Steroid balance and tissue cholesterol accumulation in germfree and conventional rats fed diets containing saturated and polyunsaturated fats

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Abstract

Steroid balance studies were conducted on 24 conventional and 12 germfree male rats, 90-120 days old, fed diets containing either 20% safflower or 20% coconut oil. Both germfree and conventional rats fed the safflower oil diets had significantly lower serum cholesterol levels and significantly higher liver cholesterol levels than did the rats fed coconut oil. No significant differences in total fecal neutral sterols, coprostanol, 5α-cholest-7-en-3β-ol, or total fecal bile acid excretion were seen between dietary groups of rats of either status. There was no evidence of qualitative differences in fecal bile acid excretion as a function of diet. The increased liver cholesterol was in the ester form, with cholesteryl linoleate the largest single component. There was no significant difference in the cholesterol content of the skin, muscle, adipose tissue, or gastrointestinal tract. The significance of a large increase in liver cholesteryl ester, lowered serum cholesterol, and no change in steroid excretion is discussed.

Supplementary key words bile acids · coprostanol · Δ7-cholestenol · cholesteryl esters · cholesteryl linoleate · intestinal microflora · fecal steroids

Studies in our laboratory have shown that dietary carbohydrates can affect the microbiological modification of cholesterol (1) and bile acids (2), resulting in qualitatively different fecal sterol excretion. Other investigators (3) have reported that feeding polyunsaturated fats resulted in an increased excretion of "nondigitonin-precipitable sterols," possibly coprostanol. Further studies (4) showed that feeding dietary linoleic acid increased the fecal excretion of coprostanol, which is formed from cholesterol by microbial action in the gut. Others have questioned whether changes in bacterial activity on steroids in the gut may have been responsible for the observed differences (5) in cholesterol metabolism as a response to fatty acid changes in the diet.

The studies reported in this paper investigate (a) if there is any evidence of differences in microbially modified fecal steroids that are related to serum cholesterol lowering by polyunsaturated fats, (b) if similar systematic effects of polyunsaturated fat diets are seen in germfree and conventional rats, and (c) what changes in tissue and serum cholesterol levels and forms occur as a result of consumption of different dietary fats by rats.

METHODS

Experiments 1 and 2

Lobund strain rats (of Wistar origin), 90–120 days old, were placed in metabolism cages for 28 days for stabilization to diet and quarters. The germfree rats were fed a modification of diet 474-E-12 (6), a semipurified cholesterol-free diet based on casein, rice starch, 5% corn oil, and crystalline vitamins and minerals. In these experiments, 20% by weight of starch was replaced by either coconut oil or safflower oil. Both of these fats were analyzed by GLC for fatty acids and neutral sterols. The analysis indicated that the fats were pure and unadulterated. The assayed cholesterol content of both diets was less than 0.01 mg/g of diet, probably from the casein. The safflower oil diet contained 0.28 mg of β-sitosterol/g and the coconut oil diet 0.16 mg of β-sitosterol/g (from the added oils).

After the 28-day preexperimental period, feces were quantitatively collected for a 5-day period for steroid balance studies. Food consumed and the weights of the rats were measured for the steroid balance studies during the collection period. The rats were fasted overnight and exsanguinated by heart puncture under Nembutal anesthesia, and the liver was taken for cholesterol analysis.

Abbreviations: cholesterol, cholest-5-ene-3β-ol; coprostanol, 5β-cholest-7-en-3β-ol; Δ7-cholestenol, 5α-cholest-7-en-3β-ol; β-sitosterol, 24β-ethyl-cholest-5-ene-3β-ol; GLC, gas-liquid chromatography; TLC, thin-layer chromatography; TMS, trimethylsilyl.

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Cholesterol assays

Serum cholesterol was analyzed by the Caraway (7) modification of the FeC13 method. Skin, fat, muscle, gastrointestinal tract, and liver were extracted by the method of Radin (8), and cholesterol was assayed on the extract by the same colorimetric method as the serum assays above. An aliquot of the liver extract was taken for cholesterol ester hydrolysis and assay. Liver and serum cholesterol were also analyzed by GLC (see below). Cholesterol was the only sterol detected in the serum and liver.

