# Age-related changes in cholesterol and bile acid metabolism in rats

**Kiyohisa Uchida, Yasuharu Nomura, Masumi Kadowaki, Haruto Takase, Kyoji Takano, and Nozomu Takeuchi**

Shionogi Research Laboratory, Fukushima-ku, Osaka 553; and Third Department of Internal Medicine, Osaka University Hospital,[1] Fukushima-ku, Osaka 553, Japan

**Abstract** Age-related changes in serum and liver cholesterol, phospholipid and triglyceride levels, serum lipoproteins, biliary secretion of cholesterol, phospholipid and bile acids, and fecal excretion of sterols and bile acids were examined in Sprague-Dawley and Wistar strain male rats, 7 to 106 weeks in age. Serum and liver lipid levels increased with age in both strains but the liver phospholipid level remained unchanged. The proportion of serum $\alpha$-lipoprotein increased and that of $\beta$- and pre$\beta$-lipoproteins slightly decreased. Cholesterol and phospholipids in low density lipoprotein and cholesterol in high density lipoprotein fractions also increased with age. Bile flow and biliary secretion of cholesterol and bile acids decreased in aged rats, but when they were expressed in terms of units per rat they were almost constant without regard to age. Pool size, synthesis, secretion, and turnover frequency of bile acids also did not change when they were expressed per rat, though 7-week-old rats showed a low value for turnover frequency. Biliary secretion of phospholipid, however, increased in aged rats. Biliary secretion of chenodeoxycholic and $\alpha$-muricholic acids decreased but that of cholic and hyodeoxycholic acids increased. Daily excretion of feces and fecal neutral sterols decreased in aged rats but the excretion of bile acids remained constant regardless of age. The ratio of coprostanol and cholesterol in the total sterols was not affected. Fecal lithocholic, $\beta$-muricholic and P10 (probably $\omega$-muricholic) acids were decreased with age but the other bile acid components were not changed or were slightly increased.

**Supplementary key words** aging · serum and liver lipids · serum lipoproteins · bile flow · biliary lipids · fecal sterols · fecal bile acids

Serum and tissue cholesterol levels increase with age in humans (1, 2) and animals (3–7). In relation to the changes in cholesterol metabolism of aged animals, many phenomena have been reported, such as a decrease in cholesterol synthesis (5, 8–12), a decrease of cholesterol turnover (13–15), a decrease in biliary and fecal excretion of cholesterol and bile acids (5, 15), a decrease in hepatic cholesterol 7$\alpha$-hydroxylase activity (16), a decrease in cholesterol absorption (5), and a decrease in the transport maximum of bile acid through the liver (17). Some of these changes can be used to explain hypercholesterolemia in aged rats but others, such as a decrease in cholesterol synthesis or absorption, cannot.

Dietary cholesterol (18–20) and bile acids (21, 22) have been reported to affect cholesterol synthesis and degradation to bile acids. The composition and amount of bile acids passing through the liver influences cholesterol synthesis and possibly bile acid synthesis. Peric-Golia and Socic (23), using developing sheep from 1 day to 12 months of age postulated that biliary chenodeoxycholic acid decreases while deoxycholic acid increases with age.

In the present experiments, we examined chronological changes in biliary and fecal excretion of cholesterol, phospholipid, and bile acids in male rats of various ages, in addition to the changes in serum and liver lipid levels.

## MATERIALS AND METHODS

### Animals and maintenance

Sprague-Dawley and Wistar strain male rats at various ages from 7 to 106 weeks were used. They were maintained in an air-conditioned room (25 ± 1°C, 50–60% humidity) with free access to chow diet (Japan CLEA CA-1 diet, Tokyo, Japan) and bottled water. The composition of the diet was as follows: protein 25.5%, lipids 4.0%, carbohydrate 53.5%, fibers 4.0%, ash 7.0%, and water 6.0%. The content of cholesterol was 0.04–0.05%. The diet also contained phytosterols such as campesterol and $\beta$-sitosterol but the amounts were far less than that of cholesterol.

Rats were individually caged for weeks before being killed and 2-day feces collections were made as described previously (20). Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the bile duct was cannulated with PE-10 polyethylene tubing

[1] Dr. Takeuchi.



Downloaded from www.jlr.org by guest, on June 19, 2012

to collect bile for 30 min. Next, blood was withdrawn by heart puncture and the liver was removed for lipid determination.

### Serum and liver lipid determination

Serum was separated by centrifugation at 3000 rpm for 15 min after the blood had stood for at least 30 min at room temperature. A portion of the largest lobus of the liver (*lobus sinistra externa*) was homogenized with 9 volumes of ice-chilled physiological saline utilizing an ULTRA-TURRAX Type TP 18-10 (IKE-WERK, Jank & Kunkel KG, West Germany).

Serum and liver homogenates were extracted in 10 volumes of ethanol by refluxing for 20 min at 90–95°C. Total cholesterol levels were determined with portions of the lipid extracts as previously reported (24, 25). Phospholipids were determined by the method of Gomori (26) and triglycerides by that of Hanahan and Olley (27) or Fletcher (28) after isopropanol extraction.

### Biliary lipid determination

Bile was extracted with ethanol; one volume of bile was poured into 10–20 volumes of ethanol, boiled for several minutes, and filtered through Toyo Filter Paper No. 2 (Toyo Roshi Co. Ltd., Tokyo, Japan) after cooling to room temperature. An aliquot of the filtrate was evaporated to dryness under a stream of nitrogen, and the residue was hydrolyzed in 2–4 ml of 1.25 N sodium hydroxide solution for 6 hr at 120°C. Sterols were extracted with diethyl ether; bile acids were then extracted after acidification with 2 N hydrochloric acid solution (29). Sterols were determined spectrophotometrically (24, 25). Bile acids were methylated with freshly prepared diazomethane and then trifluoroacetylated with trifluoroacetic anhydride. The bile acid derivatives were quantified by gas–liquid chromatography utilizing a Shimadzu gas chromatograph Model GC-4BPF (Shimadzu Co. Ltd., Kyoto, Japan) equipped with hydrogen flame ionization detector (29). A 1.5 m × 4 mm ID glass column packed with 3% QF-1 on 60–80 mesh Chromosorb W was used. The operation temperature was 235°C for the column and 290°C for the detector. When sterols were analyzed by gas–liquid chromatography, a column packed with 1.5% SE-30 on 60–80 mesh Chromosorb W was used at 230°C. Biliary phospholipid was determined by the method of Gomori (26).

