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-26-14C the animals were housed in a plastic metabolism chamber for 72 hr. Expired CO2 was collected throughout the period. The conventional rats released 50% more 14C as 14CO2 than the germfree animals. The total amount of the label recovered as 14CO2 during the 72 hr period amounted to 30% and 19% respectively, of the original dose. In both conventional and germfree rats the release of 14CO2 accounted for approximately 75% of the 14C recovered in forms other than the original cholesterol-26-14C; 15-20% was found incorporated in water-soluble and fat-soluble fractions other than 3β-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 14CO2 from cholesterol-26-14C in the intact rat is a suitable and convenient indicator of the oxidative catabolism of cholesterol.

KEY WORDS cholesterol-26-14C, 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-26-14C 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 26-14C was expired as 14CO2. Thus the 14CO2 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-26-14C in germfree and conventional rats, with special emphasis on the distribution of the label within the body. Previous reports (9, 10) had indicated that 14C-labeled propionate and acetone were major products formed during the oxidative cleavage of cholesterol-26-14C 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 14CO2, 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 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 14C 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 X 100 ml of petroleum ether; the remaining alcohol-water phase containing approximately equal parts of alcohol and water was retained. The 14C 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 X 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 14C 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 (3β-OH sterols) was followed. The washed precipitate was dissolved in pyridine for determination of 14C. The collected feces were extracted with alcohol according to the method of Siperstein and Chaikoff (8) and 14C 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 3β-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 CO2 and 14CO2 expired by the rat during the 72 hr experimental period following the in-
sterol, then slightly more than 67% (the combined fraction termed "residual," is in the form of 3β-OH cholesterol). The germfree, compared with 30% for the conventional animals. 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 3β-OH sterol. These findings suggest that some reincorporation of 14C could have occurred in these fractions and that, theoretically at least, the possibility of reincorporation of 14C 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 of 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 14CO2, 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 14C found in the alcoholic extract of the feces is higher in the conventional group, while the "residual" 14C 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 14C 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 14C 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-26-14C, in both experimental groups liver and carcass contain approximately 4% of the 14C in a non-lipid 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 14C content of the total lipid fraction is slightly higher than the value found in the nonsaponifiable fraction, which in turn shows a somewhat higher 14C content than the 3β-OH sterols. These findings suggest that some reincorporation of 14C could have occurred in these fractions and that, theoretically at least, the possibility of reincorporation of 14C into the cholesterol fraction must be considered.

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

<table>
<thead>
<tr>
<th></th>
<th>Germfree</th>
<th>Conventional</th>
<th>P</th>
<th>Germfree</th>
<th>Conventional</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>52.6 ± 1.9</td>
<td>46.6 ± 1.4</td>
<td>0.04</td>
<td>11.7 ± 0.8</td>
<td>9.7 ± 0.4</td>
<td>0.05</td>
</tr>
<tr>
<td>Soluble in aqueous alcohol*</td>
<td>1.7 ± 0.2</td>
<td>2.0 ± 0.5</td>
<td>N.S.</td>
<td>0.2 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>N.S.</td>
</tr>
<tr>
<td>Lipid</td>
<td>50.8 ± 2.2</td>
<td>43.1 ± 2.2</td>
<td>&lt;0.01</td>
<td>10.7 ± 0.7</td>
<td>8.6 ± 0.3</td>
<td>0.01</td>
</tr>
<tr>
<td>Unaponifiable</td>
<td>50.1 ± 2.1</td>
<td>43.1 ± 1.8</td>
<td>0.03</td>
<td>10.2 ± 0.6</td>
<td>8.2 ± 0.4</td>
<td>0.02</td>
</tr>
<tr>
<td>3β-OH sterol</td>
<td>48.9 ± 1.7</td>
<td>42.5 ± 1.7</td>
<td>0.02</td>
<td>10.0 ± 0.6</td>
<td>7.5 ± 0.3</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

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-26-14C

<table>
<thead>
<tr>
<th></th>
<th>Carcass</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/100 g</td>
<td>mg</td>
</tr>
<tr>
<td>Cholesterol Concentration</td>
<td>132 ± 8</td>
<td>122 ± 9</td>
</tr>
<tr>
<td>Germfree</td>
<td>117 ± 6</td>
<td>106 ± 5</td>
</tr>
<tr>
<td>Conventional</td>
<td>N.S.</td>
<td>0.12</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>N.S.</td>
</tr>
<tr>
<td>Plasm</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood-liver pool§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germfree</td>
<td>394 ± 14</td>
<td>13.2 ± 0.5</td>
</tr>
<tr>
<td>Conventional</td>
<td>336 ± 19</td>
<td>13.4 ± 0.7</td>
</tr>
<tr>
<td>P</td>
<td>0.03</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Conditions as in Table 1. Mean values ± SEM.
* Calculated per 100 g body weight.
† % dose in 3β-OH sterol fraction × body weight × 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. 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-26-14C the specific activity of the cholesterol fraction in the carcass had not yet reached the values found in the liver. With cholesterol-26-14C 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 14C 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 14C not in the form of 3β-OH sterol can be accounted for as “expired 14CO2.” Thus, the amount of 14CO2 expired by the rat after intravenous cholesterol-26-14C 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. 14C is found in forms soluble in
dilute alcohol, presumably representing both carbonate ions, and C4 and C3 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 14C 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 3β-OH sterol fraction, could reflect the occurrence of slight losses of cholesterol-26-14C in saponification and precipitation procedures. It could also indicate, however, that some reincorporation of 14C into the various lipid fractions has taken place. In the liver the amount of 14C in lipids other than 3β-OH sterol was approximately 10% of the total radioactivity in that organ. Since on a balanced diet 3β-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 14C into the 3β-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 14C into the 3β-OH sterol fraction of the carcass. The amount of 14C found in the 3β-OH sterol fraction of liver and carcass represents therefore, for all practical purposes, unchanged cholesterol-26-14C.

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 14CO2 and the decreased retention of original cholesterol-26-14C 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-26-14C, 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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