Origins of fecal neutral steroids in rats

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Abstract The origins of rat fecal neutral steroids were studied in male and female animals fed a sterol-free diet and maintained in an isotopic steady state. The specific activity of fecal cholesterol was found to be consistently lower than that of plasma cholesterol and of the fecal bile acids, indicating that a considerable portion of the fecal neutral steroids was derived from cholesterol not in equilibrium with the rapidly exchangeable pool of body cholesterol. This non-exchanging fraction of neutral steroids was larger in male than in female rats; it appeared to have at least two origins: skin surface lipids licked off fur, and sterols newly synthesized by the intestinal mucosa and secreted into the gut lumen. When the ingestion of skin sterols rich in cholesterol precursors was minimized, the proportion of the non-exchanging fraction of fecal neutral sterols fell somewhat, but the output of cholesterol precursors dropped markedly. This suggests that a significant portion of the non-exchanging fecal cholesterol fraction originated in the intestinal wall by secretion. It can be concluded that the fecal neutral steroids of rats originate primarily from three sources: *1*) de novo cholesterol synthesis by the intestinal mucosa, *2*) ingested dietary, skin, and fecal sterols, and *3*) a rapidly exchangeable cholesterol pool excreted through bile, the intestinal wall, or both.—**Miettinen, T. A., A. Proia, and D. J. McNamara.** Origins of fecal neutral steroids in rats. *J. Lipid Res.* 1981. **22:** 485–495.

Supplementary key words cholesterol · plant sterols · cholesterol precursors · sterol balance · intestinal sterol synthesis

The measurements of cholesterol synthesis, turnover, and absorption *in man* can be achieved by application of the isotopic kinetic (1) and/or chromatographic (2, 3) balance methods (4). Many investigators have attempted to measure the key parameters of cholesterol metabolism *in the rat* by either method alone or by a combination of both methods (5–7). This approach has been based on the fact that, in man, after the isotopic steady state for cholesterol is achieved, the fecal neutral steroids and bile acids are in isotopic equilibrium with the plasma cholesterol pool (4, 8); all fractions appear to be derived from endogenous sources and can be called "exchangeable".

However, the application of the isotope balance method in the rat has been criticized due to reports that, in the isotopic steady state, animals fed a sterol-free diet exhibited significantly lower specific activities of fecal neutral steroids than of plasma cholesterol (9–11). They have reported that a considerable dilution of "exchangeable" cholesterol occurs in the intestinal lumen of the rat by cholesterol *not* in equilibrium with the plasma pool; this cholesterol can be referred to as "non-exchanging". However, Wilson (12) found no evidence in rats for a non-exchanging fraction in the fecal neutral steroids and concluded that the secretion of newly synthesized (non-exchanging) cholesterol by the gut wall does not occur. In later experiments, Wilson and Reinke (13) obtained evidence for a minor dilution of cholesterol in intestinal contents by non-exchanging sterols originating in the skin and ingested during fur-licking; nevertheless, he reported no evidence for an input of non-exchanging cholesterol from the gut wall.

In view of these disparities, a series of experiments in rats was designed to clarify the question; the isotopic balance method developed in this laboratory (1, 4, 8) and the isotopic steady state balance procedure described by Chevallier (14) and improved by Wilson (12) were applied. To these methods we have added techniques (15) for the isolation, quantification, and identification of neutral steroids in rat feces, skin, and diet. To clarify the study's objectives, **Table 1** categorizes the various possible origins of fecal cholesterol in the rat and explains our terminology. The sources of unlabeled sterol during the isotopic steady state include: biliary cholesterol arising from newly synthesized cholesterol (16), cholesterol synthesized by the intestinal mucosa and secreted directly into the gut lumen, unabsorbed dietary cholesterol, skin sterols not in equilibrium with plasma cholesterol (17) and ingested by fur-licking, and any of the above sources of fecal steroids ingested by coprophagy. Sterols originating from cholesterol in equilibrium with the plasma (i.e.,

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

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TABLE 1. Sources of fecal neutral steroids in the rat in the isotopic steady state

Radioactivity	Source	Designation
Specific activity equal to plasma cholesterol	1) Biliary cholesterol derived from a rapidly miscible pool 2) Intestinal secretion and/or cholesterol from mucosal cell turnover 3) Fur-licking 4) Coprophagy	Exchangeable
Unlabeled	1) Biliary cholesterol derived from newly synthesized cholesterol 2) De novo intestinal synthesis and secretion 3) Unabsorbed dietary cholesterol 4) Fur-licking 5) Coprophagy	Non-exchanging

having the same specific activity) include biliary cholesterol not arising from new synthesis of cholesterol, cholesterol secreted from the intestinal mucosal cells that has not been synthesized locally, skin sterols originating from the plasma via exchange or secretion, and any of the above sources ingested by coprophagy. The unlabeled sterols are designated "non-exchanging" since they have not equilibrated with plasma cholesterol; all sources of radiolabeled sterols designated "exchangeable" are in equilibrium with the plasma.

