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Fecal Steroid Excretion in Chickens with Hereditary Hyperlipidemia (42589)

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Abstract. Plasma lipids (cholesterol, triglycerides, and phospholipids; mg/dl) and the fecal excretion (mg/day) of neutral steroids and bile acids were studied in layers (L), hereditary nonlayer hens (NL), and roosters (R) fed a basal cholesterol-free grain diet *ad libitum*. Each group had significantly ($P < 0.05$) different levels of plasma cholesterol, triglycerides, and phospholipids when compared to the other groups. The highest lipid values were found in the NL group (cholesterol, 798 ± 89; triglycerides, 8914 ± 679; phopholipids, 2458 ± 112). There was no difference in the fecal excretion of neutral steroids between L and NL; however, fecal bile acid excretion by these two groups was significantly different ($P < 0.05$) (L, 13.1 ± 1.7 vs NL, 26.9 ± 3.4). Fecal neutral steroid excretion by R was significantly greater ($P < 0.05$) than that by either L or NL (L, 6.4 ± 1.3; NL, 6.0 ± 1.4; R, 14.4 ± 1.2). While fecal excretion of bile acids by R (36.1 ± 4.0) was also greater than that by either L or NL, only the difference between R and L was statistically significant ($P < 0.05$). Since, in the steady state, fecal bile acid excretion is equal to its synthesis, these results suggest that bile acid metabolism in these animals can be affected by both sex and egg-laying status.

In many species, the buildup of tissue cholesterol tends to be prevented by an increased fecal excretion of both acidic and neutral sterols. There are reports that in the humans (1), dogs (2), and rats (3) fed high levels of dietary cholesterol, there is a concomitant increase in the fecal excretion of not only cholesterol, but also bile acids. In addition, changes in the fecal excretion of bile acids and neutral steroids have been shown to affect plasma cholesterol levels. For example, treatment with cholestyramine (an unabsorbable, anion-exchange resin which increases the fecal excretion of bile acids) has been shown to lower plasma cholesterol in humans (4), rabbits (5), and guinea pigs (6). On the other hand, reduction in the fecal excretion of bile acids and neutral steroids, such as that induced by casein, has been shown to result in hypercholesterolemia in the rabbit (7).

Previous studies from this and other laboratories (8–10) showed that hereditary nonlaying hens (NL) become hyperlipidemic following the attainment of puberty. The persistent hypertriglyceridemia and hypercholesterolemia in these animals is of endogenous origin and probably reflects an altered lipid metabolism (9, 11). However, it is not known if changes in the fecal excretion of bile acids and neutral steroids contribute to the hypercholesterolemia. Thus, the purpose of this study was to examine the fecal excretion of neutral steroids and bile acids in NL chickens and compare that with the fecal neutral steroids and bile acids in normal laying chickens (L) and roosters (R).

Materials and Methods. *Chemicals.* [4-^{14}C]Cholesterol was obtained from New England Nuclear (Boston, MA), diluted with unlabeled cholesterol (99+%; Sigma Chemical Co., St. Louis, MO) and purified by thin-layer chromatography (TLC) on preparative (500-μm-thick) thin-layer plates (Analtech, Inc., Newark, DE) developed in benzene/ethyl acetate (2:3, v/v). The specific activity of the purified labeled cholesterol was 1.3×10^{12} dpm/mole. Hyocholic acid, the internal standard for bile acid analysis, was obtained from Calbiochem-Behring (San Diego, CA). All other bile acid standards for gas–liquid chromatography (GLC), cholic acid, chenodeoxycholic acid, 3β-Δ^5-cholenoic acid, deoxycholic acid, lithocholic acid, 7-ketolithocholic acid, β-muricholic acid, and urso- and hyodeoxycholic acid, were obtained from Steraloids (Wilton, NH). All solvents were of reagent grade and used as supplied.

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Animals and diet. The eggs of white leghorn hens (*Gallus domesticus*) fertilized by roosters carrying an abnormal gene for restricted ovulation were obtained from the University of Wisconsin at Madison. These eggs were hatched and the chickens were raised on commercial chicken mash *ad libitum* at the University of Illinois Poultry Farm, Urbana, as described previously (10). The grain diet consisted of 23% crude protein, 3% fat, 5% crude fiber, over 65% carbohydrate, and a mineral and vitamin premix. Layer hens which produced five or six eggs weekly and nonlayers, which had not produced a single egg up to the age of 1 year, were used.

Fecal excretion of neutral steroids and bile acids. Among flocks of year-old L, NL, and R, animals were randomly selected and quantitative fecal collections were made individually for 3 days and frozen until analyzed. Food intake during the 3 days was also recorded. Even though the animals were of the same age, they consumed different amounts of food and had different feed efficiency. In view of this, fecal excretion data were expressed as milligrams per/day to provide more complete information on total daily turnover of cholesterol via the fecal route.

