# Catabolism and elimination of cholesterol in germfree rats

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ABSTRACT Three-month old germfree and conventional male rats were maintained on a complete steam-sterilized, semisynthetic diet. After intravenous injection of cholesterol-26-$^{14}$C the animals were housed in a plastic metabolism chamber for 72 hr. Expired $CO_2$ was collected throughout the period. The conventional rats released 50% more $^{14}$C as $^{14}CO_2$ than the germfree animals. The total amount of the label recovered as $^{14}CO_2$ during the 72 hr period amounted to 30% and 19% respectively, of the original dose. In both conventional and germfree rats the release of $^{14}CO_2$ accounted for approximately 75% of the $^{14}$C recovered in forms other than the original cholesterol-26-$^{14}$C; 15–20% was found incorporated in water-soluble and fat-soluble fractions other than 3$\beta$-OH sterol of liver and carcass while the remainder was excreted with feces and urine. After the 72 hr period the specific activities of the cholesterol in plasma and liver were lower in conventional than in germfree animals.

The data express the accelerating effect of the intestinal microflora on systemic cholesterol catabolism. They demonstrate that the release of $^{14}CO_2$ from cholesterol-26-$^{14}$C in the intact rat is a suitable and convenient indicator of the oxidative catabolism of cholesterol.

KEY WORDS cholesterol-26-$^{14}$C · oxidative catabolism · germfree · rat · intestinal microflora · bile acids

DANIELSSON AND GUSTAFSSON reported that rats reared in a germfree environment showed higher serum cholesterol levels than comparable conventional animals (1). Wostmann and Wiech (2) found that in the absence of a microflora, serum cholesterol levels of 3-month old rats were slightly lower than those found in conventional controls, but that liver cholesterol concentrations in the germfree animal were higher at all ages.

In both germfree (3) and antibiotic-treated conventional (4) rats, the rate of fecal excretion of bile acids was greatly decreased. All bile acids found in the feces of the germfree rats were conjugated and unmodified from the biliary bile acids (5). The half-life of taurocholic acid in the germfree group was 2–3 times longer than in the conventional animals.

It has been proposed (6) that a feedback mechanism controlled by the bile acid turnover rate might affect cholesterol biosynthesis. Since in the absence of an intestinal flora the bile acid turnover time was prolonged, presumably owing to a more effective reabsorption of the intact conjugated acid, the oxidative catabolism of cholesterol would be retarded and would lead to the higher cholesterol concentrations observed in the liver of the germfree rat (2). The germfree rat also lacks the bacterial conversion mechanism which normally changes cholesterol to less absorbable sterols (1, 7). At least 82% and probably more of the sterols excreted in the feces of the germfree rat were in the form of unchanged cholesterol (1). This would lead to a more effective recirculation of cholesterol and would amplify the effect of the germfree state on liver cholesterol concentration.

The availability of germfree animals makes it possible to obtain a baseline for systemic cholesterol and bile acid metabolism, and then to study the effect of the intestinal microflora or of its isolated components on sterol metabolism.

The use of cholesterol-26-$^{14}$C for the study of the catabolism of cholesterol has the advantage that in the process of its conversion to bile acids the label is liberated from the steroid moiety. Siperstein and Chaikoff (8) administered cholesterol labeled both in the 4- and the 26-positions to bile duct-cannulated rats. In their experiments over 90% of the administered cholesterol was converted to bile acids, while most of the 26-$^{14}$C was expired as $^{14}CO_2$. Thus the $^{14}CO_2$ content of the expired air appeared to be a suitable indicator of the rate of oxidative catabolism of cholesterol in the rat.

Combined with gnotobiotic techniques this method should provide an ideal test system to study the effect of the intestinal microflora and its components on this conversion.

In this report we present data on the metabolism of cholesterol-26-$^{14}$C in germfree and conventional rats, with special emphasis on the distribution of the label within the body. Previous reports (9, 10) had indicated that $^{14}$C-labeled propionate and acetone were major products formed during the oxidative cleavage of cholesterol-26-$^{14}$C in vitro by liver mitochondria, and that extensive reincorporation of the label occurred. The present data show that upon oxidative degradation of the cholesterol molecule in the intact rat more than 75% of the label in the 26-position appears as expired $^{14}CO_2$, and that the presence of a "normal" intestinal microflora increases the rate of the oxidative conversion by at least 40% over the values found in the germfree animal.

