovered in bile from gerbil B in 6.5 days.

Bile from gerbil A was pooled and hydrolyzed, and the distribution of ^{14}C was as follows: neutral fraction, 27%; water wash from neutral fraction, 2%; acid fraction, 37%; water wash from acid fraction, 18%; and residual ^{14}C in hydrolyzed extracted bile, 14%. The radioactive material in the neutral fraction was shown by chromatography and isotope dilution to be unchanged cholestanol. The sizeable amount of unchanged sterol recovered may be a reflection of the large amount of sterol administered, or it may represent a significant difference in metabolism between the gerbil and other species. The bile acid fraction was chromatographed on a partition column (Fig. 2). The distribution of radioactive metabolites paralleled that of mass, with 83% of the radioactivity in fractions 80-1 and 80-2, in which cholic acid is eluted, and 7.4% in fractions 20-4 to 40-3, in which dihydroxy bile acids are usually eluted. The ratio of tri- to dihydroxy metabolites is thus 11:1, comparable with that of the bile acids normally found in gerbil bile, but contrasted with a ratio of 6:1 for these metabolites in rat bile (12).

Material from fractions 80-1 and 80-2 was methylated and subjected to isotope dilution studies after addition of

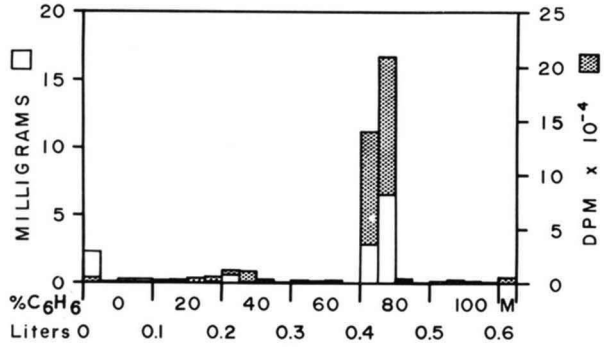

FIG. 2. Partition chromatography of the biliary acid fraction obtained after the administration of [4-^{14}C]cholestanol to gerbil A. The height of the open bars measured *from the baseline* denotes the amount of mass in the fractions; the height of the stippled bars measured *from the baseline* denotes the amount of ^{14}C in the fractions. The last fraction, *M*, represents methanol wash.

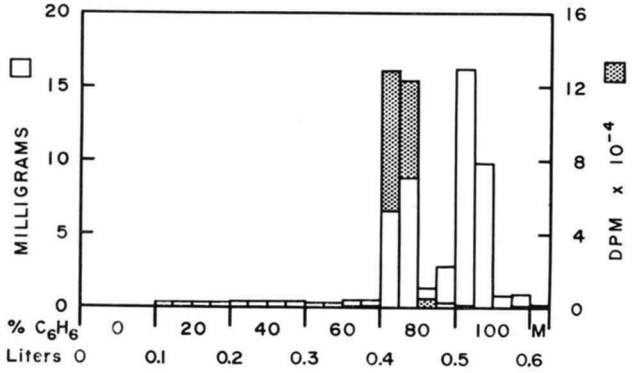

FIG. 3. Partition chromatography of the radioactive metabolite from fractions 80-1 and 80-2 (Fig. 2) to remove 3β,7α,12α-trihydroxy-5α-cholanoic acid present as a result of isotope dilution. Bar heights and fraction *M* are defined in legend to Fig. 2.

methyl 3β,7α,12α-trihydroxy-5α-cholanoate. After four successive crystallizations from a mixture of acetone and hexane, with a decrease in crystalline mass from 192.4 mg to 120 mg, the specific activity dropped from 8.61 $\times$ 10^5 dpm/mmole to 1.45 dpm/mmole. The residues from the mother liquors were combined with the last crop of crystals and hydrolyzed to the free acid. In five successive crystallizations from a mixture of acetone and hexane, with a decrease in crystalline mass from 180 mg to 83 mg, the specific activity changed from 7.17 $\times$ 10^5 dpm/mmole to 0.037 $\times$ 10^5 dpm/mmole, confirming the nonidentity of the radioactive metabolite with the added 5α-cholanate.