### Fecal lipid determination

Fecal sterols and bile acids were determined as reported previously (20). Briefly, dried and powdered feces were extracted with absolute ethanol and hydrolyzed in sodium hydroxide solution at 120°C under pressure; the sterols and bile acids were then quantified by gas–liquid chromatography. Fecal sterols comprised cholesterol, coprostanol, $\Delta^7$-cholestenol and phytosterols such as campesterol, $\beta$-sitosterol and their metabolites (20). In the present experiment, $\Delta^7$-cholestenol and phytosterols were not included in the fecal sterols. $\Delta^7$-Cholestenol was small in amount, and changed almost in parallel with cholesterol; the origin of this compound was obscure. Phytosterols probably came from the diet, and interconversion between cholesterol and phytosterol was unlikely.

Acids designated as P8 and P10 were not identified but seemed to correspond to hyocholic and $\omega$-muricholic acids, respectively, on the basis of analysis by gas–liquid chromatography and gas–liquid chromatography–mass spectrometry.

### Pool size, synthesis, secretion, and turnover frequency of bile acids

Pool size, synthesis, secretion, and turnover frequency of bile acids were estimated by making a total bile fistula and draining out all the bile salts in the enterohepatic circulation, as described by Mok, Perry, and Dowling (30). Turnover frequency was calculated by dividing the cumulative amount of bile acids being secreted during 24 hr at the initial secretion rate by pool size (30).

### Serum lipoprotein and lipoprotein lipid analysis

Polyacrylamide gel electrophoresis of serum lipoproteins was performed by the method of Fings, Foster, and Cohen (31) with a slight modification (32).

Ultracentrifugation was performed according to the method described by Havel, Eder, and Bragdon (33) and Bragdon, Havel, and Boyle (34), with separation into four fractions; chylomicrons ($d < 1.006$ g/ml), very low density lipoprotein (VLDL, d 1.006–1.019 g/ml), low density lipoprotein (LDL, d 1.019–1.063 g/ml) and high density lipoprotein (HDL, $d > 1.063$ g/ml). Each fraction was extracted with chloroform–methanol 2:1 according to the method of Folch, Lees, and Sloane Stanley (35) for the determination of lipid levels.

## RESULTS

### Serum and liver lipids

Changes in serum and liver lipid levels are given in **Table 1.** Serum cholesterol level gradually increased from 70 to 80 mg/dl at 8–23 weeks to 130–150 mg/dl at 63–102 weeks. The serum phospholipid level changed almost in parallel with the serum cholesterol level, but the increase was less than that of cholesterol,



Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 1. Serum and liver lipid levels in male rats of different ages

| | Sprague-Dawley Rats | | | | | Wistar Rats | |
|---|---|---|---|---|---|---|---|
| | 8 wk | 10–11 wk | 17–23 wk | 48–51 wk | 63–69 wk | 17–23 wk | 100–102 wk |
| No. of rats | 4 | 9 | 10 | 7 | 12 | 10 | 4 |
| Body weight (g) | 276 ± 15.7[a] | 374 ± 3.9[b] | 414 ± 12.9[b] | 739 ± 17.0[b] | 751 ± 29.5[b] | 423 ± 9.8[a] | 545 ± 31.6 |
| Serum cholesterol (mg/100 ml) | 67 ± 3.3 | 70 ± 3.2 | 73 ± 4.3 | 123 ± 10.2[b] | 131 ± 9.2[b] | 82 ± 5.7 | 147 ± 7.2[c] |
| phospholipid (mg/100 ml) | 126 ± 9.5 | 127 ± 5.0 | 130 ± 4.7 | 179 ± 11.7[b] | 185 ± 6.7[b] | 150 ± 4.9 | 192 ± 11.5[c] |
| triglyceride (mg/100 ml) | 95 ± 23.9 | 113 ± 13.5 | 145 ± 14.0 | 172 ± 16.4[b] | 149 ± 18.6 | 127 ± 17.6 | 94 ± 21.0 |
| C/P ratio[d] | 0.54 ± 0.017 | 0.55 ± 0.018 | 0.55 ± 0.016 | 0.68 ± 0.043[b] | 0.71 ± 0.035[b] | 0.54 ± 0.024 | 0.77 ± 0.047[c] |
| Liver weight (g/100 g BW) | 4.77 ± 0.168 | 4.18 ± 0.115[b] | 4.01 ± 0.092[b] | 3.13 ± 0.111[b] | 3.06 ± 0.099[b] | 3.42 ± 0.110 | 2.75 ± 0.151[c] |
| cholesterol (mg/g) | 4.00 ± 0.203 | 3.78 ± 0.241 | 4.14 ± 0.098 | 6.27 ± 0.310[b] | 6.12 ± 0.383[b] | 3.72 ± 0.267 | 4.35 ± 0.095[c] |
| phospholipid (mg/g) | 42.9 ± 0.38 | 41.4 ± 1.06 | 42.4 ± 0.68 | 43.4 ± 0.97 | 42.4 ± 1.14 | 43.5 ± 0.81 | 38.1 ± 1.29[c] |
| triglyceride (mg/g) | 6.8 ± 1.10 | 11.8 ± 0.73[b] | 14.6 ± 1.84[b] | 33.2 ± 3.92[b] | 27.9 ± 3.13[b] | 13.5 ± 2.08 | 10.9 ± 1.32 |

[a] Mean ± SE.
[b] Statistically significant against 8-week-old rats ($P < 0.05$).
[c] Statistically significant against 17–23 week-old rats ($P < 0.05$).
[d] C/P ratio: cholesterol/phospholipid ratio.

resulting in a gradual increase in the cholesterol–phospholipid ratio. The triglyceride level increased rapidly with age; it attained the highest level during the period of 48 to 51 weeks, and then slightly decreased. The decrease of triglyceride in aged Wistar rats was partially due to the decrease of diet intake, since aged rats often refused the diet when in the individual cages, although they had been housed for several weeks in the same cages.

The liver weight also increased with age, but proportionately less than the increase in body weight. Thus the liver weight per unit body weight decreased with age. Liver cholesterol and triglyceride levels also increased with age, but the phospholipid level remained almost constant. In aged Wistar rats about 2 years old, a decrease was found in the liver phospholipid concentration.

No significant difference was found between the Sprague-Dawley and Wistar strains. Serum cholesterol and phospholipid levels were slightly higher in Wistar rats but liver weight and liver cholesterol level were higher in Sprague-Dawley rats.