The present study demonstrates that, in the rat, isotopic balance methods significantly underestimate whole body sterol synthesis. Fecal neutral steroids consist of cholesterol and its bacterial breakdown products derived from the rapidly miscible pool of cholesterol in the body (exchangeable), but they also originate by intestinal synthesis of cholesterol and by inclusion of skin surface sterols ingested during fur-licking, unabsorbed dietary cholesterol, and newly-synthesized biliary cholesterol (non-exchanging).

MATERIALS AND METHODS

Animal treatment

Male and female rats of the Sprague-Dawley strain (Holtzman, Madison, WI) were fed ad lib either regular Purina Laboratory chow (Ralston Purina Co., St. Louis, MO) or a "sterol-free diet" (ICN Life Sciences Group, Cleveland, OH) with a composition by weight: 25% casein, 65% sucrose, 6% cellulose, 4% USP XIV salt mix to which was added sterol-free "coconut oil" (5% by weight) synthesized into triglycerides from the appropriate fatty acids by Dr. Fred Mattson of Proctor and Gamble Co. (15). Both diets contained small amounts of sterols: as seen in Table 2, the total daily sterol intake on the chow diet approximates 47 mg per kg body weight per day and on the "sterol-free" diet 3.3 mg/kg per day. Rats were housed either individually or by sex in groups of five in cages with wire-net bottoms.

Feces

Under most conditions, 2-day stool collections from each animal of the group were collected and analyzed separately. Occasionally, feces of individual rats were pooled and, in two studies, feces were collected from all five animals simultaneously. In all cases, food and hair were removed from the feces, and after drying in a vacuum dessiccator for 2 days at room temperature, the dried feces were weighed and ground to a fine powder in a mortar and pestle.

Isotopic labeling

To obtain the isotopic steady state, [4-^{14}C]cholesterol was implanted subcutaneously in gelatin capsules, as described by Wilson (12). To obtain specific activity-decay curves for kinetic analysis, a single intraperitoneal injection of albumin-stabilized [4-^{14}C]cholesterol was given. In all cases, [4-^{14}C]cholesterol (New England Nuclear Corp., Boston, MA) was used after purification by thin-layer chromatography (TLC) on silver nitrate-impregnated silica gel G to remove traces of cholestanol. In preparing gelatin capsules, the purified isotopic cholesterol was diluted with similarly purified unlabeled cholesterol to a final specific activity of 120,000 dpm/mg. For intraperitoneal injection, an ethanolic solution of purified isotopic cholesterol was

diluted 10-fold with 1% bovine albumin in normal saline, and a volume of 1 ml containing 1.4 μCi was injected intraperitoneally.

Plasma cholesterol

Blood samples (0.5 ml) from a tail vein were drawn into heparin tubes at the end of each 2-day stool collection period while the animals were under light ether anesthesia. Plasma cholesterol concentrations and radioactivity were measured separately as previously described (3).

Radioactivity measurements

These were performed with a Packard Tri-Carb Liquid Scintillation Spectrophotometer (Model 3003) using Liquifluor (New England Nuclear, Boston, MA). Quench corrections were performed using [^{3}H]- or [^{14}C]toluene (Packard Instrument Co.) as internal standards. Counting efficiencies were approximately 85% for ^{14}C and 26% for ^{3}H.

Fecal neutral and acidic steroids

Fecal steroids were determined as previously described (2, 15). Since in all experiments the acidic sterols were labeled, the total radioactivity contained in the initial chloroform–methanol extract of the bile acids served as a reference against which to calculate final recoveries of the fecal bile acids in the aliquots analyzed by GLC. Percentage recoveries of neutral steroids were measured by adding known amounts of purified [7α-^{3}H]cholesterol (New England Nuclear Corp.) to the sample of fecal homogenate under analysis; they always exceeded 85%.

Fecal bile acids were quantified as a group as previously described (2), and fecal neutral steroids as subgroups after separation by TLC into three fractions (3, 15): "cholesterol" (Δ^5 sterols and 5α-sterols), "coprostanol" (ring-saturated 5β-sterols plus Δ^7, Δ^8, and $\Delta^{8,24}$ sterols), and "coprostanone". The characterization and identification of the sterol composition of each fraction, including the plant sterol analogues, are discussed in detail in the accompanying manuscript (15). In GLC analyses of the "coprostanol" fraction, the methostenol-lanosterol area contained some 4-methyl sterols, and thus were probably overestimated. The 5β-derivatives of the three plant sterols were also somewhat overestimated, owing to the similar retention times of Δ^8-methostenol and other cholesterol precursors (15).

The evidence to be presented below demonstrates that negligible losses of plant sterols occurred during intestinal transit in rats. Thus, it was unnecessary to correct the fecal steroid data for neutral sterol degradation during intestinal transit as is required in studies of man (18). Analysis of the sterol content in the stomach of three randomly selected rats showed no coprostanol, indicating that coprophagy was minimal.

Calculation of non-exchanging cholesterol

The amount of cholesterol and its bacterial degradation products in fecal neutral steroids that was derived from cholesterol in equilibrium with plasma cholesterol was determined by dividing the total radioactivity found in those samples by the specific activity of plasma cholesterol. This fraction represented the total mg of exchangeable cholesterol in the sample. The amount of non-exchanging cholesterol was obtained as the difference between the total (measured chromatographically) and the exchangeable (measured radioisotopically) cholesterol.