Feces were analyzed for neutral steroids and bile acids by the method of Miettinen *et al.* (12) and Subbiah (13), respectively. To a weighed aliquot of fecal homegenate, hyocholic acid was added as an internal standard and the feces were saponified and extracted for bile acids as described (13). The fecal bile acids were methylated with freshly prepared diazomethane, and the bile acid methyl esters were acetylated using 1 ml of a mixture of acetic anhydride, acetic acid, and perchloric acid (5:5:0.05, v/v/v) at room temperature for 1 hr (14). The bile acid methyl ester acetates were quantitated by GLC using hyocholic acid as the internal standard. A Perkin–Elmer Sigma 2000 (Norwalk, CT) gas–liquid chromatograph equipped with a 6-ft column of 1% OV-225 on Gas-Chrom Q (100/120 mesh) (Supelco, Bellefonte, PA) was used (15). The operating conditions were column, 240°C; injector, 250°C; and detector (FID), 260°C. Helium was used as the carrier gas at 30 ml/min. Identification of the individual bile acids was based on comparison to relative retention times of the authentic standards obtained from Steraloids (Wilton, NH).

The major steroid groups in the neutral steroid extract were separated by TLC as described by Miettinen *et al.* (12). After elution from the gel with diethyl ether, the steroids in each group were quantitated by GLC using 5α-cholestane (Steraloids) as the internal standard. A Hewlett–Packard Model 5840A (Palo Alto, CA) gas–liquid chromatograph equipped with a 6-ft column of 3% OV-17 on Gas-Chrom Q (100/120 mesh) (Applied Science, State College, PA) was used. The operating conditions were column, 255°C; injector, 265°C; and detector (FID), 300°C. Nitrogen was used as the carrier gas at a flow rate of 50 ml/min. [4-^{14}C]Cholesterol was used to correct for procedural losses of neutral steroids. Fecal excretion of neutral steroids was corrected for variations in fecal flow and steroid losses during intestinal transit by the fecal recovery of dietary β-sitosterol as described previously (16).

Plasma lipid analysis. After the fecal collection, the birds were fasted overnight and blood samples were drawn from wing veins by means of butterfly needles (Abbott Hospital, Inc., Chicago, IL) into heparinized tubes. Plasma was separated by centrifugation at 1500*g* for 30 min at 4°C. Total plasma cholesterol was determined enzymatically (17) by using the A-Gent cholesterol reagent (Abbott Laboratories, N. Chicago, IL). The value of plasma triglyceride was measured by a colorimetric Hantzsch condensation method of Foster and Dunn (18). For the determination of plasma phospholipid content, total lipid material was extracted from an aliquot of plasma according to the method of Folch *et al.* (19), and then the total phospholipid value was determined from aliquots of lipid extracts after perchloric acid digestion (20).

All data were analyzed by ANOVA procedure of the SAS (21) in conjunction with Fisher's LSD multiple comparison test (22).

Results. The data for body weight, feed consumption, and fecal output of the three types of chickens studied are given in Table I. Even though the animals were of the same age, NL weighed significantly more ($P < 0.05$) than either L or R. There was no difference in body weights between L and R. Layers and NL con-

TABLE I. BODY WEIGHT, FEED CONSUMPTION, AND FECAL OUTPUT OF LAYERS, NONLAYERS AND ROOSTERS[1]

	Body weight[2] (g)	Feed consumption (g/day)	Fecal output (g/day)
Layer ($N = 3$)[3]	1511 ± 169^a	59.0 ± 1.7^a	15.4 ± 0.7^a
Nonlayer ($N = 4$)	2248 ± 43^b	62.3 ± 7.6^a	17.3 ± 3.4^a
Rooster ($N = 4$)	1755 ± 73^a	101.1 ± 13.5^b	31.4 ± 3.3^b

[1] Values are means ± SE.
[2] Means in the same column not sharing a common superscript letter differ significantly ($P < 0.05$).
[3] Number of animals (N).

sumed similar amounts of food, while food consumption by R was nearly twofold greater ($P < 0.05$) than that of either L or NL. Fecal output reflected the feed intake pattern. Thus, fecal output by R was nearly twofold greater ($P < 0.05$) than that by either L or NL.

It is evident from Table II that NL were grossly hyperlipidemic compared to plasma lipid levels in either L or R. While R had a significantly higher ($P < 0.05$) plasma cholesterol level than L, this value is normal for R. A comparison of the differences between means showed that all differences were statistically significant ($P < 0.05$).