TABLE 2. Serum lipoproteins in rats of different ages

| | Age (wk) | No. of Rats | Serum Lipoprotein[a] (%) α | β | preβ |
|---|---|---|---|---|---|
| Aburahi[c] | 8–10 | 9 | 55 ± 3.1[b] | 27 ± 2.1 | 18 ± 3.9 |
| Wistar | 13–15 | 23 | 62 ± 1.4 | 23 ± 0.9 | 15 ± 1.2 |
| male | 20–32 | 11 | 64 ± 2.2 | 21 ± 2.9 | 15 ± 2.4 |
| | 48–68 | 8 | 74 ± 1.5[e] | 17 ± 1.4[e] | 9 ± 1.9 |
| JCL[d] | 3–4 | 2 | 58 | 26 | 16 |
| Wistar | 11–13 | 14 | 64 ± 2.1 | 15 ± 0.9 | 22 ± 2.0 |
| male | 64 | 4 | 77 ± 1.0 | 16 ± 0.9 | 8 ± 0.5 |
| JCL[d] | | | | | |
| Sprague- | 9–11 | 38 | 55 ± 1.2 | 18 ± 0.8 | 27 ± 1.4 |
| Dawley | 52–63 | 11 | 62 ± 3.2 | 15 ± 1.7 | 24 ± 2.3 |

[a] The ratios of serum lipoproteins were analyzed by polyacrylamide gel electrophoresis (31).
[b] Mean ± SE.
[c] Rats bred in our laboratory.
[d] Rats obtained from Japan CLEA Co. (Tokyo, Japan).
[e] Statistically significant against 8–10 week-old rats ($P < 0.05$).

## Serum lipoproteins and lipoprotein lipids

**Table 2** shows the changes of serum lipoproteins with age, as judged by polyacrylamide gel electrophoresis. The proportion of α-lipoprotein increased with age but that of β- and preβ-lipoproteins slightly decreased.

Serum samples from rats of various ages (4–99 weeks) were separated into chylomicron, VLDL, LDL, and HDL fractions by ultracentrifugation, and the cholesterol, phospholipid, and triglyceride levels in these fractions were determined (**Table 3**). Cholesterol and phospholipid levels in chylomicron and VLDL were similar for all ages but those in LDL and cholesterol in HDL were significantly higher in aged rats.

When the patterns in polyacrylamide gel electrophoresis of VLDL, LDL, and HDL fractions obtained by ultracentrifugation were examined, VLDL and HDL gave a single peak corresponding to preβ- and α-lipoproteins, respectively, but LDL showed two peaks corresponding to β- and preβ-lipoproteins. Chylomicrons did not migrate under the present experimental conditions (32).



Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 3. Serum lipoprotein lipids in Wistar male rats of different ages

| | No. of Rats | Chylo | VLDL | LDL | HDL | Total |
|---|---|---|---|---|---|---|
| | | | | *μg/ml serum* | | |
| Total cholesterol | | | | | | |
| 4 wk | 2 | 34 | 103 | 208 | 464 | 808 |
| 11–16 wk | 6 | 14 ± 4.1[a] | 194 ± 21.2 | 113 ± 42.6 | 560 ± 93.4 | 881 ± 114.0 |
| 63–99 wk | 11 | 12 ± 2.3 | 140 ± 12.3 | 351 ± 23.8 | 643 ± 45.1 | 1146 ± 69.8 |
| Phospholipid | | | | | | |
| 4 wk | 2 | 87 | 149 | 183 | 1054 | 1472 |
| 11–16 wk | 6 | 55 ± 10.0 | 354 ± 44.0 | 120 ± 31.8 | 1140 ± 46.9 | 1655 ± 109.6 |
| 63–99 wk | 11 | 48 ± 9.7 | 239 ± 30.8 | 350 ± 37.8 | 1213 ± 68.4 | 1849 ± 92.1 |
| Triglyceride | | | | | | |
| 4 wk | 2 | 46 | 258 | 59 | 58 | 422 |
| 11–16 wk | 6 | 87 ± 18.0 | 793 ± 72.1 | 40 ± 8.1 | 68 ± 26.3 | 989 ± 90.0 |
| 63–99 wk | 11 | 24 ± 5.9 | 378 ± 40.4 | 50 ± 6.4 | 25 ± 3.3 | 477 ± 48.1 |

[a] Means ± SE.

## Biliary cholesterol and bile acids

**Table 4** shows the changes in bile flow and biliary secretion of cholesterol and bile acids. These values were apparently decreased in aged rats but when they were expressed in terms of per rat instead of per kg body weight, such an age-dependent change was not found. They remained almost constant without regard to the increase of body weight.

When the bile acid composition was examined, a striking age-dependent change was found. Chenodeoxycholic and α-muricholic acids decreased, while cholic acid slightly increased. Deoxycholic acid decreased but lithocholic and hyodeoxycholic acids remained unchanged. In aged Wistar rats, the decrease of chenodeoxycholic acid was significant but an increase of cholic acid was not found. In these rats, there was a marked increase in hyodeoxycholic acid and another unidentified bile acid, probably hyocholic acid (P8), indicating that secretion of secondary bile acids increased in these 2-year-old rats.

TABLE 4. Biliary secretion of sterols and bile acids in male rats of different ages