## MATERIALS AND METHODS

Germfree male rats of Wistar origin (Lobund strain) were reared in the Trexler plastic germfree system and were fed a sterilized semisynthetic diet, L-356 (11) from weaning. This formula contains less than 25 mg of cholesterol per 100 g of diet (2). Genetically closely related conventional animals were housed in the open animal colony and fed the same sterilized diet. Each experimental group consisted of 10 animals. At approximately 100 days of age, each animal was anesthetized with sodium pentobarbital and injected intravenously via the femoral vein with 1 ml of a solution containing approximately 5 $\mu$c of cholesterol-26-$^{14}$C and 200 $\mu$g of carrier cholesterol in saline–Tween detergent, prepared according to the method of Meier, Siperstein, and Chaikoff (12). An aliquot of this solution was assayed to determine the total dose (in counts per minute) which each individual animal received.

Immediately after injection each rat was placed in a closed system plastic metabolism chamber similar to that described by Edwards, Edwards, and Gadsden (13), with ad libitum access to both food and water. During the entire 72 hr metabolic period, $CO_2$-free air was circulated through the chamber at the rate of 2–2.5 liters/min. The expired $CO_2$ was collected in 5 N NaOH. Aliquots of the $Na_2CO_3$ were converted to $BaCO_3$ by the method of Braasch, Levenson, Crowley, Vinecour, and Vinecour (14), and the derived barium carbonate was assayed for $^{14}$C with a thin-window gas flow Geiger-Müller counter. Necessary corrections for background and self-absorption were made in all determinations. The total $CO_2$ expired was calculated from $BaCO_3$ weights.

During the metabolic period both urine and feces were collected. At the end of the 72 hr experimental period the animals were anesthetized with sodium pentobarbital and exsanguinated via cardiac puncture. Plasma was collected using heparin. The liver and cecal contents were removed and homogenized in saline, as was the remainder of the carcass. All homogenates were stored at −20°. Aliquots of plasma and urine, cecal, the liver, and carcass homogenates, and aliquots of the liver and carcass extractions (described in detail below) were brought on planchets, dried, and assayed for $^{14}$C content as indicated above.

Aliquots of both liver and carcass homogenates were extracted with 250 ml of Bloor's mixture according to the procedure described by Entenman (15) and the resulting filtrate was divided into two equal portions of 100 ml. Both portions were concentrated to approximately 6 ml. Lipid material was extracted from one portion with 3 × 100 ml of petroleum ether; the remaining alcohol–water phase containing approximately equal parts of alcohol and water was retained. The $^{14}$C content of the lipid phase was determined after the petroleum ether extract had been evaporated to dryness in a stream of nitrogen and the residue taken up in redistilled chloroform. The radioactivity in the alcohol–water phase was also determined. The other portion of the concentrated Bloor extract was saponified with 5 ml of 30% KOH for 5 hr and the nonsaponifiable material was extracted according to a modification of the procedure of Siperstein and Chaikoff (8) with 4 × 100 ml of petroleum ether. The combined extracts were evaporated to dryness in a stream of nitrogen and the residue was taken up in acetone–alcohol 1:1 (v/v). The $^{14}$C of the nonsaponifiable material was determined directly on an aliquot of alcohol–acetone solution. Another aliquot was treated with a 2% solution of digitonin in 96% alcohol and the Sperry procedure (16) for isolation of digitonin-precipitable material (3$\beta$-OH sterols) was followed. The washed precipitate was dissolved in pyridine for determination of $^{14}$C. The collected feces were extracted with alcohol according to the method of Siperstein and Chaikoff (8) and $^{14}$C was determined directly in the extract. "Specific activity" values were calculated in each case by dividing the percentage of the total dose found in the 3$\beta$-OH sterol fraction by its total cholesterol content. This value was normalized to a body weight of 100 g and the resulting figure was arbitrarily multiplied by 1000.

Total cholesterol content of the plasma, carcass, and liver was determined according to procedures previously described (2).

## RESULTS

Data regarding the $CO_2$ and $^{14}CO_2$ expired by the rat during the 72 hr experimental period following the in-

TABLE 1 $CO_2$ AND $^{14}CO_2$ PRODUCED BY GERMFREE AND CONVENTIONAL MALE RATS AFTER INTRAVENOUS ADMINISTRATION OF CHOLESTEROL-26-$^{14}$C

| | Germfree | Conventional | P |
|---|---|---|---|
| Body weight, *g* | 308 ± 10 | 291 ± 15 | N.S. |
| Liver weight, % *body weight* | 3.35 ± 0.06 | 4.01 ± 0.1 | <0.01 |
| Total $CO_2$, *g/100 g body weight* | 10.5 ± 0.6 | 12.2 ± 1.3 | 0.14 |
| $^{14}C$ in $CO_2$, % *total dose* | 19.0 ± 1.2 | 29.7 ± 2.0 | <0.01 |

Experimental period: 72 hr. Diet: Semisynthetic L-356 (11). 10 animals per group. Mean values ± SEM.