The fecal excretion of neutral steroids and bile acids by the various groups is given in Table III. Both L and NL excreted similar amounts of neutral steroids. However, bile acid excretion by NL was nearly twofold greater ($P < 0.05$) than that by L. Thus, total fecal steroid excretion by NL was also significantly greater ($P < 0.05$) than that by L. Neutral steroid excretion by R was significantly greater than that by either L or NL. While bile excretion by R was also greater than that by L or NL, only the difference between L and R was statistically significant ($P < 0.05$). Total fecal steroid excretion by R, however, was significantly greater ($P < 0.05$) than that by either L or NL.

Compositional analysis of the major fecal bile acids is given in Table IV. Chenodeoxycholic acid was the major (69–77% of total) bile acid in all groups. Excretion of chenodeoxycholic acid by L was significantly lower ($P < 0.05$) than that by NL or L. The excretion of 7-ketolithocholic acid by R was significantly greater ($P < 0.05$) than that by either L or NL and constituted approximately 24% of total fecal bile acids in R. Cholic acid excretion by NL constituted approximately 11% of total fecal bile acids in NL and was significantly greater ($P < 0.05$) than that by either L or R. While only a trace amount of urso-/hyodeoxycholic acid was found in L and NL, this bile acid constituted approximately 6% of total fecal bile acids in R.

Discussion. A very interesting aspect of this study was the finding that feed efficiency in all three types of chickens was considerably different. NL had the highest feed efficiency while R had the lowest. Whether such metabolic differences affect plasma lipid values is not known. However, this finding does suggest that both sex and egg-laying status probably affect overall metabolism in these animals.

TABLE II. PLASMA LIPIDS IN LAYERS, NONLAYERS, AND ROOSTERS[1]

	Cholesterol[2]	Triglycerides	Phospholipids
Layer ($N = 3$)[3]	90.3 ± 5.5^a	714.0 ± 67.1^a	346.9 ± 25.0^a
Nonlayer ($N = 4$)	797.8 ± 89.2^b	$8{,}913.6 \pm 679.4^b$	$2{,}457.6 \pm 111.9^b$
Rooster ($N = 4$)	141.4 ± 18.8^c	75.5 ± 2.3^c	185.3 ± 10.9^c

[1] Values are means (mg/dl) ± SE.
[2] Means in each column not sharing a common superscript letter differ significantly ($P < 0.05$).
[3] Number of animals (N).

TABLE III. FECAL STEROID EXCRETION IN LAYERS, NONLAYERS, AND ROOSTERS[1]

	Steroids (mg/day)		
	Neutral steroids[2]	Bile acids	Total
Layer ($N = 3$)[3]	6.44 ± 1.29^a	13.13 ± 1.69^a	19.57 ± 2.92^a
Nonlayer ($N = 4$)	5.96 ± 1.35^a	26.94 ± 3.43^b	32.90 ± 3.17^b
Rooster ($N = 4$)	14.42 ± 1.20^b	36.07 ± 4.01^b	50.56 ± 4.23^c

[1] Values are means ± SE. Means in each column not sharing a common superscript letter differ significantly ($P < 0.05$).
[2] Approximately 97% cholesterol. Minor component, coprostanol.
[3] Number of animals (N).

Fecal bile acid composition showed that the predominant bile acid was chenodeoxycholic acid. Chenodeoxycholic acid is a primary bile acid in many species (23) including the chicken (24). Generally, however, fecal bile acids represent bacterial transformation products (2° bile acids) of primary (1°) bile acids (23). The fact that 2° bile acids such as 7-ketolithocholic acid, deoxycholic acid, and lithocholic acid were detected in the feces suggests that bacterial transformation of primary bile acids does occur in these chickens. It may well be that, in these animals, the intestinal transit time is sufficiently rapid thereby preventing a more complete bacterial transformation of the 1° bile acids. The identification of ursodeoxycholic acid, β-muricholic acid, and hyodeoxycholic acid must remain tentative pending additional proof of structure by more rigorous methods.

Previous studies had demonstrated a persistent gross elevation of plasma triglycerides, phospholipids, and cholesterol in hereditary NL after sexual maturity (8–10). The plasma of sexually mature L showed a mild hyperlipidemia which did not persist beyond 35 weeks of age (10). The plasma of mature roosters was normolipidemic. In the present study, plasma lipid values from 1-year-old animals were similar to that found previously (8, 9). Since cholesterogenesis is reportedly reduced in NL (9, 11), we were interested in determining if any other adaptive changes, tending to reduce the degree of hypercholesterolemia, had occurred in NL.