| | Sprague-Dawley Rats | | | | | Wistar Rats | |
|---|---|---|---|---|---|---|---|
| | 8 wk | 10–11 wk | 17–23 wk | 48–51 wk | 63–69 wk | 17–23 wk | 100–102 wk |
| No. of rats | 4 | 9 | 10 | 7 | 12 | 10 | 4 |
| Bile flow (ml/hr/kg) | 5.06 ± 0.044[a] | 3.92 ± 0.152[b] | 3.63 ± 0.115[b] | 2.23 ± 0.138[b] | 2.17 ± 0.140[b] | 3.22 ± 0.137 | 2.30 ± 0.170[c] |
| Cholesterol (mg/hr/kg) | 1.02 ± 0.151 | 0.91 ± 0.081 | 0.93 ± 0.054 | 0.38 ± 0.022[b] | 0.38 ± 0.029[b] | 0.55 ± 0.099 | 0.46 ± 0.036 |
| Bile acids (mg/hr/kg) | 36.9 ± 8.12 | 31.4 ± 2.59 | 38.0 ± 4.19 | 15.3 ± 1.06[b] | 13.9 ± 1.11[b] | 24.5 ± 2.07 | 19.3 ± 2.66 |
| Bile flow (ml/hr/rat) | 1.53 ± 0.183 | 1.47 ± 0.064 | 1.50 ± 0.052 | 1.63 ± 0.111 | 1.61 ± 0.095 | 1.40 ± 0.084 | 1.41 ± 0.037 |
| Cholesterol (mg/hr/rat) | 0.35 ± 0.083 | 0.34 ± 0.034 | 0.34 ± 0.043 | 0.28 ± 0.017 | 0.29 ± 0.030 | 0.23 ± 0.014 | 0.28 ± 0.011 |
| Bile acids (mg/hr/rat) | 9.58 ± 1.337 | 11.71 ± 0.928 | 15.31 ± 1.432[b] | 11.18 ± 0.756 | 10.35 ± 0.776 | 10.30 ± 0.935 | 10.47 ± 1.589 |
| Bile acid composition (%) | | | | | | | |
| 3α | 0.5 ± 0.20 | 0.5 ± 0.04 | 0.3 ± 0.04 | 0.3 ± 0.06 | 0.3 ± 0.04 | 0.4 ± 0.09 | 0.6 ± 0.25 |
| 3α12α | 3.1 ± 1.11 | 3.6 ± 0.49 | 2.7 ± 0.23 | 2.6 ± 0.71 | 2.1 ± 0.19 | 2.8 ± 0.19 | 2.2 ± 0.38 |
| 3α6β7α | 4.9 ± 0.80 | 3.3 ± 0.43 | 1.6 ± 0.15[b] | 3.0 ± 0.43 | 2.0 ± 0.30[b] | 3.1 ± 0.20 | 2.6 ± 0.47 |
| 3α7α | 7.4 ± 0.97 | 7.1 ± 0.50 | 4.2 ± 0.19[b] | 4.3 ± 0.38[b] | 3.6 ± 0.36[b] | 7.5 ± 0.68 | 4.6 ± 1.19 |
| 3α6α | 3.9 ± 1.11 | 4.2 ± 0.73 | 4.0 ± 0.35 | 4.6 ± 0.40 | 4.5 ± 0.47 | 3.6 ± 0.33 | 8.4 ± 1.01[c] |
| 3α7α12α | 58.2 ± 6.00 | 68.3 ± 2.23 | 78.3 ± 1.48[b] | 69.4 ± 3.25 | 70.7 ± 0.29 | 65.3 ± 1.77 | 53.6 ± 1.36 |
| P8[d] | 12.5 ± 3.04 | 7.4 ± 1.77 | 3.3 ± 0.78[b] | 9.4 ± 1.77 | 8.5 ± 0.22 | 10.5 ± 0.65 | 14.7 ± 3.64 |
| Others[e] | 8.3 ± 1.48 | 5.6 ± 0.70 | 6.6 ± 1.08 | 6.3 ± 1.08 | 8.3 ± 0.95 | 7.1 ± 0.58 | 10.2 ± 0.93 |

[a] Mean ± SE.
[b] Statistically significant against 8-week old rats ($P < 0.05$).
[c] Statistically significant against 17–23-week old rats ($P < 0.05$).
[d] P8, unidentified peak in gas–liquid chromatogram, but presumably hyocholic acid.
[e] Others comprises unidentified peaks other than P8, and keto bile acids.



Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 5. Diet intake and fecal excretion of sterols and bile acids in male rats of different ages

| | Sprague-Dawley Rats | | | Wistar Rats | |
|---|---|---|---|---|---|
| | 10–11 wk | 17–23 wk | 63–69 wk | 17–23 wk | 100–102 wk |
| No. of rats | 9 | 6 | 4 | 5 | 4 |
| Diet intake (g/day/rat) | 25 ± 0.6[a] | 20 ± 0.7[b] | 21 ± 1.0[b] | 23 ± 1.0 | 11 ± 1.9[c] |
| No. of rats | 9 | 10 | 12 | 10 | 4 |
| Feces wt. (g/day/rat) | 6.00 ± 0.199[a] | 4.66 ± 0.179 | 3.92 ± 0.262[b] | 5.58 ± 0.243 | 3.56 ± 0.263[c] |
| Total sterols | 15.35 ± 1.285 | 11.00 ± 0.976[b] | 9.73 ± 0.660[b] | 11.79 ± 2.991 | 9.87 ± 1.496 |
| Coprostanol | 7.29 ± 0.661 | 5.66 ± 0.538 | 4.54 ± 0.368[b] | 4.78 ± 1.200 | 4.72 ± 0.630 |
| Cholesterol | 8.06 ± 0.877 | 5.34 ± 0.496[b] | 5.20 ± 0.413[b] | 7.01 ± 1.848 | 5.15 ± 0.881 |
| Total bile acids | 13.58 ± 0.968 | 12.05 ± 0.495 | 13.50 ± 0.447 | 9.10 ± 0.614 | 12.65 ± 1.761 |
| 3α | 1.53 ± 0.125 | 0.84 ± 0.076[b] | 0.82 ± 0.181[b] | 0.83 ± 0.096 | 1.30 ± 0.282 |
| 3α12α | 4.02 ± 0.340 | 3.42 ± 0.201 | 4.25 ± 0.507 | 2.36 ± 0.187 | 2.20 ± 0.373 |
| 3α6β7α | 0.22 ± 0.041 | 0.22 ± 0.023 | 0.22 ± 0.035 | 0.07 ± 0.032 | 0.13 ± 0.039 |
| 3α6α | 3.30 ± 0.574 | 3.71 ± 0.322 | 4.35 ± 0.532 | 3.38 ± 0.274 | 5.09 ± 0.999 |
| 3α6β7β | 1.84 ± 0.258 | 0.76 ± 0.158[b] | 0.88 ± 0.232[b] | 0.64 ± 0.135 | 0.65 ± 0.187 |
| P10[d] | 2.53 ± 0.341 | 2.27 ± 0.191 | 1.59 ± 0.221[b] | 1.75 ± 0.277 | 0.81 ± 0.135[c] |
| Others[e] | 0.53 ± 0.081 | 0.65 ± 0.113 | 0.35 ± 0.057 | 0.63 ± 0.070 | 2.47 ± 0.354[c] |

[a] Mean ± SE.
[b] Statistically significant against 10–11-week old rats ($P < 0.05$).
[c] Statistically significant against 17–23-week old rats ($P < 0.05$).
[d] P10, unidentified peak in gas–liquid chromatogram, but presumably ω-muricholic acid.
[e] Others comprises unidentified peaks other than P10, and keto bile acids.