RESULTS

Isotopic steady state

Preliminary experiments measuring the specific activities of fecal cholesterol, coprostanone, and coprostanol during the isotopic steady state in rats fed a "sterol-free" diet demonstrated that fecal cholesterol and coprostanone exhibited identical specific activities, whereas that of coprostanol was routinely 3–5% lower. In order to test whether this decrease represented the presence of non-labeled steroids in the coprostanol fraction or a true difference in specific activity, cholesterol and coprostanol were rechromatographed on silica gel G and silver nitrate-impregnated silica gel G TLC plates as previously described (15), followed by crystallization of each sterol from methanol and measurement of their respective specific activities. The specific activity of cholesterol remained unchanged; the cholestanol separated from cholesterol by this procedure had the same specific activity as cholesterol. These data suggest that the presence of cholestanol in the original cholesterol fraction did not result in an erroneous determination of cholesterol specific activity, and that the cholestanol was derived from cholesterol, since the implanted [4-^{14}C]cholesterol was free of contaminating cholestanol.

Upon crystallization, the coprostanol specific activity was found to be identical to that of the fecal cholesterol. The observed decreased coprostanol specific activity results from the fact that the coprostanol fraction contains Δ^7-coprostenol and other steroids that are unlabeled and as yet not identified (15). Their presence as contaminants results in a 3–5% underestimation of the specific activity of coprostanol isolated by TLC on Florisil. These results indicate that any observed difference in specific activity of fecal

sterols (particularly coprostanol) that is greater than 5% represents a real difference that should not be ascribed to technical problems of steroid separation.

Sex differences in fecal steroid excretion

Male and female rats fed the low-sterol diet excreted similar amounts of fecal bile acids (approximately 18.5 mg/kg per day) and total fecal neutral steroids derived from cholesterol (about 10.8 mg/kg per day) (**Table 2**). Both sexes fed the low-sterol diet excreted substantially less total fecal steroids than male rats fed regular rat chow; of course, the latter diet contains significant amounts of cholesterol and plant sterols.

The data in Table 2 illustrate the fact that losses of the non-absorbable plant sterols during intestinal transit were negligible. In animals fed the "sterol-free" diet there was a slightly *positive* balance for plant sterols, owing to overestimation of 5β-saturated plant sterols by contamination with cholesterol precursors (15). In animals fed the chow diet, the difference between intake (30.75 mg/kg per day) and output (28.12 mg/kg per day) was 2.63 mg, a loss due to neutral sterol degradation during intestinal transit of only 8.5%. The data in Table 2 also illustrate the fact that the excretion of cholesterol precursors was significantly greater in males than in females on the "sterol-free" diet, totaling 4.4 and 1.7 mg/kg per day, respectively.

Chromatographic measurement of fecal steroids in all three groups of rats demonstrated that the total excretion of neutral and acidic steroids (derived from cholesterol) was much larger than the intake; this difference represents the amount due to new synthesis. On the "sterol-free" diet, males had a net negative sterol balance of 26.8 mg/kg per day; the chow-fed male rats had a negative balance of 46.0 mg/kg per day. Despite the higher cholesterol intake, the males fed chow synthesized significantly more cholesterol than those fed the "sterol-free" diet; the very much larger excretion of bile acids was previously noted by Wilson (12). These results support the previously reported observation that net sterol balance is significantly decreased in rats fed a semi-synthetic diet as compared to the chow-fed rat, this decreased fecal steroid output by semi-synthetic diets has been ascribed to the fiber

TABLE 2. Influence of diet and sex on sterol balance in the rat[a]

	Diet		
	Chow	"Sterol-free"	
	Male (5)	Male (5)	Female (5)
Body weight (g)	335 ± 15	329 ± 13	279 ± 14
Dietary intake (mg/kg per day)			
Cholesterol	16.27	2.13	2.08
Plant sterols	30.75	1.22	1.18
Stool weight (g/kg per day)	16.42	9.42 ± 1.52	8.96 ± 1.79
Cholesterol output (mg/kg per day)			
Cholesterol	14.93	6.81 ± 1.40	6.52 ± 1.15
Coprostanol	6.90	4.56 ± 1.52	3.51 ± 1.54
Coprostanone	0.30	0.15 ± 1.70	0.11 ± 0.04
Total	22.12	11.52 ± 1.70	10.14 ± 2.15
Bile acid output (mg/kg per day)	40.18	17.39 ± 7.29	19.57 ± 5.27
Plant sterol output (mg/kg per day)[b]			
"Cholesterol fraction"	20.24	0.82 ± 0.18	0.82 ± 0.18
"Coprostanol fraction"	7.55	0.94 ± 0.21	0.61 ± 0.14
"Coprostanone fraction"	0.33	ND[d]	ND[d]
Total	28.12	1.76	1.43
Cholesterol precursor output (mg/kg per day)			
Δ^7-Cholestenol	4.27	2.37 ± 0.58	0.75 ± 0.22
"Methostenol"[c]	2.24	1.22 ± 0.27	0.43 ± 0.11
"Lanosterol"[c]	1.31	0.79 ± 0.46	0.47 ± 0.29
Total	7.82	4.38	1.65

[a] Steroids were chromatographically analyzed from three individual 2-day fecal pools except for animals fed standard chow, which were collected from all five animals in one cage. Number in parentheses indicates number of animals in each group. Data presented as mean ± SD.