TABLE 2 PERCENTAGE DISTRIBUTION OF $^{14}C$ AFTER INTRAVENOUS ADMINISTRATION OF CHOLESTEROL-26-$^{14}$C TO GERMFREE AND CONVENTIONAL MALE RATS

| | Germfree | Conventional | P |
|---|---|---|---|
| Expired air | 19.0 ± 1.2 | 29.7 ± 2.0 | 0.01 |
| Carcass: 3β-OH sterol | 48.9 ± 1.7 | 42.5 ± 1.7 | 0.02 |
| Other | 3.7 ± 1.1 | 4.1 ± 1.2 | N.S. |
| Liver: 3β-OH sterol | 10.0 ± 0.6 | 7.5 ± 0.3 | 0.01 |
| Other | 1.7 ± 0.4 | 2.2 ± 0.5 | N.S. |
| Fecal extract | 2.6 ± 0.4 | 4.6 ± 0.4 | 0.01 |
| Residual (urine, cecal contents, blood, etc.) | 3.2 ± 0.1 | 2.3 ± 0.1 | 0.01 |
| Total recovery of original dose | 89.1 ± 1.8 | 92.9 ± 2.0 | N.S. |

Conditions as in Table 1. Mean values ± SEM.

travenous administration of cholesterol-26-$^{14}$C are given in Table 1. They show that the germfree rats expired on the average 19% of the label originally contained in the cholesterol, compared with 30% for the conventional animals. The data for the conventional animals are similar to those reported by Siperstein and Chaikoff (8). Total $CO_2$ generated by the germfree animals was slightly, but not significantly, lower than that found for the conventional group.

The data also demonstrate a slight difference in liver weight as a percentage of body weight between the two experimental groups which was not observed in earlier work (2, 17). However, when total liver weights are compared, no significant difference is found. The greater cecal weights of the germfree animals may be the cause of the slightly higher body weight in this group.

Table 2 shows the distribution and recovery of $^{14}C$ 72 hr after administration of cholesterol-26-$^{14}$C. Compared to the germfree animal, the rat harboring a "normal" flora releases 50% more $^{14}CO_2$ with the expired air. Recovery of the label in the 3β-OH sterol fraction of the liver and carcass of the conventional animals is lower than in their germfree counterparts. Assuming that part of the $^{14}C$ found in the alcoholic fecal extract and in the fraction termed "residual," is in the form of 3β-OH sterol, then slightly more than 67% (the combined values in liver and carcass) of the label recovered in the germfree group is present as 3β-OH sterol, presumably unchanged cholesterol, against somewhat more than 54% in the conventional animal. In both experimental groups the liver contains 14–16% of the unconverted cholesterol, while the carcass contains approximately 80%. Of the label recovered from the germfree and conventional rats, respectively, in forms other than 3β-OH sterol, approximately three-fourths is expired as $^{14}CO_2$, while one-fourth remains in the body or is excreted with feces or urine. From 6 to 7% is found in liver and carcass. The total amount of $^{14}C$ found in the alcoholic extract of the feces is higher in the conventional group, while the "residual" $^{14}C$ value is higher in germfree animals, because of the greater amount of the label present in the more voluminous cecal contents of the germfree rat. The total amount of $^{14}C$ excreted in feces and urine amounts to 3.6% of the recovered dose in the germfree and 5.6% in the conventional animal (approximately 0.7% is excreted with the urine in both groups).

Table 3 depicts a more detailed distribution of $^{14}C$ in carcass and liver. The data demonstrate the greater percentage of label recovered from the germfree animal. They further show that 72 hr after the administration of cholesterol-26-$^{14}$C, in both experimental groups liver and carcass contain approximately 4% of the $^{14}C$ in a nonlipid form soluble in dilute alcohol. In the carcass 92% and in the liver approximately 80% of the label is present in the form of 3β-OH sterol. In all series the $^{14}C$ content of the total lipid fraction is slightly higher than the value found in the nonsaponifiable fraction, which in turn shows a somewhat higher $^{14}C$ content than the 3β-OH sterols. These findings suggest that some reincorporation of $^{14}C$ could have occurred in these fractions and that, theoretically at least, the possibility of reincorporation of $^{14}C$ into the cholesterol fraction must be considered.