## Fecal excretion of sterols and bile acids

Fecal excretion of sterols and bile acids in rats of various ages is shown in **Table 5.** Daily excretion of feces and total sterols decreased with age, but the excretion of total bile acids remained rather constant regardless of age. The ratios of coprostanol and cholesterol in the total sterols were not appreciably affected by age. In regard to the bile acid composition, aged Sprague-Dawley rats showed a decrease in lithocholic, β-muricholic, and P10 acids. The P10 acid was probably ω-muricholic acid. However, such changes in bile acid composition were not clearly found in Wistar rats. An increase in ketonic bile acids was observed in aged Wistar rats.

Preliminary data on diet and water intake of our rats (36) indicated that the daily amounts consumed by a rat were almost constant through life after 2–3 months of age (**Fig. 1**). In the present experiment, the diet intake was not obtained in all of the rats used but the data showed that it was decreased in aged rats. The aged Sprague-Dawley rats consumed daily about 20 g of diet while the youngest rats ate 25 g, but no significant difference was found in rats 17–23 weeks old and 63–69 weeks old. The diet intake of aged Wistar rats was extremely low. These rats refused to take enough food when housed individually in metabolic cages. Therefore, the experiments on the oldest rats were performed only in a state of reduced diet intake. The amount of feces was decreased but the levels of sterols and bile acids were comparable with those of the younger Wistar rats.

High values for the amount of feces and fecal sterols in the youngest Sprague-Dawley rats were probably due to a large intake of diet. In these rats, fecal bile acids were not increased and levels were comparable in value with those of the aged rats.

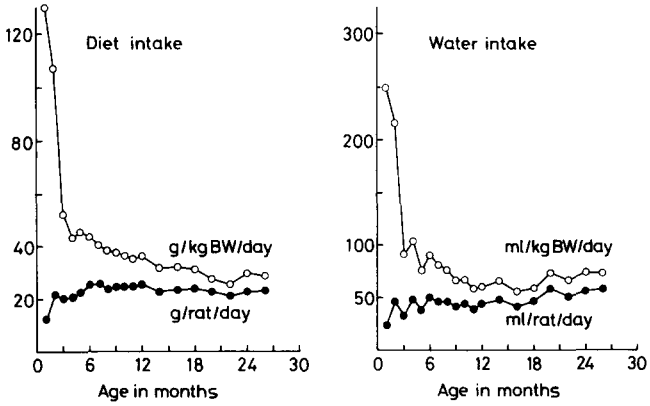


**Fig. 1.** Changes in diet and water intake in Sprague-Dawley male rats (36).

## Changes of liver weight and bile flow in relation to body weight

**Fig. 2** shows the relationships between body weight and liver weight, and between body weight and bile flow. Individual values from the present experiments along with values from other unpublished experiments[2] are shown. Liver weight slightly increased with age but to a far less extent than body weight; twice the increase in body weight corresponded to

[2] Uchida, K., M. Kadowaki, Y. Nomura, and H. Takase, unpublished data.



Downloaded from www.jlr.org by guest, on June 19, 2012

about a 50% increase in liver weight. Bile flow seemed to increase with age when rats were young but remained almost constant in rats weighing 300 g or more (2–3 months old).

### Pool size, secretion rate, synthesis, and turnover frequency of bile acids, and secretion rates of cholesterol and phospholipids

Pool size, secretion rate, synthesis, and turnover frequency of bile acids, and secretion rates of cholesterol and phospholipids in rats of various ages were determined by the washout method and are shown in **Table 6.** Although the youngest rats showed a low value for bile acid turnover frequency, the pool size, synthesis, secretion, and turnover frequency remained almost constant without regard to age when they were expressed as per rat instead of per 100 g body weight. The secretion of bile acids, cholesterol, and phospholipid in the 7-week-old rats was lower than that of the others but the differences were statistically insignificant ($P > 0.05$). On the other hand, the secretion of cholesterol in the oldest rats was significantly higher than that in the others. Biliary secretion of cholesterol and phospholipid gradually increased with age.

## DISCUSSION

The present experiments demonstrated that serum and liver lipid levels, except liver phospholipid, increase with age in both Sprague-Dawley and Wistar rats. The changes are in good agreement with previous reports (3–7).

In contrast to the serum and liver lipid levels, biliary and fecal excretion rates of bile acids were unaffected by age, when expressed in terms of the amount of daily excretion per rat, mg/rat (Table 4). This was confirmed by the washout experiment (Table 6). Rats

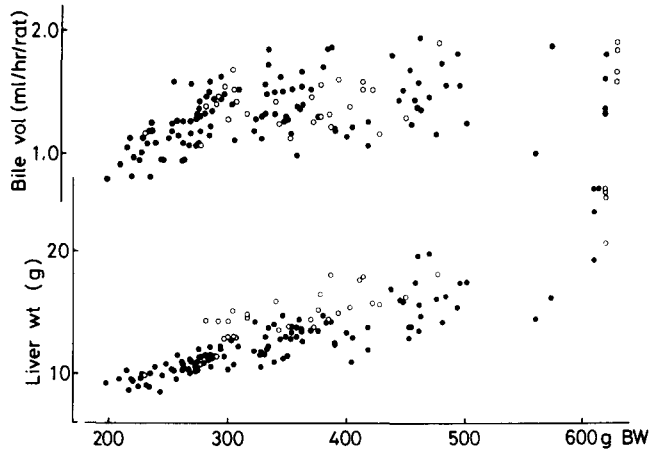


**Fig. 2.** Relationships between liver weight and body weight, and between bile flow and body weight. ○, Sprague-Dawley rats; ●, Wistar rats.

of various ages showed similar values for pool size, secretion, synthesis, and turnover frequency of bile acids. The procedures of evaluating these components of enterohepatic circulation are clearly explained by Mok, Perry, and Dowling (30). The rates of bile acid synthesis obtained by the washout method were higher than those expected from the values of fecal excretion. Fecal excretion of bile acids is considered to represent the degree of bile acid synthesis. The daily values obtained by the washout method were about 18 mg/rat (Table 6) but those from the fecal excretion were around 13 mg/rat (Table 5). We obtained low-point values that correspond to the values of hepatic synthesis 15–18 hr after bile fistula in most of the animals, but Mok et al. (30) have indicated that the low point occurs within 10 hr after cannulation. Therefore, the hepatic synthesis of bile acids in our washout experiment may have been elevated to some extent from the basal level, since bile acid inhibits bile acid synthesis by a negative feedback mechanism