[b] Sum of all plant sterols (campesterol, stigmasterol, sitosterol) and their respective 5β-saturated and ketonic products.

[c] Includes compounds with identical retention times.

[d] Not determined in all samples, values ranged from 2–9 μg/day.

(19), protein (20), and/or fat (21) composition of the diet.

When [4-^{14}C]cholesterol was administered intraperitoneally to male and female rats on "sterol-free" diets, a precursor–product relationship between the plasma and fecal cholesterol specific activity-decay curves was not observed in either sex; cholesterol turnover was slightly more rapid in female than in male animals (**Fig. 1**). The failure to demonstrate a relationship between the two time-curves rules against a simple relationship between plasma cholesterol as precursor and total fecal cholesterol as product. However, the comparisons are only qualitatively suggestive: the plasma cholesterol specific activities were obtained on blood samples drawn at the end of 2-day stool collection periods, rather than at the beginning or middle of the collection period, thus falsely accentuating any differences ascribable merely to the time required for biliary (or intestinal) cholesterol to pass through the intestinal tract. The faster die-away curve in females could not have been due to more rapid synthesis, however, since (as will be shown below) the net synthesis of "exchangeable" cholesterol plus bile acids was unaffected by sex. More likely the steeper curve in females was due to a smaller exchangeable pool of cholesterol in the females, since they were smaller in body weight than the males (Table 2).

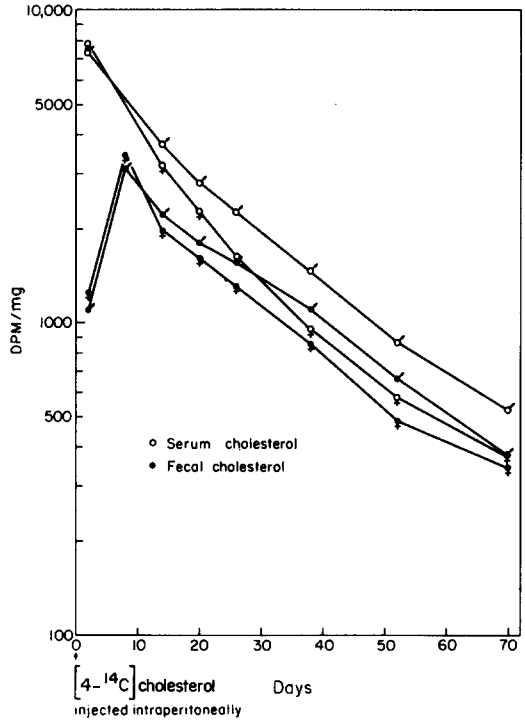

Fig. 1. Specific activity curves of plasma and fecal cholesterol after a single intraperitoneal injection of [4-^{14}C]cholesterol in male and female rats on "sterol-free" diets. Each point represents the mean of the data obtained in five animals. The specific activities of plasma cholesterol were plotted at the end of each 2-day stool collection period.

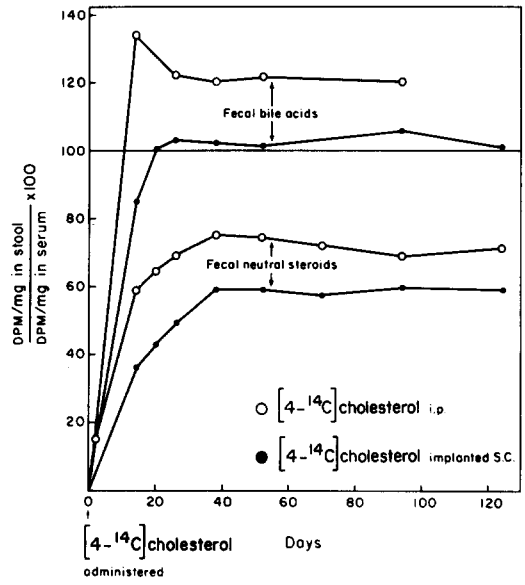

Fig. 2. Specific activity curves of fecal bile acids and neutral steroids of endogenous origin are presented as percent of plasma cholesterol specific activity following a single intraperitoneal (i.p.) injection of [4-^{14}C]cholesterol (○), or after subcutaneous (s.c.) implantation of a gelatin capsule containing crystalline [4-^{14}C]cholesterol (●) in male rats fed a "sterol-free" diet. Each point represents the mean of the data obtained in five animals. The specific activities of plasma cholesterol were determined at the end of 2-day stool collection periods.

Measurement of non-exchanging cholesterol in fecal steroids

Quantification of the contribution of non-exchanging cholesterol to total fecal neutral steroid output is theoretically valid, provided that a constant relationship between the specific activity of fecal steroids and plasma cholesterol has been achieved. The time-course of attainment of this steady state by intraperitoneal injection versus subcutaneous implantation of labeled cholesterol into male and female rats fed the "sterol-free" diet is shown in **Fig. 2,** in which the specific activity of plasma cholesterol was taken as 100 and the radioactivities of fecal bile acids and fecal neutral steroids by endogenous origin were expressed as percentages of the plasma cholesterol specific radioactivity at stated intervals.