Liver cholesteryl esters, triglycerides, and cholesterol were separated, as outlined by Radin (8), by TLC on silica gel G plates developed in CHCl3-benzene 1:1. After detection by rhodamine 6G, the cholesteryl esters and free cholesterol were eluted with CHCl3. The esters were saponified and the cholesterol was analyzed by GLC as the TMS ether. The fatty acids from the esters and the triglycerides were methylated and analyzed by GLC on a 10% EGA column at 180°C. The GLC system was verified by analyzing quantitative fatty acid standard mixture KD obtained from Applied Science Laboratories, State College, Pa. Our results agreed with the published analysis within 1% (wt %). (This standard contains methyl esters of 14:0, 16:0, 16:1, 18:0, and 18:1 fatty acids.)

Experiment 3

The rats for this experiment were CFN (Wistar) male rats obtained from Carworth (Portage, Mich.). The rats were randomly allotted to experimental groups and housed individually. The diet was identical with that used in experiments 1 and 2, except rice starch was not available and cornstarch was substituted for the carbohydrate portion of the diet. After 28 days on the experimental diets the rats were killed, and samples of skin, abdominal muscle, visceral fat, liver, total gastrointestinal tract, and serum were taken for cholesterol analysis.

Steroid balance studies

The feces were analyzed for neutral sterols and bile acids as described elsewhere (9-11). The only changes were the use of a Hewlett-Packard 7610A gas chromatograph equipped with a model 3700B electronic digital integrator and teletypewriter. The fecal steroids were identified by comparison of GLC and TLC properties with pure standards and with the published work of others (9-11).

An estimate of the amount of microbiologically modified bile acid was obtained by our previously published method (9). This consists of comparing by GLC on SE-30 columns the retention times of TMS ethers of bile acids from germfree and conventional rats (9). Those peaks with similar retention times are assumed to be primary bile acids, and those with different retention times to be secondary (microbiologically modified) bile acids. This “index” is not an absolute value, but it is a useful indication of microbiologically modified bile acids for comparison between groups of animals.

RESULTS

Experiment 1

Two groups of 12 conventional rats each were studied individually by steroid balance techniques. The results are given in Table 1. Serum cholesterol levels were significantly lower in the safflower oil-fed group than in the coconut oil-fed group. The liver cholesterol levels, however, were significantly higher in the safflower-fed group. The total serum plus liver cholesterol pool was also significantly higher in the safflower-fed rats.

The endogenous fecal neutral sterol excretion was virtually identical in the two groups, and there was no difference in the Δ7-cholestenol or the microbiologically formed fecal coprostanol. The β-sitosterol recoveries for the safflower oil and coconut oil-fed groups were 68% and 72%, respectively. Since the recoveries for the germfree groups were similar (70 and 71%, respectively) and should have been ≈ 100% (92.4% in our previous paper, Ref. 9), we believe that our recovery figures of approximately 70%...
are the result of an analytical error in the \( \beta \)-sitosterol analysis of our diet. Since the mean recoveries were all inside a range of 68-72\%, the results should still be valid for comparison between groups. Due to the uncertainty of the source of these low recoveries, corrections were not applied to fecal neutral sterol excretions to correct for possible plant sterol losses.

The fecal bile acid excretion was the same for both dietary groups, and there was no apparent difference in the levels of microbiological modification of bile acids as estimated by our previous method (9).

**Experiment 2**

The same experimental protocol was used in studies with germfree rats. Again, the serum cholesterol levels were significantly lower and the liver total cholesterol was significantly higher in the safflower oil-fed rats than in the coconut oil-fed group (see Table 2). The liver cholesteryl esters of the safflower oil group were over five times higher (842 mg vs. 162 mg) than those of the coconut oil-fed group. As with the conventional rats, there was no significant difference between the dietary treatments in endogenous fecal neutral sterol or fecal bile acid excretion.