Cholesterol content and "specific activity" are given in Table 4. The data confirm the earlier observation (2) that the cholesterol concentration in the liver of the germfree rat is approximately 20% higher than the value found in the conventional animal. They also corroborate the slightly lower concentration in the serum of the 3 month old germfree male rat (2), although with the present number of animals this small difference is not statistically significant. The total cholesterol contents of liver, carcass, and of the blood–liver pool show little difference, although the carcass cholesterol and the total body cholesterol calculated from these data could be slightly higher in the germfree group.

The "specific activity" of the 3β-OH sterol fraction in the liver of the germfree animal is significantly higher than the value found in the conventional group, demonstrating the faster turnover of cholesterol in the presence

TABLE 3 PERCENTAGE DISTRIBUTION OF $^{14}C$ IN LIVER AND CARCASS OF GERMFREE AND CONVENTIONAL MALE RATS AFTER INTRAVENOUS ADMINISTRATION OF CHOLESTEROL-26-$^{14}C$

| | Carcass | | | Liver | | |
|---|---|---|---|---|---|---|
| | Germfree | Conventional | *P* | Germfree | Conventional | *P* |
| Total | 52.6 ± 1.9 | 46.6 ± 1.4 | 0.04 | 11.7 ± 0.8 | 9.7 ± 0.4 | 0.05 |
| Soluble in aqueous alcohol* | 1.7 ± 0.2 | 2.0 ± 0.5 | N. S. | 0.2 ± 0.1 | 0.4 ± 0.1 | N. S. |
| Lipid | 50.8 ± 2.2 | 43.1 ± 2.2 | <0.01 | 10.7 ± 0.7 | 8.6 ± 0.3 | 0.01 |
| Unsaponifiable | 50.1 ± 2.1 | 43.1 ± 1.8 | 0.03 | 10.2 ± 0.6 | 8.2 ± 0.4 | 0.02 |
| 3β-OH sterol | 48.9 ± 1.7 | 42.5 ± 1.7 | 0.02 | 10.0 ± 0.6 | 7.5 ± 0.3 | <0.01 |

Conditions as in Table 1. Mean values ± SEM.
* Soluble in approximately 50% alcohol (v/v); see text.

TABLE 4 AMOUNT AND SPECIFIC ACTIVITY OF CHOLESTEROL OF GERMFREE AND CONVENTIONAL MALE RATS AFTER INTRAVENOUS ADMINISTRATION OF CHOLESTEROL-26-$^{14}C$

| | Cholesterol Concentration | Total Cholesterol* | "Specific Activity"† | Activity/mg Cholesterol‡ |
|---|---|---|---|---|
| | *mg*/100 *g* | *mg* | | |
| Carcass | | | | |
| Germfree | 132 ± 8 | 122 ± 9 | 431 ± 42 | — |
| Conventional | 117 ± 6 | 106 ± 5 | 422 ± 36 | — |
| *P* | N.S. | 0.12 | N.S. | — |
| Liver | | | | |
| Germfree | 394 ± 14 | 13.2 ± 0.5 | 760 ± 49 | 892 ± 57 |
| Conventional | 336 ± 19 | 13.4 ± 0.7 | 589 ± 37 | 693 ± 43 |
| *P* | 0.03 | N.S. | <0.01 | 0.02 |
| | *mg*/100 *ml* | | | |
| Plasma | | | | |
| Germfree | 110 ± 2 | — | — | 960 ± 74 |
| Conventional | 119 ± 6 | — | — | 685 ± 54 |
| *P* | N.S. | — | — | 0.01 |
| Blood–liver pool§ | | | | |
| Germfree | — | 20.4 ± 0.6 | — | — |
| Conventional | — | 21.1 ± 0.5 | — | — |
| *P* | — | N.S. | — | — |

Conditions as in Table 1. Mean values ± SEM.
* Calculated per 100 g body weight.
† $\frac{\%\text{ dose in 3}\beta\text{-OH sterol fraction}}{\text{mg total cholesterol}} \times \frac{\text{body weight}}{100} \times 1000.$
‡ $\frac{\%\text{ dose in tissue}}{\text{mg total cholesterol}} \times \frac{\text{body weight}}{100} \times 1000.$
§ Calculated on the basis of a blood volume of 6% of body weight.

of an intestinal microflora. Carcass cholesterol specific activity values lie markedly below the liver values.

## DISCUSSION

Cholesterol solubilized as a Tween–cholesterol complex and injected into the circulation is rapidly taken up by the liver and subsequently enters the various metabolic cycles.[1] In the case of labeled cholesterol, rapid equilibrium within the blood–liver pool can be expected (18). Equilibration with other tissue spaces is apparently less rapid (19). The data in Table 4 indicate that 72 hr after administration of cholesterol-26-$^{14}C$ the specific activity of the cholesterol fraction in the carcass had not yet reached the values found in the liver. With cholesterol-26-$^{14}C$ being constantly catabolized and the specific activity values in the blood–liver pool decreasing accordingly, the average specific activity of the cholesterol found in the rest of the body had not attained its potential maximum value after the 72 hr period.