Both methods caused a more rapid labeling of fecal bile acids than of fecal neutral steroids: the activity of the neutral steroids reached the steady state at the 35th day, compared to the 20th day for bile acids. The significantly higher relative specific activity of fecal bile acids, found when labeling was carried out by intraperitoneal injection, is probably ascribable to an exten-

sive enterohepatic recirculation of bile acids of high relative specific activity prior to their excretion in the feces, and to the fact that the plasma specific activities were determined on blood samples drawn at the end of 2-day stool collection periods. Such a difference would not be observed in animals with the subcutaneous implant in which the cholesterol and bile acid specific activities have reached a steady state. It is also possible that intraperitoneal dosing may have led to a more rapid delivery of labeled cholesterol from the peritoneum, compared to a slower rate of absorption from a subcutaneous site.

Fig. 2 shows that, while there was no significant time difference between the two methods in terms of the labeling of plasma cholesterol (as precursor) and fecal bile acids (as product) in rats in the steady state; the specific activity of the fecal neutral steroids was only 60% of that of plasma cholesterol. Thus, 40% of the fecal neutral steroids were not in equilibrium with plasma cholesterol and were, by definition, non-exchanging.

Calculations of the relative contributions of the exchangeable and non-exchanging cholesterol pools to the excretion of the total fecal neutral steroids were made in two ways. First, the specific activity of fecal neutral sterols was divided by the specific activity of plasma cholesterol at the end of a 2-day stool collection period, and second, fecal neutral sterol specific activity was divided by the specific activity of fecal bile acids isolated from the same feces. Numerical data derived from experiments in male and female rats 49 days after isotope administration are given in **Table 3.** In agreement with Table 2, there was no significant difference in total cholesterol excretion in male and female rats; however, according to both calculations, there was a considerable contribution from non-exchanging pools in both sexes, but more in males than in females.

The close agreement in the two calculations of exchangeable cholesterol in male rats in which labeled cholesterol had been implanted subcutaneously attests to the attainment of the isotopic steady state in these animals and to the validity of the assumptions upon which the calculations were based. On the other hand, in animals given [4-^{14}C]cholesterol by intraperitoneal injection, we found a significant difference ($P < 0.02$) in the two calculations of excretion from the exchangeable cholesterol pools.

Thus, the assembled data suggest that the calculation of excretion from the exchangeable cholesterol pools in intraperitoneally injected animals is more validly based on the specific activity of fecal bile acids than on that of plasma cholesterol. This conclusion is also supported by the fact that the specific activity of fecal neutral steroid output was found to be approximately 60% of that of the fecal bile acids by both labeling methods (Fig. 2 and Table 3).

Table 3 also shows that the amount of the fecal neutral steroids derived from exchangeable cholesterol pools was not significantly different in the two sexes. Not shown are the data for total exchangeable cholesterol balance (including bile acids) (27.5 mg/kg per day for females, 24.1 for males, a non-significant difference). The contribution of non-exchanging cholesterol to the total fecal neutral steroid output cannot be accounted for by the small amount of dietary cholesterol which equaled 2.1 mg/kg per day, irrespective of its absorption.

The data presented in Tables 2 and 3 indicated that *1*) male and female rats excreted similar amounts of neutral and acidic steroids in the feces, *2*) the two sexes had comparable rates of synthesis of cholesterol, *3*) male rats excreted a larger proportion of neutral steroids from non-exchanging cholesterol pools, and *4*) males excreted more sterol precursors in feces than females.

TABLE 3. Fecal excretion of exchangeable and non-exchanging cholesterol by male and female rats[a]

Source of Excretion of Fecal Steroids	[4-^{14}C]Cholesterol Administered Intraperitoneally				[4-^{14}C]Cholesterol Implanted Male (5)	
	Female (5)		Male (5)			
	A	B	A	B	A	B
	(mg/kg/day)					
Exchangeable pools	9.07 ± 0.75	7.56 ± 0.79	8.84 ± 1.70	7.36 ± 1.46	7.33 ± 1.16	7.14 ± 1.22
Non-exchanging pools[b]	1.00 ± 0.75	2.51 ± 0.82	3.28 ± 2.04	4.77 ± 1.79	4.95 ± 0.94	5.14 ± 1.06
Total[c]	10.07 ± 0.43		12.12 ± 3.04		12.28 ± 1.34	
% Contribution from exchangeable pools	90%	75%	73%	61%	60%	58%

[a] Sterols were analyzed for mass and specific activity in animals fed a "sterol-free" diet. Labeling had been carried out 49 days prior to these analyses. Exchangeable cholesterol was calculated as follows: A: [total DPM in stool/day]/[plasma cholesterol specific activity (dpm/mg)]; B: [total DPM in stool/day]/[fecal bile acid specific activity (dpm/mg)].

[b] Difference between total and exchangeable cholesterol.

[c] Determined chromatographically.