The mixture of free acids was chromatographed on the partition column, whereupon 3β,7α,12α-trihydroxy-5α-cholanoic acid (fractions 80-4 to 100-2) was separated from the metabolite and cholic acid (Fig. 3). The radioactive material (2.52 $\times$ 10^5 dpm) in fractions 80-1 and 80-2 was methylated with diazomethane and separated from methyl cholate by repeated PLC in

TABLE 1. Isotope dilution of the methylated metabolite from fractions 80-1 and 80-2 (Fig. 2) with methyl allocholate

Crystallization No. and Solvent	Weight of Crystals	Weight in Mother Liquor	Specific Activity	
	mg	*mg*	*dpm/mg* ($\times$ 10^{-3})	*dpm/mmole* ($\times$ 10^{-5})
Methyl allocholate				
Calculated value[a]	102.4		1.27	5.36
1. Acetone–hexane	76.9	29.1	1.22	5.16
2. Acetone–hexane	67.0	8.5	1.19	5.03
3. Acetone–hexane	52.7	14.5	1.20	5.07
4. Ethyl acetate–benzene	40.3	10.7	1.19	5.03
Allocholic acid[b]				
5. Acetone–hexane	27.8	6.3	1.23	5.03

[a] A portion (1.30 $\times$ 10^5 dpm, 33%) of the metabolite from fractions 80-1 and 80-2 (Fig. 2) was freed from 3β,7α,12α-trihydroxy-5α-cholanoic acid by partition chromatography (Fig. 3), methylated, and separated from methyl cholate by PLC; 102.4 mg of authentic methyl allocholate was added to the purified material.

[b] The fourth crop of crystals was hydrolyzed and submitted to partition chromatography (Fig. 4) to show coincident elution of ^{14}C and mass. Fractions 80-1 to 80-3 were combined for crystallization.

benzene–acetone (1:4) (19) to provide 2 mg of material containing 30% of the radioactivity used for the initial isotope dilution. This material was diluted with methyl allocholate and crystallized four times with little change in specific activity (Table 1). After hydrolysis and partition chromatography of the free acids, coincident elution of mass and radioactivity was observed (Fig. 4). Fractions 80-1 through 80-3 were combined and crystallized with no change in specific activity (Table 1). Allocholic acid accounted for 94% of the ^{14}C in the diluted isotopic sample. Although only 30% of the radio-

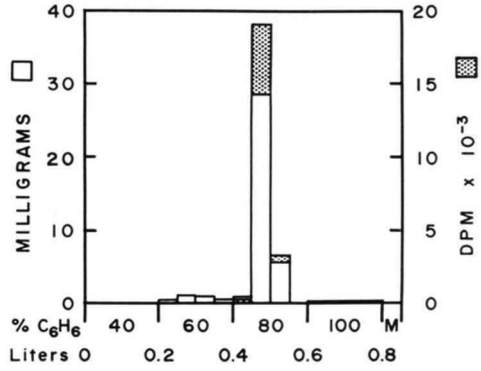

FIG. 4. Partition chromatography of the metabolite of [4-^{14}C]-cholestanol obtained from fractions 80-1 and 80-2 (Fig. 3) after methylation, isotope dilution with methyl allocholate (Table 1), PLC to remove methyl cholate, four crystallizations, and hydrolysis to the free acids. The coincident elution of radioactivity and mass is demonstrated. Bar heights and fraction *M* are defined in legend to Fig. 2.

activity from fractions 80-1 and 80-2 (Fig. 1) was recovered and diluted with allocholate, the unrecovered radioactivity probably had the same composition. Thus, allocholic acid is the principal acidic biliary metabolite in the gerbil.

Identification of the metabolite(s) in fractions 20-3 through 40-2 awaits the accumulation of sufficient material. It should be noted that allochenodeoxycholic acid was identified as a biliary metabolite of cholestanol in rats made hyperthyroid in order to produce more dihydroxy bile acids (14).

Distribution of ^{14}C

Because of the recovery of rather small amounts of the administered ^{14}C, tissues of gerbil B were assayed for radioactivity. The various tissues were removed, solubilized in Soluene (Nuclear-Chicago), and assayed in Bray's solution. Of the total ^{14}C recovered in these tissues, 1% appeared in brain, 14% in fat, 27% in liver, 34% in the intestine, 6% in blood, 2% in the spleen, 6% in kidney, 0.004% in the pituitary, 0.08% in the heart, and 10% in the adrenals. If the ^{14}C is present as the original sterol, the rather large amount present in the adrenals may be compared with the observation of Werbin and Chaikoff and coworkers (28, 29), who reported 10–12% of the sterols of guinea pig adrenals as cholestanol.

