Intestinal Microflora and Cholesterol Catabolism^{1,2}

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Although the direct relationship between serum cholesterol and atherosclerosis remains obscure, recent data again indicate that most, if not all, of the cholesterol found in aortic lesions is derived from the circulating blood (1). In the etiology of coronary heart disease high cholesterol levels are considered a danger sign and the reduction of body cholesterol pools is regarded as being of major importance in both prophylaxis and treatment (2).

The loss of cholesterol from the body pools is mainly determined by its oxidative conversion to bile acids and by the loss of cholesterol and its bacterial conversion products in the feces. The main catabolic pathway, the conversion to bile acids, presumably is controlled by the size of the bile acid pools (3). The turnover of bile acid pools is strongly reduced in the absence of an intestinal microflora (4). Thus the metabolism of cholesterol is influenced in diverse ways by the various components of the intestinal flora, but the sum of these effects would presumably increase the turnover of cholesterol in the body pools because of an increased loss of cholesterol's major metabolite, the bile acids. This seems to be reflected in the higher liver cholesterol values found in germfree rats of all ages (5).

The purpose of the study presented here was the development of a system in which the above presumptions could be tested. As a first approach we studied the turnover of cholesterol -26-C¹⁴, both in the presence and in the absence of a "normal" intestinal microflora. The results indicate an increase of 50% in cholesterol catabolism over "germfree" values in the presence of an intestinal microflora. A more complete report of this work will be published elsewhere.

Materials and Methods

Three to 5 months old male germfree and conventional rats of the Lobund strain (of Wistar origin) were used. The diet was L-356, a semisynthetic formula containing 20% casein, 60% rice flour, 7% corn oil, yeast, liver extract, vitamins and minerals (6). After injection of approximately 6-7 μ C of cholesterol-26-C¹⁴, solubilized with Tween 20, into the femoral vein, the experimental animal was housed in a plexiglas, gas-tight metabolism chamber (20 x 20 x 16.5 cm), provided with an inlet and outlet for air. Incoming air was dried and freed of CO₂. Outgoing air was conducted through three wash bottles containing 5 N NaOH to trap expired CO₂. In the germfree experiments this system was enclosed in a sterile plastic isolator and the air passing through the metabolism chamber was sterilized by passage through a

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fiberglas filter. In all experiments an airflow of 2.0-2.5 liter/min. was maintained. The experimental period was 72 hours, after which the animals were removed from the metabolic chamber and sacrificed for organ analysis. The expired trapped CO_2 was precipitated as $BaCO_3$ and plated on planchets for determination of radioactivity. Liver and carcass were homogenized and the separation into water soluble, lipid and unsaponifiable fractions was achieved following generally accepted methods. The beta sterol fraction was isolated after saponification via digitonin precipitation (7). Aliquots of these fractions were used for the determination of radioactivity.

The specific activity of total cholesterol in liver and carcass was obtained by dividing the counts in the digitonin precipitated samples by the amount of total cholesterol as determined by our modification of the Liebermann-Burchard method (5). Only in the case of plasma was the specific activity calculated by taking the total radioactivity of the sample divided by its cholesterol content.

Results and Discussion

After a 72 hour experimental period, on the average $92 \pm 1\%$ of the C¹⁴ label could be recovered in expired air, liver, carcass, cecal contents, and excreta. Calculated on the total recovered dose, the

TABLE I

C¹⁴ distribution after cholesterol-26-C¹⁴ administration to adult germfree and conventional male rats. Time: 72 hours.

Diet: L-356.			
	Germfree	Conventio	nal
Total	100	100	
Expired air	22	32	s
Carcass beta-sterol	55	46	s
other	3	4	
Liver beta-sterol	11	8	s
other	2	2	
Excreted in feces	3	5	s
Balance (urine, blood, cecal content etc.)	4	3	

distribution of the label is given in Table I. The data indicate a 50% increase in oxidative cholesterol conversion over germfree values, obviously caused by the presence of the intestinal microflora. A correspondingly lower retention of the originally administered cholesterol- $26-C^{14}$ in indicated by the lower values found in the carcass and liver beta sterol fraction of the conventional rats. The significantly higher excretion of label with the feces in these animals presumably represents an increased loss of cholesterol as coprostanol or other bacterial conversion products in which the side chain remains intact.