TABLE 4. Excretion of fecal and skin cholesterol and cholesterol precursors by male rats

	Total Sterol				
	Total Cholesterol, Coprostanol, and Coprostanone	Exchangeable Cholesterol	Non-exchanging Cholesterol	Δ^7-Cholestenol	Methostenol + Lanosterol
			(mg/k/day)		
Acetone-washed[a]					
Skin	6.20 ± 0.63	3.10 ± 0.18	3.10 ± 0.18	5.00 ± 0.96	1.99 ± 0.75
Feces (1)	8.73	5.99	2.74	0.93	0.33
Not acetone-washed[b]					
Feces (5)	12.17 ± 1.33	7.26 ± 1.14	4.91 ± 0.93	2.37 ± 0.58	2.01 ± 0.78

[a] Five male animals on a "sterol-free" diet were washed with acetone for 5 consecutive days and placed together in a single cage. A single 24-hr stool collection was obtained and analyzed for sterol composition. Skin sterol data represent the mean ± S.D. of the last acetone wash assayed individually in the five rats.
[b] Five male rats handled as described in footnote *a*, but not acetone-washed.

Non-exchanging cholesterol in skin surface lipids

Five male rats maintained on the "sterol-free" diet and in the isotopic steady state (subcutaneous implantation of [4-^{14}C]cholesterol) were lightly anesthesized, then dipped into acetone (35°C) up to the head on 5 successive days. The animals were dried manually and placed in a 37°C box until completely dry. Feces, plasma, and skin lipids from the last acetone wash were separately analyzed for neutral steroid mass and specific activity; these data were compared to values obtained prior to acetone washing in the same animals.

The specific activity of cholesterol in the skin surface lipids was found to be half that found in plasma cholesterol; thus, 50% of the cholesterol in skin surface lipids was derived from a non-exchanging source and presumably was synthesized de novo by the skin, as has previously been shown in man (17). As shown in **Table 4,** sterol precursors were found in the skin surface lipids in amounts slightly larger than cholesterol itself. Feces contained a reduced amount of sterol precursors (cf. Table 2) but, despite an acetone wash per day for 5 days, the fraction of fecal neutral steroids derived from non-exchanging cholesterol pools remained high (31% in acetone-washed versus 40% in controls, Table 3).

Effect of preventing fur-licking

A second attempt to decrease the ingestion of skin surface sterols consisted of restraining five male rats with subcutaneous implants of [4-^{14}C]cholesterol in individual cylindrical wire cages constructed in such a way that the animals had ready access to food and water but could not lick their fur. Feces and blood were obtained before and at the end of this 6-day experiment. The daily excretion of cholesterol fell from 11.93 to 6.75 mg/kg per day (**Table 5**), primarily due to the reduction of food intake during the restraint period. The percentage of total fecal neutral steroids made up by sterol precursors was also markedly reduced, from 4.46 to 0.57 mg/kg per day. As predicted from the results in Table 4, the contribution of sterols from the exchanging and non-exchanging cholesterol pools was not significantly changed, with the proportion derived from the non-exchanging cholesterol pools remaining constant at 32–40% in the rats prevented from fur-licking as well as in rats subjected to acetone washes.

Thus, the results of Tables 4 and 5 suggest that the bulk of the non-exchanging cholesterol in the feces arose not from ingested skin sterols but from de novo synthesis by the intestinal wall, or from cholesterol

TABLE 5. Effect of preventing fur-licking on fecal neutral excretion[a]

Test Period	Fecal Neutral Steroids[b]					
	Cholesterol		Δ^7-Cholestenol		Methostenol plus Lanosterol	
	mg/kg/day	*sp act*[c]	*mg/kg/day*	*%*[d]	*mg/kg/day*	*%*[d]
Prior to caging	11.93 ± 0.72	60 ± 10	2.92 ± 0.60	24 ± 6	1.54 ± 0.78	13 ± 4
During caging	6.75 ± 1.48	68 ± 7	0.39 ± 0.15	5 ± 1	0.18 ± 0.09	3 ± 1

[a] Male rats in the isotopic steady state were placed individually in cylindrical cages to prevent fur-licking. Two-day stool collections were performed prior to restriction and during the last 2 days of restriction.
[b] Mean excretion ± S.D. (n = 5).
[c] Given as % of the plasma cholesterol specific activity.
[d] Percent of total fecal neutral steroids.

newly synthesized in the liver and passing into the bile without equilibrating with plasma cholesterol.

Site of intestinal cholesterol synthesis

An attempt was made to determine the intestinal level at which non-exchanging cholesterol was contributed to the lumen contents. Three male rats in the isotopic steady state were washed with acetone for 5 days, then a 24-hr collection of feces was made, blood was sampled, and the animals were killed. Skin sterol data were obtained in the final acetone wash, and the specific activities of cholesterol from different sources were related to that of plasma cholesterol.

The data in **Table 6** demonstrate that radioactivity was present in the stomach contents as well as at all levels throughout the small and large intestine. The highest relative specific activity was found in the small bowel mucosa, but the percentage of non-exchanging cholesterol was not strikingly different in the ileal and cecal contents and feces. Thus the level of the intestine where maximal secretion of non-exchanging cholesterol occurs remains unclear.