In the steady state, on a cholesterol-free diet, total daily fecal steroid (neutral steroid and bile acids) excretion is a good approximation of daily cholesterol turnover (25). In the present study, this would be true for NL and R. For L, the daily transfer of cholesterol into the egg must also be considered. However, daily fecal bile acid excretion in all three groups is a good approximation of daily bile acid syn-

TABLE IV. EXCRETION OF FECAL BILE ACIDS IN LAYERS, NONLAYERS, AND ROOSTERS[1]

Bile acids	Layers ($N = 3$)[2]	Nonlayers ($N = 4$)	Roosters ($N = 4$)
Lithocholic acid	0.17 ± 0.17^a	0.28 ± 0.18^a	t
3β-Δ^5-Cholenoic acid	t[3]	t	t
Deoxycholic acid	0.16 ± 0.11^a	0.42 ± 0.08^a	t
Chenodeoxycholic acid	10.11 ± 0.81^a	20.31 ± 2.97^b	25.02 ± 3.27^b
Urso- and hyodeoxycholic acid	t	t	2.14 ± 1.24
7-Ketolithocholic acid	1.39 ± 0.53^a	2.66 ± 0.47^a	8.70 ± 3.84^b
Cholic acid	1.18 ± 0.36^a	2.86 ± 0.46^b	0.21 ± 0.21^a
β-Muricholic acid	0.12 ± 0.09^a	0.41 ± 0.31^a	t
Total	13.13 ± 1.69^a	26.94 ± 3.43^b	36.07 ± 4.01^b

[1] Values are means (mg/day) ± SE. Means in horizontal rows not sharing a common superscript letter differ significantly ($P < 0.05$).
[2] Number of animals (N).
[3] t, values were less than 1% of total.

thesis. Since the conversion of cholesterol to bile acids is quantitatively a very important pathway of removal of cholesterol from the body (26), then estimation of daily fecal bile acid excretion provides important information regarding the catabolism of cholesterol via this route. Thus the major objective of this study was to determine the differences, if any, in the fecal excretion of neutral steroids and bile acids among L, NL, and R, and from that information gain some insight into differences in cholesterol metabolism in these animals.

It is evident from Table III that egg-laying status does not affect fecal excretion of neutral steroids. However, since the roosters excreted nearly twice as much neutral steroids as did L and NL, it suggests that the sex of the animals may affect fecal neutral steroid excretion. Since there appears to be a relationship between food consumption and fecal output, it is tempting to speculate that the increased amount of nondigestible ("fiber") matter in the food was responsible for the increased fecal excretion of neutral steroids and bile acids in R. However, while both L and NL consumed similar amounts of food, fecal bile acid excretion by NL was nearly two fold greater than that by L. Thus, it appears unlikely that feed intake influenced fecal excretion of neutral steroids and bile acids in the roosters.

The lack of relationship between egg-laying capacity and fecal neutral steroid excretion is at odds with the findings of Burczak *et al.* (27), who reported a positive correlation between egg-laying capacity and fecal neutral steroids. The reason for this discrepancy may lie in the fact that Burczak *et al.* (27) monitored the variation in neutral steroid excretion with advancing age whereas, in the present study, all animals were of the same age.

The dramatic difference in fecal bile acid excretion between L and NL and between L and R suggests that both sex and egg-laying status affect fecal bile acid excretion. Increased fecal bile acid excretion in NL implies that the daily synthesis of bile acids in NL is also enhanced, thereby increasing the rate of removal of cholesterol. This change, coupled with the documented reduction in cholesterogenesis in NL (9, 11), appears to be an adaptive response designed to combat the hypercholesterolemia resulting from the loss of the usual route of excretion of cholesterol. Clearly, these changes were inadequate in normalizing plasma cholesterol levels. Although not obtained in this study, data on hepatic cholesterol 7α-hydroxylase activity and bile acid pool size would have been useful additional information in support of the data on fecal bile acid excretion.

While there are numerous studies, of both humans and animals (23), documenting the role of sex in cholesterol metabolism, the mechanism for the apparent change in bile acid biosynthesis in NL is not known. In humans, various forms of hyperlipoproteinemia have been reported to affect the bile acid metabolism (23, 28, 29). Patients with type II hyperlipoproteinemia (high serum cholesterol, phospholipid, and β-lipoprotein concentrations) excreted a reduced amount of fecal bile acid with prolonged cholate half-life (28), whereas type IV hyperlipoproteinemics (high serum triglycerides and pre-β-lipoprotein) displayed increased fecal bile acid excretion (29). However, the data on the correlation between specific lipoproteins and fecal bile acid excretion exhibit a marked heterogeneity and appear controversial at best (30).

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