**Experiment 3**

In the third experiment, additional tissues were examined for cholesterol content. There was no significant difference in the cholesterol content of skin, serum, muscle, adipose tissue, or total gastrointestinal tract between the two dietary treatments (Table 3). Total liver cholesterol was again significantly higher in the safflower oil-fed group; the increase, as in experiment 2, was caused by a marked rise in liver cholesteryl esters.

### TABLE 2. Experimental data from mature male germfree rats fed 20% dietary coconut oil or safflower oil

<table>
<thead>
<tr>
<th></th>
<th>Coconut Oil (n = 6)</th>
<th>Safflower Oil (n = 6)</th>
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<tbody>
<tr>
<td>Average final body weight (g)</td>
<td>365 ± 22e</td>
<td>375 ± 33f</td>
</tr>
<tr>
<td>Diet consumed/day/rat (g)</td>
<td>16.5 ± 2.7</td>
<td>18.4 ± 2.7</td>
</tr>
<tr>
<td>Serum cholesterol (mg/100 ml)</td>
<td>83.8 ± 12.3h</td>
<td>70.9 ± 8.0</td>
</tr>
<tr>
<td>Liver cholesterol (mg/100 g)</td>
<td>396 ± 120c</td>
<td>1003 ± 271c</td>
</tr>
<tr>
<td>Liver cholesteryl ester (%/100 g)</td>
<td>41 ± 12</td>
<td>84 ± 11</td>
</tr>
<tr>
<td>Serum + liver pool (mg)</td>
<td>48.7 ± 9.1j</td>
<td>104.4 ± 18.0</td>
</tr>
<tr>
<td>Total endogenous fecal neutral sterol excretion (mg/kg body wt/day)</td>
<td>12.08 ± 2.11m</td>
<td>14.53 ± 3.23</td>
</tr>
<tr>
<td>Fecal bile acid excretion (mg/kg body wt/day)</td>
<td>8.26 ± 1.5</td>
<td>6.53 ± 2.22</td>
</tr>
</tbody>
</table>

* Mean ± standard deviation.

1 \( P < 0.06. \)

2 \( P < 0.001. \)

3 Serum pool = 3.1% body wt.

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The fatty acid composition of the liver cholesteryl esters of conventional rats is given in Table 4. The cholesteryl linoleate content of the safflower oil-fed group is over 21 times higher than that of the coconut oil-fed group. For comparison, the fatty acid composition of the liver triglycerides is also reported. Owing to technical difficulties, comparable data from germfree animals are unavailable.

**DISCUSSION**

The endogenous fecal neutral sterol and bile acid excretions of the germfree rats were all lower than the comparable excretions of the conventional rats. This is in agreement with our previous findings (9). Comparison of Tables 1 and 2 also reveals that the serum and liver cholesterol levels were higher in the germfree groups. The effect of microbiological status on serum and liver cholesterol levels has been reviewed by Kellogg and Wostmann (12).

The lower serum and higher liver cholesterol levels of rats fed polyunsaturated (safflower) versus saturated (coconut) fats is in agreement with reports of other groups (13, 14). The increased liver cholesterol was shown to be due to an increase in cholesteryl esters, as has been reported by others (13, 14).

In the first experiment (Table 1), no significant difference in total endogenous neutral sterol excretion or bile acid excretion was seen between the two dietary treatments. This similarity of quantitative fecal steroid excretion on differing dietary fat sources has been observed by other investigators in human subjects (5, 15) and in rats (3).

There was no significant difference in the fecal excretion of coprostanol, the microbiologically saturated form of
cholesterol, indicating that microbial modification of the neutral sterols in the gut was not a function of type of dietary fat.

A calculated index of microbiological modification of the bile acid molecule (9) indicated no significant difference in the degree of microbiological activity on the bile acids in the gut.