TABLE 6. Effect of age on bile acid metabolism in rats (washout method)[a]

| | 7 wk | 22–23 wk | 60–61 wk | 104–106 wk |
|---|---|---|---|---|
| No. of rats | 3 | 4 | 4 | 4 |
| Body weight (g) | 204 ± 3.1[b] | 419 ± 20.3 | 607 ± 21.1 | 684 ± 42.1 |
| Pool size (mg) | 44.17 ± 3.041 | 45.42 ± 4.298 | 45.89 ± 2.891 | 42.56 ± 4.426 |
| Synthesis (mg/day) | 18.08 ± 1.162 | 19.92 ± 3.261 | 23.22 ± 3.322 | 15.60 ± 2.113 |
| Secretion (mg/hr) | 7.87 ± 0.965 | 13.21 ± 1.645 | 12.18 ± 1.953 | 14.69 ± 3.332 |
| Turnover frequency[c] | 4.4 ± 0.78[d] | 7.4 ± 1.59 | 6.6 ± 1.36 | 8.0 ± 1.03 |
| Cholesterol (μg/hr) | 137 ± 4.3[d] | 175 ± 12.5[d] | 198 ± 19.0[d] | 268 ± 10.7[e] |
| Phospholipid (mg/hr) | 4.06 ± 0.591[d] | 4.32 ± 0.202[d] | 6.26 ± 0.797 | 7.82 ± 0.613 |

[a] The values were determined by the washout method (30).
[b] Mean ± SE.
[c] Turnover frequency was calculated by dividing the cumulative amount of bile acids being secreted during 24 hrs at the initial secretion rate by pool size.
[d] Statistically significant against 104–106-week old rats ($P < 0.05$).
[e] Statistically significant against the other groups ($P < 0.05$).



Downloaded from www.jlr.org by guest, on June 19, 2012

and withdrawal of bile acid from the enterohepatic circulation results in an increase of hepatic synthesis (37). The present fecal bile acid values were close to those reported by Grundy, Ahrens, and Miettinen (38).

Biliary bile acid composition, however, changed with age. The most pronounced change was a decrease of chenodeoxycholic acid. Cholic and chenodeoxycholic acids are both primary bile acids but they have very different biological effects. Absorption of cholesterol is known to be enhanced in the presence of bile acids. Cholic acid administered with cholesterol results in elevated serum and liver cholesterol levels but chenodeoxycholic acid has almost no effect (39–41). Similarly, deoxycholic acid, which is derived from cholic acid, causes accumulation of cholesterol, but secondary bile acids from chenodeoxycholic acid, such as lithocholic and hyodeoxycholic acids, do not increase the serum and liver cholesterol levels (39, 40). Howe and Hutchison (42) and Beher, Baker, and Penney (43) have also suggested that the effect of bile acid on serum and tissue cholesterol levels differs with the structure of the bile acid. Conceivably, the decrease of chenodeoxycholic acid is a factor causing hypercholesterolemia in aged rats.

In our experiments (39, 40) we used free bile acids. Reicht, Cohen, and Mosbach (41) used taurine-conjugated bile acids and obtained a similar result; taurocholate produced hypercholesterolemia but taurochenodeoxycholate did not. However, Gallo-Torres, Miller, and Hamilton (44) have mentioned that taurochenodeoxycholate is as effective as taurocholate in cholesterol absorption, while glycine-conjugated and free bile acids are less effective. These data suggest that the conjugation of the bile acids is as important as their chemical structure. If taurine-conjugated chenodeoxycholate is as effective as taurocholate in cholesterol absorption, our proposal that the decrease of chenodeoxycholic acid in aged rats enhances cholesterol absorption, which results in hypercholesterolemia, may not be plausible.

On the other hand, there is apparent evidence indicating that cholic acid is more easily absorbed than chenodeoxycholic acid and the latter is more easily excreted in feces. When biliary and fecal excretion of bile acids were examined after feeding of exogenous cholic and chenodeoxycholic acid for 1 week, the fecal excretion rates were similar to each other but the biliary excretion of cholic acid was greater than that of chenodeoxycholic acid (39, 45). When similar comparisons were done in rats without feeding exogenous bile acids, the ratio of chenodeoxycholic acid to cholic acid (CDCA/CA) in the bile was about 0.25–0.33 but the ratio in the feces was >1 (39, 45). The half-life of chenodeoxycholic acid is shorter than that of cholic acid in rats (46). Based on the above, we postulate that, as a result of a decrease of chenodeoxycholic acid in aged rats, total excretion of cholesterol is delayed, resulting in hypercholesterolemia.

In regard to fecal bile acids, lithocholic, β-muricholic and P10 (probably ω-muricholic) acids were decreased in aged Sprague-Dawley rats. This decrease corresponded well with the decrease of chenodeoxycholic acid in the bile. However, such changes were not found in Wistar rats. The aged Wistar rats showed an increase of keto bile acids included in the term "other" (Tables 4 and 5). It is not known whether such a species difference is real, but a decrease of diet intake in the aged Wistar rats may be involved.

Fasting reduces biliary secretion of bile acids and results in an increase of deoxycholic acid in rats (47). The restriction of diet intake to about 70% for a year also reduced biliary and fecal excretion of bile acids, but, in this case, no remarkable change was found in the bile acid composition, except a decrease of hyodeoxycholic acid in the feces.[3] Therefore, since the pattern of bile acid composition was different between aged rats and fasted or diet-restricted rats, the changes found in the present experiment were not merely due to the decrease of food.

Biliary and fecal excretion of bile acids and the components of enterohepatic circulation such as pool size, secretion, synthesis, and turnover frequency remained almost constant, except in the youngest rats, when they were expressed in terms of the values per rat. The turnover frequency in the rats of 7 weeks of age was lower than that of the oldest rats.

Body weight usually increases with age in rats. Weights of adipose tissues, liver, kidneys, spleen, heart, and digestive tissues increase as body weight increases, but most of the endocrine and related organs such as testes, pituitary, adrenals, ventral prostate, and seminal vesicles remained constant in mature rats over about 6 months old.

The increase in adipose tissue nearly paralleled that of body weight, but increases of liver and kidney weights were proportionately less than the increase in body weight. Therefore, the relative weight of the adipose tissue was unchanged but those of the liver, kidneys, or endocrine organs decreased with age. Thus, if a function of the liver is corrected for body weight, the function may seem to be depressed in aged rats. In addition, if the function is under the influence of endocrine organs, it may be further decreased.

[3] Uchida, K., M. Kadowaki, Y. Nomura, H. Takase, and H. Sakai, unpublished data.



Downloaded from www.jlr.org by guest, on June 19, 2012

In the present experiments, bile acid metabolism remained unchanged in aged rats, when it was expressed as per rat, though liver weight increased with age (Fig. 2). These data suggest that the liver function in regard to bile acid metabolism is constant as a whole but decreases with age. The participation of endocrine organs such as the thyroid, gonad, or adrenals is also conceivable.