DISCUSSION

Roscoe and Fahrenbach (5) administered [4-^{14}C]-cholesterol intraperitoneally to male gerbils and observed excretion of 60% of the ^{14}C in 2 wk: 54% in feces and 6% in urine. 50% of the administered radioactivity was excreted by the 8th to 9th day in contrast to 4–5 days in the rat (30). Of the total radioactivity excreted by the gerbil, 90% appeared in feces and 10% in urine, in contrast to 1% excreted in rat urine. These results suggested a lower turnover rate of cholesterol in the gerbil and a greater need for the synthesis of steroid hormones from this sterol. The urinary excretion of 10% of the cholesterol label is similar to the 16% observed after labeled cholestanol.

The presence of small amounts of dihydroxy acids in gerbil bile, particularly chenodeoxycholic acid reported here, confirms a difference in sterol metabolism in this species compared with the rat. Subsequent to our preliminary report (15), Beher et al. (31) indicated by TLC the presence of "muricholic acids" and small amounts of chenodeoxycholic acid in the bile acid pool of gerbils as obtained from gall bladders, bile, and small intestines, but no comparisons with reference muricholates were reported. Analysis by GLC has failed in our hands to show the presence of either α- or β-muricholic acid. However, the short duration of bile flow suggests obstruction in the cannula or the bile duct, which may have resulted in urinary excretion of these derivatives (25, 27). The appearance in urine of 10% of the metabolites of cholesterol (5) and 16% from cholestanol in these experiments lends credence to this proposal. The half-life and turnover time of chenodeoxycholic acid was reported (31) to be shorter than cholic acid, but the gerbil has a relatively high rate of synthesis of cholic acid compared with the rat, which accounts for the predominance of cholic acid in this species. In their comparative studies on rodents, Beher et al. (31) reported urinary excretion of administered [24-^{14}C]cholic acid to be less than 7% in all cases but did not state the exact value for the gerbil.

From their experience with [4-^{14}C]cholesterol administered intraperitoneally, Roscoe and Fahrenbach (5) presented evidence that 92% of the material was absorbed within 4 hr and 95% within 24 hr. In our experiments with [4-^{14}C]cholestanol, approximately half of the ^{14}C was recovered in bile over the same period of time. The specific activity of the available [4-^{14}C]-cholestanol was such that about eight times as much ^{14}C was administered to gerbil A, as in the experiments of Roscoe and Fahrenbach with [4-^{14}C]cholesterol, with the objective of acquiring sufficient radioactivity in acidic biliary metabolites for identification. Because of the poor recovery in bile, positive identification was possible only with the trihydroxy acid obtained.

Identification of allocholic acid was undertaken by isotope dilution prior to the absolute identification of synthetic allocholic acid and its 3β-isomer (32). It is clear from these studies that the 3β- and 3α-isomers of these acids are distinctly separable by partition chromatography and that the metabolite is not the 3β-isomer. A subsequent paper will demonstrate that the third unidentified biliary metabolite of cholestanol previously reported in the rat (12) is the 3β-isomer of allocholic acid. Apparently, the enzyme systems of gerbil liver also differ from the rat in this respect, since no appreciable ^{14}C appeared in those chromatographic fractions associated with 3β,7α,12α-trihydroxy-5α-cholanoic acid.

The limited survey of residual ^{14}C retained by a gerbil following administration of [4-^{14}C]cholestanol suggests significance of the adrenal glands regarding storage of the sterol or its derivatives. Roscoe and Fahrenbach (5) have also referred to the significance of the adrenals based upon the relatively large amount of urinary ^{14}C (10%) from administered [4-^{14}C]cholesterol and upon the relative size of the adrenals of the gerbil compared with the rat. These differences may be related to the need of the gerbil to retain salts and water in its normally arid surroundings.

The results of the present study confirm a difference in sterol metabolism in the gerbil and in the rat. Within the limitations of these experiments (bile flow never longer than 7 days) the ratio of cholic to chenodeoxycholic acid was 23:1, compared with 4:1 in the rat on a comparable diet (33). Allocholic acid is identified as the major acidic biliary metabolite derived from cholestanol, and the adrenal gland is suggested as an important organ in this species as a site for sterol storage.

A portion of the material presented herein is taken from the dissertation submitted to the Graduate School of St. Louis University by Burton W. Noll in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1970.

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