Δ7-Cholestanol (lathosterol) excretion has been used as an indicator of intestinal cell sloughing and/or secretion (9). In this study, no difference was seen in its fecal excretion, suggesting that these processes were not affected differently by the dietary fats.

The absence of any dietary differences in (a) fecal coprostanol excretion (a microbially modified neutral sterol) and (b) extent of microbial modification of bile acids, while the serum and liver cholesterol levels were both changed significantly, supports a conclusion that the mode of action of type of dietary fat on serum cholesterol levels is independent of the activity of the intestinal microflora on neutral sterols or bile acids.

This independence was proved by the second experiment utilizing germfree rats (Table 2). The percentage decreases in serum levels were virtually the same, 15% and 16%, in the conventional and germfree safflower oil-fed groups compared with the coconut oil-fed groups. The increases in liver cholesterol levels (195% in conventional and 251% in germfree) were similar. Since the sizes of the effects of dietary lipid on serum and liver cholesterol levels were essentially the same in both conventional and germfree rats, the cause of these effects must be independent of, and not modulated by, the activity of the intestinal microflora. Both the germfree and conventional studies reveal an increased level of liver cholesterol in polyunsaturated fat-fed rats with no change in fecal steroid excretion. This difference in liver cholesterol is entirely due to the difference in liver cholesteryl ester (520% greater in the germfree group and 989% greater in the conventional group). Avigan and Steinberg (13) first reported increased liver cholesteryl ester levels when feeding corn oil as opposed to coconut oil. They also showed an increased incorporation of [1-14C]acetate into liver nonsaponifiable lipid in the rats fed corn oil. Bloomfield (14) reported that rats fed cholesterol (0.64%) and safflower oil at 20% of diet had over three times as much liver cholesteryl ester as did a group fed cholesterol and butter. This was accompanied by an inverse movement in the serum cholesterol levels. His studies also revealed no change in cholesterol levels of other tissues, an observation confirmed by the data in Table 3.

Seen together, Bloomfield’s studies (14) and ours indicate that rats fed polyunsaturated fats as compared with saturated fat (a) increase cholesterol synthesis, (b) do not increase cholesterol excretion either as bile acids or as neutral sterols, (c) accumulate a larger proportion of their cholesterol in the liver at the expense of the serum, and (d) accumulate the increased liver cholesterol as the ester.

Table 4 contains the fatty acid compositions of the liver cholesteryl esters of conventional rats and, for comparative purposes, the liver triglycerides of conventional rats. The liver cholesteryl esters of the safflower oil-fed group were composed of significantly less myristic and oleic acids and much more linoleic acid than the coconut oil-fed group. On a mg/100 g basis, cholesteryl linoleate was over 21 times higher (Table 4).

Quarfordt and Goodman (16) have shown that the uptake of chylomicron cholesteryl esters by the liver is essen-
dietary corn oil, the liver cholesteryl ester fatty acid content observed in our safflower oil-fed rats was significantly less myristic, palmitic, and oleic acids and more linoleic acids as compared to the coconut oil group. This 3.7-times higher cholesteryl linoleate content in the liver triglycerides of the safflower oil-fed group was the same as the increase seen in the coconut oil-fed group (3.8 times, on a wt % basis). However, expressed on a mg/100 g liver basis, the cholesteryl linoleate in the liver of the safflower oil-fed groups is 90.6 mg/100 g (40% of the total liver cholesteryl ester) compared with 4.25 mg/100 g (18% of the total) for the coconut oil-fed group, over 21 times higher. A correlation between serum cholesteryl ester concentration and cholesteryl linoleate levels in serum has been reported (20). It has also been observed (21) that serum cholesterol-reducing drugs increase cholesteryl ester formation in the liver with oleate and linoleate esters being most stimulated.

These reports coupled with the high levels of liver cholesteryl linoleate observed in our safflower oil-fed rats suggest a role for this ester in the polyunsaturated fat-induced changes in cholesterol distribution in rats.

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