DISCUSSION

Any attempt to measure the key regulatory parameters of sterol metabolism (absorption, synthesis, tissue flux, conversion to bile acids, and excretion) requires the precise measurement of sterol balance in the living organism. Previous studies in this laboratory have validated a system of chromatographic procedures for the analysis of fecal steroids in man, and these procedures have been applied in various measurements of whole body cholesterol metabolism in man.

In man, the measurement of cholesterol synthesis rates by the isotopic and chromatographic balance methods demonstrated excellent agreement between the two methods (4), since the fecal steroids exhibited the same specific activity as that of plasma cholesterol (4, 8). In the rat, however, numerous investigators have reported differences between fecal and plasma steroid specific activity (9–11) that, if true, would invalidate any attempt at measurement of whole body cholesterol synthesis or of cholesterol absorption in the rat when performed by isotopic balance methods (4, 22). If cholesterol synthesized in the liver or intestinal mucosa is excreted into the intestinal lumen before it reaches isotopic equilibrium with that in the plasma (11), the radioactivity of sterols in the lumen, and eventually in the feces, will be diluted to some undefined degree. In addition, any sterols ingested through the licking of fur by the rat will also dilute the radioactivity in the fecal neutral sterols. In an attempt to define the relative contributions of intestinal cholesterol synthesis and of ingested skin sterols on sterol balance in the rat, we carried out sterol balance measurements in rats during the isotopic steady state by both the isotopic (12) and chromatographic (15) methods under conditions where skin sterols were and were not ingested.

Fecal cholesterol

The specific radioactivity of fecal cholesterol was found to be markedly lower than that of plasma cholesterol and of fecal bile acids in rats fed a sterol-free diet; this finding was independent of whether [4-^{14}C]cholesterol was injected in a bolus or delivered continuously by subcutaneous implantation. The data clearly demonstrate that cholesterol not in equilibrium with the plasma enters the intestinal lumen and contributes to total fecal neutral sterol output. Thus, 40–50% of fecal neutral sterols in rats fed a sterol-free diet arise from a source of non-exchanging cholesterol, whether the calculations are based on the specific radioactivity of plasma cholesterol or of fecal bile acids.

TABLE 6. Neutral steroids in the gastrointestinal tract of acetone-washed rats[a]

	Cholesterol Precursors		
	Δ^7-Cholestenol	Methostenol plus Lanosterol	Cholesterol Specific Activity
	(% of cholesterol mass)		*(% plasma cholesterol specific activity)*[b]
Skin	78.0 ± 10.5	33.0 ± 8.2	52.0 ± 2.6
Stomach contents	24.0 ± 10.8	4.3 ± 1.5	44.3 ± 6.5
Ileal contents	5.0 ± 0.9	2.3 ± 0.6	73.0 ± 8.5
Small bowel mucosa	0.7 ± 0.2	0.3 ± 0.1	90.0 ± 2.6
Cecum content	5.7 ± 1.5	2.3 ± 0.6	76.7 ± 2.1
Feces	10.7 ± 1.5	3.3 ± 0.6	69.3 ± 0.6

[a] Three male rats in the isotopic steady state, acetone-washed for 5 days, then killed. Fur-licking was not prevented.
[b] Using the plasma data at the time of killing.

These results confirm the findings of Danielsson (9), Chevallier (10), and Peng, Ho, and Taylor (11).

In addition, the data suggest that female rats have a lower output of non-exchanging cholesterol. The excretion of exchangeable cholesterol appears to be independent of sex, however. Under conditions that prevent fur-licking, either by acetone-washing of the animals or by physical restraint, the contribution of non-exchanging cholesterol to the total fecal neutral sterol output was approximately 33%: it must have originated either from cholesterol synthesized in the intestinal mucosa and secreted directly into the gut lumen (11, 23), or from cholesterol newly synthesized in the liver and secreted directly into the bile without mixing with labeled cholesterol pools in the liver (24).

Fecal cholesterol precursors

The fecal output of cholesterol precursors was lower in female than in male rats and was reduced by 80% in both sexes when fur-licking was prevented. Cholesterol precursors have been found in the intestinal mucosa of the rat (25), yet in the acetone-washed animals the concentration of cholesterol precursors in the intestinal mucosa was significantly reduced, as was the fecal output of these precursors. In a similar fashion, plant sterols are easily detected in the rat intestinal mucosa of animals fed a regular chow diet containing plant sterols (15, 26), but are undetectable in the mucosa of rats fed a sterol-free diet.[4] While it is probable that the intestinal mucosa synthesizes cholesterol precursors, the bulk of these steroids in the feces has its origin in fur-licking. Partial absorption into the mucosa of these precursors may explain their reported presence in the mucosa (26).

Sterol balance measurements in the rat

Sterol balance measurements in the rat present a number of technical differences from those carried out in man. Cohen et al., (27) have shown that in measuring fecal bile acids by GLC, as much as 25% of the total methylated mass can be nonsteroidal hydroxy fatty acids, which, if included in the calculations, cause a significant overestimation of bile acid output.