In vitro studies by Suld et al., suggest the possibility of the loss of the last 3 carbon atoms of the side chain as a unit which then, for the

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most part, would enter the tricarboxylic acid cycle and lead to extensive reincorporation of the tracer (8). Such an effect would seriously endanger the interpretation of experiments in which the liberation of $C^{14}O_2$ from cholesterol-26- C^{14} would be taken as a measure of the rate of oxidative catabolism of cholesterol. However, the data in Table

TABLE II

C¹⁴ distribution in liver after cholesterol-26-C¹⁴ administration to adult germfree and conventional male rats. Time: 72 hours. Diet: L-356. SDM values given.

Per cent dose in:	Germfree (7)	Conventional (10)
total water sol. lipid unsapon. beta-sterol Total liver cholesterol/	$egin{array}{llllllllllllllllllllllllllllllllllll$	9.7 ± 0.4 0.1 ± 0.1 8.6 ± 0.6 8.2 ± 0.5 7.5 ± 0.3 s
100 gm body weight Spec. activity*	$13.0,\pm0.6$ 744	$13.3 \pm 0.6 \\ 589 \qquad { m s}$

* % dose/mg/min./100 gm body weight x103.

II show that most of the radioactivity found in the liver can be accounted for as the originally injected labelled cholesterol (beta sterol fraction). Some C^{14} containing water soluble metabolites apparently occur and a slight reincorporation into other lipid fractions, like the fatty acids, may be indicated. However, as the data in Table III indicate,

TABLE III

C¹⁴ distribution after cholesterol-26-C¹⁴ administration to adult germfree and conventional male rats. Values in per cent dose. SDM values given. Time: 72 hours. Diet: L-356.

	Germfree (8)	Conventional (10)
Expired air	19.3 ± 1.5	29.7 ± 1.7 s
Liver (beta-sterol fract.)	9.8 ± 0.7	7.5 ± 0.1 s
Carcass (beta-sterol fract.)	48.3 ± 2.1	$42.5\pm1.7~ m s$
Total	77.4	79.7

expired $C^{14}O_2$ plus the original cholesterol-26- C^{14} in liver and carcass account for about 78% of the originally injected dose (and for 87% of the recovered dose). This calculation leaves the partially unidentified form of the label, as present in feces, urine, blood and cecal contents still unaccounted for. Thus, it would seem that in the intact rat the expired $C^{14}O_2$ is a good indicator of the cholesterol-26-C¹⁴ cholic acid conversion.

All data indicate an increase of oxidative conversion of cholesterol in the conventional animal and a correspondingly lower retention in its liver and carcass, while body cholesterol pools are approximately the same size in both experimental categories. This leads to the differences in specific activity of the cholesterol in liver and plasma as shown in Table IV. The data also demonstrate that the equilibration of the

TABLE IV

Specific activity of beta-sterols after cholesterol-26-C¹⁴ administration to adult germfree and conventional male rats. Values in % dose/mg./ min./100 gm body weight x10³. SDM values given.

Time: 72 hours. Diet: L-356.

	Germfree (8)	Conventional (10)
Plasma*	889 ± 74	$685\pm 64~ m s$
Liver	744 ± 63	$589\pm37~ m s$
Carcass	478 ± 31	422 ± 35

* Total activity.

carcass cholesterol fraction proceeds relatively slow, causing the specific activity in this pool to lag behind that of the liver.

The main mechanism involved in the increased cholesterol catabolism seems to be the bacterial clearage of the conjugated bile acids (4), and possibly the dehydroxylation in the 7-position (9), both effects that tend to make the bile acids less available for reabsorption. By fecal elimination of bile acids, a faster oxidative catabolism of cholesterol becomes possible, which brings body cholesterol pools to a lower level.

Thus intestinal microorganisms help to eliminate a potentially dangerous metabolite. A next step will be to determine, with the help of gnotobiotic techniques, which components of the intestinal microflora are instrumental in increasing cholesterol catabolism. And in the distant future we foresee the possibility that dietary and other means will regulate the composition of the microflora to a point where the presence of bacterial species with a potentially beneficial effect, like the one illustrated above, could be enhanced.

Summary

The degradation of cholesterol was studied in germfree and conventional rats with the help of cholesterol-26-C¹⁴. The major part of the tracer liberated from the cholesterol molecule during the oxidative conversion could be recovered as $C^{14}O_2$ from the expired air. The results indicate a 50% greater degradation of cholesterol in the presence of a "normal" intestinal microflora.

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