In the steady state, the fecal excretion of bile acids is presumed to correspond to the hepatic synthetic rate of bile acids. Thus, we concluded from the present experiment that the net amount of bile acid does not decrease with age. However, this does not agree with the previous reports. Yamamoto and Yamamura (5) and Hrůza and Zbukorá (15) have shown that the fecal excretion of intraperitoneally or intravenously injected [$^{14}$C]- or [$^{3}$H]cholesterol was decreased in aged rats. Hrůza and his coworkers (13–15) have shown a decrease of cholesterol turnover. Story and Kritchevsky (16) have reported the decrease of cholesterol 7$\alpha$-hydroxylase activity in aged rats. These reports indicate a decrease of hepatic activity of bile acid synthesis in aged rats.

In our experiment, liver weight slightly but continuously increased with the increase of body weight, while the bile flow remained rather constant, especially in rats weighing more than 300 g (Fig. 2). This suggests that the total liver function remains unchanged but the unit activity decreases with age.

Thus, we concluded that the metabolic turnover of cholesterol decreases in aged rats but the net amount of excretion of bile acids remains constant, as shown in the present experiments. As often observed in metabolic events, individual rates generally decrease in aged rats while overall levels remain unchanged. Cholesterol and bile acid metabolism are examples of such phenomena. In in vivo experiments using the tracer technique, many factors such as absorption, transport in blood and body fluid, or membrane passage may affect the results in addition to the metabolic conversion rates. Therefore, such factors can be responsible for the depression of bile acid formation in aged rats. In any case, the decrease in the metabolic turnover ratio is postulated as a cause for age-related hypercholesterolemia.

Decreases in cholesterol synthesis (5, 8–12) and cholesterol absorption (5) have been reported in aged rats, but their actual participation in regulating serum cholesterol levels has not been estimated.

When estradiol was subcutaneously injected for weeks into male rats, hepatic cholesterol synthesis and serum cholesterol levels increased and showed a significant positive correlation (48). From this, an increase of hepatic cholesterol synthesis was concluded to increase the serum cholesterol level, but when hepatic cholesterol synthesis was inhibited by feeding cholesterol or sodium cholate, no reduction in serum cholesterol level was found in these estradiol-treated rats (49). Thus, cholesterol synthesis in the liver does not seem to be the sole factor regulating the serum cholesterol level. Serum lipoproteins seem to be another and probably more important factor; this has not been fully analyzed yet.

Kitani et al. (17) have shown that when bile salts are infused the transport maximum (Tm) of bile salts through the liver is deceased in aged rats. However, the secretion rate of bile acids in rats in the present experiment was 10–15 mg/hr per rat, which is lower than the Tm value of old rats (0.58 $\mu$mol/min per 100 g body weight). This suggests that the decrease of Tm cannot be a factor affecting the bile acid clearance time.

We thank Mr. Y. Muraoka in our laboratory for kindly supplying the data on diet and water intake by rats from the same colony as that we used.

*Manuscript received 16 May 1977; accepted 16 December 1977.*

## REFERENCES

1. Adlersberg, D., L. E. Schaefer, A. G. Steinberg, and C. I. Wang. 1956. Age, sex, serum lipids, and coronary atherosclerosis. *J. Amer. Med. Assoc.* **162:** 619–622.
2. Hallberg, L., A. M. Högdahl, A. Svanborg, and O. Vikrot. 1966. Plasma lipids in women. Variation in cholesterol, phospholipids and triglycerides at different ages in a random population sample. *Acta Med. Scand.* **180:** 697–707.
3. Carlson, L. A., S. O. Fröberg, and E. R. Nye. 1968. Effect of age on blood and tissue lipid levels in the male rat. *Gerontologia* **14:** 65–79.
4. Kritchevsky, D., and S. A. Tepper. 1964. Serum cholesterol levels of inbred rats. *Amer. J. Physiol.* **207:** 631–633.
5. Yamamoto, M., and Y. Yamamura. 1971. Changes of cholesterol metabolism in the ageing rat. *Atherosclerosis* **13:** 365–374.
6. Dupont, J., M. M. Mathias, and N. B. Cabacungan. 1972. Dietary lipid, fatty acid synthesis and cholesterol metabolism in aging rats. *Lipids* **7:** 576–589.
7. Story, J. A., S. A. Tepper, and D. Kritchevsky. 1976. Age-related changes in the lipid metabolism of Fisher 344 rats. *Lipids* **11:** 623–627.
8. Bloch, K. E., E. Borek, and D. Rittenberg. 1946. Synthesis of cholesterol in surviving liver. *J. Biol. Chem.* **162:** 441–449.
9. Rosenman, R. H., and E. Shibata. 1952. Effect of age upon hepatic synthesis of cholesterol in rat. *Proc. Soc. Exp. Biol. Med.* **81:** 296–298.
10. Trout, E. C., Jr., K. T. Kao, C. A. Hizer, and T. H. McGavack. 1962. In vitro synthesis of free and ester cholesterol in various tissues of young and old rats. *J. Gerontol.* **17:** 363–368.