The presence of cholesterol precursors in fecal extracts can cause the overestimation of coprostanol output, as previously shown (15), and the differentiation of cholesterol and its bacterial breakdown products from cholesterol precursors is more complex than that observed in human fecal steroids. It should be noted, however, that in the rat the recovery of dietary sitosterol is almost 100%, making the correction for neutral steroid degradation during intestinal transit unnecessary (18).

In the rat, newly synthesized hepatic cholesterol can become the precursor of cholesterol and bile acids in bile without equilibrating with the rapidly exchangeable cholesterol pool (16). This preferential utilization of newly synthesized cholesterol could account for the minor decrease in bile acid specific activity observed, but it fails to explain the significant decrease in the specific activity of the fecal neutral steroids that most investigators have observed.

On a sterol-free diet, the only sources of fecal neutral steroids are biliary cholesterol and cholesterol secreted into the lumen from the intestinal mucosa. Turley and Dietschy (28) have shown that the rate of biliary cholesterol output in the rat under a variety of experimental conditions is relatively constant, amounting to 18–19 mg/kg per day. The present study has shown that male rats fed the sterol-free diet excreted 11.5 mg/kg per day of cholesterol. Assuming a 60% absorption of biliary cholesterol (29), the contribution of biliary cholesterol to fecal neutral steroids can account for only 7–8 mg/kg per day, or approximately 64% of the total output. One-third of the fecal cholesterol in this study was derived from non-exchanging sources and can easily have come from non-biliary sources. One could assume that in the chow-fed rat, which has the same biliary cholesterol output and absorption and where daily fecal cholesterol excretion equals 22 mg/kg per day, the contribution of non-exchanging cholesterol to fecal excretion would be much greater, almost two-thirds (10).

The present study has shown that the measurement of sterol balance in rats by a combined chromatographic and isotopic method enables the essential distinctions to be drawn between the various exchangeable cholesterol fractions and the other fractions contributed from non-exchanging sources. However, the demonstration of multiple sources of fecal neutral steroids in the rat makes difficult a clearcut definition in the meaning of the words "endogenous" and "exogenous", since the non-exchanging cholesterol derived from the skin, from the liver, and from the gut wall are certainly of endogenous origin, yet these fractions do not contribute to the regulation of cholesterol homeostasis in the "internal environment" of the rat.

Total body cholesterol synthesis rates in the rat

Sterol balance measurements of whole body cholesterol synthesis in man requires the existence of the metabolic steady state (i.e., no net flux of cholesterol between body pools is occurring). In the rat, which under most conditions is constantly growing, this metabolic steady state does not exist; however, most in-

[4] Miettinen, T. A. Unpublished observations.

vestigators have assumed that during various interventions the rate of cholesterol deposition into growing tissues is fixed and thus equal in control and treatment groups. Recent studies have challenged this assumption and have shown that the whole body cholesterol content of the rat can be significantly altered by dietary choline deficiency (7), the experimental nephrotic syndrome (30), portacaval anastomosis (31), and diet.[5] It should also be noted that, as rats age and growth rates decrease, fecal neutral steroid excretion decreases (6), as would the rate of cholesterol deposition into tissues due to decreased growth rates.

With these considerations in mind, we can now state that accurate, quantitative sterol balance measurements in the rat require the measurement of the following variables: *1*) dietary cholesterol intake; *2*) fecal neutral sterol output measured chromatographically; *3*) fecal bile acid output measured chromatographically or in the isotopic steady state (and potential changes in bile acid pool sizes during the course of the study); and *4*) the rate of tissue flux of cholesterol (either influx or efflux during the balance period), calculated as follows: daily cholesterol synthesis (mg/kg per day) = fecal neutral steroid output + fecal bile acid output + tissue accumulation − dietary cholesterol intake. With these values at hand, the investigator can calculate the daily rate of whole body cholesterol synthesis (including that produced by the skin and gut mucosa, as well as all other internal organs and tissues). But, if knowledge is needed of that fraction of whole body synthesis that affects the internal regulation of cholesterol homeostasis, it seems imperative to quantify, not only that fraction we have called "non-exchanging", but by difference the exchangeable fraction.

Cholesterol absorption rates in the rat

Obviously, cholesterol absorption cannot be measured by isotopic equilibrium methods due to the dilution of radioactivity by non-exchanging cholesterol originating from mucosal synthesis and secretion and from fur-licking. However, the methods of Borgström (32) or Zilversmit and Hughes (29) can validly be used for measurement of absorption of dietary cholesterol, and this in turn allows the calculation of cholesterol turnover by cholesterol kinetics, and of daily cholesterol synthesis by difference between daily turnover and daily absorption.

This work was supported in part by USPHS Grants HL 06222 and HL 24190 from the National Heart, Lung, and Blood Institute; by Grants-in-Aid from the New York Heart Association, the National Dairy Council, and the New York State Dairy Council; grants from the Herman Goldman Foundation and Weight Watchers Foundation, Inc.; and by a Career Scientist Award to D.J.M. from the Irma T. Hirschl Charitable Trust. The authors express their gratitude to Dr. E. H. Ahrens, Jr. for his support and encouragement of these studies.

Manuscript received 24 June 1980 and in revised form 24 November 1980.

[5] McNamara, D. J., A. Proia, and K. D. G. Edwards. Unpublished observation.

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