Suld, Staple, and Gurin (10), working with rat liver mitochondria, have claimed that the major part of $^{14}C$ liberated by the oxidative degradation of the side chain of the cholesterol molecule is reincorporated into other metabolic cycles, especially into the Krebs cycle. Our results demonstrate that after 72 hr in both germfree and conventional rats at least 75% of the $^{14}C$ not in the form of 3β-OH sterol can be accounted for as "expired $^{14}CO_2$." Thus, the amount of $^{14}CO_2$ expired by the rat after intravenous cholesterol-26-$^{14}C$ administration is a valid indicator of the rate of oxidative catabolism of cholesterol. When used to study the effect of the intestinal flora on this conversion, it should be noted that Chaikoff et al. (20) demonstrated that no liberation of the 26-C atom by direct bacterial action took place.

The data indicate, however, that some reincorporation of the label takes place. $^{14}C$ is found in forms soluble in

[1] Wostmann, B. S., and T. F. Kellogg, unpublished results.

dilute alcohol, presumably representing both carbonate ions, and $C_2$ and $C_3$ moieties in the process of being reincorporated via the Krebs cycle (Table 3). The fact that in all series (carcass and liver values of both germfree and conventional animals) the $^{14}C$ content of the total lipid fraction is slightly higher than that found in the nonsaponifiable fraction, which in turn shows a slightly higher value than the 3$\beta$-OH sterol fraction, could reflect the occurrence of slight losses of cholesterol-26-$^{14}C$ in saponification and precipitation procedures. It could also indicate, however, that some reincorporation of $^{14}C$ into the various lipid fractions has taken place. In the liver the amount of $^{14}C$ in lipids other than 3$\beta$-OH sterol was approximately 10% of the total radioactivity in that organ. Since on a balanced diet 3$\beta$-OH sterols comprise less than 10% of the total liver lipids (21) and as it may be assumed that reincorporation of the tracer into the various lipid fractions occurs more or less at random, with rates comparable in order of magnitude, it can be concluded that a possible reincorporation of $^{14}C$ into the 3$\beta$-OH sterol fraction could amount to no more than 1% of the total radioactivity found in that organ. Similar arguments lead to an even lower level of reincorporation of $^{14}C$ into the 3$\beta$-OH sterol fraction of the carcass. The amount of $^{14}C$ found in the 3$\beta$-OH sterol fraction of liver and carcass represents therefore, for all practical purposes, unchanged cholesterol-26-$^{14}C$.

Gustafsson has demonstrated that rats harboring a "normal" intestinal microflora turn over bile acids three times faster than germfree rats (3). In the present data both the increased elimination of the tracer as $^{14}CO_2$ and the decreased retention of original cholesterol-26-$^{14}C$ in liver and carcass of the conventional animals indicate a faster oxidative catabolism of cholesterol in the conventional animal. Although Gustafsson states that little difference exists between the actual size of the bile acid pools in germfree and conventional rats (3), the present data fit the assumption that in conventional animals a lower bile acid concentration occurs at the site of the oxidative conversion of the cholesterol side chain, presumably the liver mitochondria. This would enhance the conversion of cholesterol to bile acid (22) and would act in accordance with the feedback mechanism postulated by Beher, Baker, Anthony, and Beher (6). The increased cholesterol catabolism in turn would lead to the lower concentration of liver cholesterol in conventional rats demonstrated in Table 4 and in previous studies (2). However, owing to the smaller livers found in the germfree animals, the sizes of the liver total cholesterol pools and of the blood–liver total cholesterol pools in both experimental groups were quite similar.

The present data agree with the observation that in the rat the oxidative conversion to bile acids is the major route of elimination of cholesterol (23). During the 72 hr experimental period following the administration of the cholesterol-26-$^{14}C$, germfree rats released 19% and conventional animals 30% of the terminal C atom of the cholesterol side chain. During the same period only 3.2% and 5.2% respectively of the tracer was excreted with feces and urine. These last figures represent the maximum possible amount of original cholesterol eliminated as neutral sterols.

The data demonstrate a major accelerating effect of the intestinal microflora on cholesterol catabolism. Presumably this phenomenon is caused mainly by an increase in the elimination of bile acids. Bile acid and cholesterol pool sizes are at best only slightly affected by this acceleration of the cholesterol catabolism, indicating that in the rat, cholesterol anabolism and catabolism are well balanced.

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