Downloaded from www.jlr.org by guest, on June 19, 2012

11. Shefer, S., S. Hauser, V. Lapar, and E. H. Mosbach. 1972. HMG CoA reductase of intestinal mucosa and liver of the rat. *J. Lipid Res.* **13:** 402–412.
12. Takeuchi, N., Y. Yamamura, Y. Katayama, C. Hayashi, and K. Uchida. 1976. Impairment of feedback control and induction of cholesterol synthesis in rats by aging. *Exp. Gerontol.* **11:** 121–126.
13. Hrůza, Z., and M. Wachtlova. 1969. Decrease of cholesterol turnover in old rats. *Exp. Gerontol.* **4:** 245–250.
14. Hrůza, Z. 1971. Increase of cholesterol turnover of old rats connected by parabiosis with young rats. *Exp. Gerontol.* **6:** 103–107.
15. Hrůza, Z., and V. Zbuzkora. 1973. Decrease of excretion of cholesterol during aging. *Exp. Gerontol.* **8:** 29–37.
16. Story, J. A., and D. Kritchevsky. 1974. Cholesterol oxidation by rat liver preparations: Effect of age. *Experientia.* **15:** 242–243.
17. Kitani, K., S. Tsuruoka, R. Miura, and Y. Morita. 1976. Study on the transport maximum of bile salts in the rat. 1. The variation with strain and age. *Kanzo* **17:** 76–80 (in Japanese).
18. Wilson, J. D. 1964. The quantification of cholesterol excretion and degradation in the isotopic steady state in the rat: the influence of dietary cholesterol. *J. Lipid Res.* **5:** 409–417.
19. Dietschy, J. M., and M. D. Siperstein. 1967. Effect of cholesterol feeding and fasting on sterol synthesis in seventeen tissues of the rat. *J. Lipid Res.* **8:** 97–104.
20. Uchida, K., Y. Nomura, M. Kadowaki, N. Takeuchi, and Y. Yamamura. 1977. Effect of dietary cholesterol on cholesterol and bile acid metabolism in rats. *Jpn. J. Pharmacol.* **27:** 193–204.
21. Shefer, S., S. Hauser, I. Bekersky, and E. H. Mosbach. 1969. Feedback regulation of bile acid biosynthesis in the rat. *J. Lipid Res.* **10:** 646–655.
22. Liersch, M. E. A., C. A. Barth, H. Hackenschmidt, H. L. Ullman, and K. F. A. Decker. 1973. Influence of bile salts on cholesterol synthesis in the isolated perfused rat liver. *Eur. J. Biochem.* **32:** 365–371.
23. Perric-Golia, L., and H. Socic. 1968. Biliary bile acids and cholesterol in developing sheep. *Amer. J. Physiol.* **215:** 1284–1287.
24. Uchida, K., and T. Miyake. 1961. Effect of various steroids on the plasma lipid levels of castrated male rats. *Ann. Rept. Shionogi Res. Lab.* **11:** 119–132. (in Japanese).
25. Uchida, K., M. Kadowaki, and T. Miyake. 1965. Failure of estrogen to produce hypocholesterolemic effect in immature rats. *Endocrinology* **76:** 766–770.
26. Gomori, G. 1942. A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. *J. Lab. Clin. Med.* **27:** 955–960.
27. Hanahan, D. J., and J. N. Olley. 1958. Chemical nature of monophosphoinositides. *J. Biol. Chem.* **231:** 813–828.
28. Fletcher, M. J. 1968. A colorimetric method for estimating serum triglycerides. *Clin. Chim. Acta* **22:** 393–397.
29. Uchida, K., Y. Nomura, M. Kadowaki, K. Miyata, and T. Miyake. 1970. Effects of estradiol, dietary cholesterol and l-thyroxine on biliary bile acid composition and secretory rate, and on plasma, liver and bile cholesterol levels in rats. *Endocrinol. Jpn.* **17:** 107–121.
30. Mok, H. Y. I., P. M. Perry, and R. H. Dowling. 1974. The control of bile acid pool size: Effect of jejunal resection and phenobarbitone on bile acid metabolism in the rat. *Gut* **15:** 247–253.
31. Fings, C. S., L. B. Foster, and P. S. Cohen. 1971. Electrophoretic separation of serum lipoproteins in polyacrylamide gel. *Clin. Chem.* **17:** 111–114.
32. Uchida, K., Y. Nomura, M. Kadowaki, and N. Takeuchi. 1976. Effects of synthetic progestational steroids on liver and serum lipids in rats. *Endocrinol. Jpn.* **23:** 97–108.
33. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
34. Bragdon, J. H., R. J. Havel, and E. Boyle. 1956. Human serum lipoproteins I. Chemical composition of four fractions. *J. Lab. Clin. Med.* **48:** 36–42.
35. Folch, J., M. Lees, and G. H. Sloan Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
36. Muraoka, Y., M. Ito, and Y. Hayashi. 1976. Effects of the population density on growth of SD-JCL rats. *Exp. Anim.* **25:** 283–289.
37. Eriksson, S. 1957. Biliary excretion of bile acids and cholesterol in bile fistula rat: bile acids and steroids. *Proc. Soc. Exp. Biol. Med.* **94:** 578–582.
38. Grundy, S. M., E. H. Ahrens, Jr., and T. A. Miettinen. 1965. Quantitative isolation and gas–liquid chromatographic analysis of total fecal bile acids. *J. Lipid Res.* **6:** 397–410.
39. Nomura, Y., M. Kadowaki, and K. Uchida. 1976. Effects of dietary bile acids on cholesterol and bile acid metabolism in rats. *Jpn. J. Pharmacol.* 26, Suppl. p. 74.
40. Uchida, K., and T. Miyake. Defense mechanism of rats against alimentary hypercholesterolemia. 1970. *Proc. Japanese Conf. on the Biochem. of Lipids* **16:** 167–171 (in Japanese).
41. Reicht, R. F., B. I. Cohen, and E. H. Mosbach. 1974. Effects of sodium taurochenodeoxycholate and sodium taurocholate on cholesterol absorption in the rat. *Gastroenterology* **67:** 1155–1161.
42. Howe, E. E., and C. F. Hutchison. 1962. Effect of dietary bile acids on plasma cholesterol of the rat. *J. Nutr.* **71:** 237–240.
43. Beher, W. T., G. D. Baker, and D. G. Penney. 1963. A comparative study of the effects of bile acids and cholesterol on cholesterol metabolism in the mouse, rat, hamster and guinea pig. *J. Nutr.* **79:** 523–530.
44. Gallo-Torres, H. E., O. N. Miller, and J. G. Hamilton. 1969. A comparison of the effects of bile salts on the absorption of cholesterol from the intestine of the rat. *Biochim. Biophys. Acta* **176:** 605–615.
45. Uchida, K., and Y. Nomura. 1977. Regulation of bile acid metabolism. *Seikagaku* **49:** 62–75 (in Japanese).
46. Beher, W. T., K. K. Casazza, A. M. Filus, M. E. Beher, and J. Bertasius. 1970. Effects of accumulated tissue cholesterol on bile acid metabolism in hypophysectomized rats and hamsters. *Atherosclerosis* **12:** 383–392.
47. Uchida, K., N. Takeuchi, and Y. Yamamura. 1975. Effect of glucose administration on cholesterol and bile acid metabolism in rats. *Lipids* **10:** 473–477.
48. Uchida, K., Y. Nomura, M. Kadowaki, K. Miyata, and T. Miyake. 1973. Stimulative and inhibitive effects of estrogen on cholesterol synthesis in rats. *Endocr. Jpn.* **20:** 129–134.
49. Uchida, K., and N. Takeuchi. 1977. Estradiol induced hypercholesterolemia in rats. *Folia Endocrin. Japon.* **53:** 341 (Abstr.).



Downloaded from www.jlr.org by guest, on June 19, 2012