The main pathway of cholesterol catabolism involves the conversion of cholesterol to bile acids which are eliminated via the bile and feces. As liver cholesterol is considered to exist in equilibrium with serum cholesterol, nutritional factors accelerating cholesterol catabolism may lower the serum cholesterol level. Furthermore, it is well known that hypercholesterolemia and subsequent experimental atherosclerosis can be more easily produced in chickens than in rats; the latter are extremely resistant to experimental atherosclerosis. Only extensive treatment with dietary cholesterol, thiouracil, and cholic acid causes hyperlipemia in rats.1,2

It has been observed that the feeding of essential amino acids increased cholic acid output in biliary fistulated dogs.3 However, the effect of dietary factors on cholesterol catabolism has not been previously correlated with the dynamic equilibrium of cholesterol or serum lipoproteins in vivo. The present study was designed to compare the effect of dietary protein on the rate of cholesterol-4-C14 catabolism in chicks and rats and the effect of protein on the distribution of injected C14 in bile constituents in the chick. The relationship between cholesterol catabolism and serum lipoproteins, serum cholesterol, and liver cholesterol was also studied in bile duct cannulated and bile duct ligated chicks.

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Methods

Forty day-old chicks (New Hampshire-Columbia Cross) were kept for 1 week on a purified basal diet containing 1 per cent corn oil and then divided into 4 lots of 10 birds each of approximately the same weight. Two lots were kept for 4 weeks on a diet containing 15 per cent protein, and 2 lots on a diet containing 30 per cent protein. One lot at each protein level was supplemented with 1 per cent cholesterol. At 5 weeks of age the neck of the gall bladder was ligated and the hepatic and cystic ducts cannulated with polyethylene tubing (size PE 60). The tubing was inserted by an abdominal approach while the chick was under anesthesia with sodium pentobarbital (4 mg./100 gm. body weight). An emulsion of cholesterol-4-C14 (specific activity 28.9  µc./mg.) prepared by the method of Bergstrom and Norman5 was injected intraperitoneally (2 µc./Kg. of body weight) and bile was collected at various time intervals for a total period of 72 hours.

The weanling rats used in this study were kept on a regular stock diet 6 for 5 weeks and then divided into 2 groups. One group of rats was kept on a diet which contained 8 and the other 28 per cent of casein (table 1). At the end of a 6-week feeding period, representative rats were anesthetized with ether, their bile ducts cannulated with polyethylene tubing, and circulation of the bile maintained for 24 hours by external connection of the bile cannula with polyethylene tubing (size PE 50) inserted into the distal portion of the bile duct. The rats were then injected with an emulsion containing cholesterol-4-C14 (2 µc./Kg.), the enterohepatic circulation was interrupted by disconnecting the polyethylene tubes, and bile was collected at various time intervals for a total period of 72 hours.

A 20 µl aliquot of chick or rat bile was spread as a thin film on an aluminum planchet, dried under an infrared lamp on a Spinco Spinner, and its C14 content counted with the aid of a Packard Gas Flow counter and a Baird Atomic Sealer. The values were corrected for mass absorption, and the cumulative percentage of the injected C14 of cholesterol-4-C14 excreted in bile was obtained.

Pooled samples of chick bile were also lyophilized, taken up in one-half of their original volume with 8 N aqueous sodium hydroxide solution, and saponified for 3.5 hours at 120 C. under
CHOLESTEROL METABOLISM

Table 1
Composition of Rat Diets

<table>
<thead>
<tr>
<th>Constituents</th>
<th>High protein</th>
<th>Low protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>28.0 Gm.</td>
<td>8.0 Gm.</td>
</tr>
<tr>
<td>Corelose</td>
<td>57.0 Gm.</td>
<td>77.0 Gm.</td>
</tr>
<tr>
<td>Wesson salts</td>
<td>5.0 Gm.</td>
<td>5.0 Gm.</td>
</tr>
<tr>
<td>Corn oil</td>
<td>10.0 Gm.</td>
<td>10.0 Gm.</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.2 Gm.</td>
<td>0.2 Gm.</td>
</tr>
<tr>
<td>Vitamins*</td>
<td>0.2 Gm.</td>
<td>0.2 Gm.</td>
</tr>
<tr>
<td>Vitamin†</td>
<td>0.2 ml.</td>
<td>0.2 ml.</td>
</tr>
</tbody>
</table>

*Thiamine, 1.24 Gm.; riboflavin, 1.24 Gm.; calcium pantothenate, 2.48 Gm.; folic acid, 0.30 Gm.; pyridoxine, 1.24 Gm. made up to 100 Gm. with corelose.
†Vitamin A, 1,200,000 I.U.; vitamin D₃, 12 mg.; α-tocopherol acetate, 1.42 Gm. diluted to 100 ml. with corn oil.

20 lb. pressure. After saponification, an equal volume of 95 per cent alcohol was added to the alkaline hydrolysate, and the unsaponifiable materials were extracted with Skellysolve F. The aqueous phase was acidified with dilute hydrochloric acid, the fatty acids removed by extraction with Skellysolve F and the bile acids extracted from the aqueous phase with ethyl ether. The bile acids were then separated on a partition column using 70 per cent aqueous acetic acid as the stationary phase and various mixtures of benzene and Skellysolve B equilibrated with 70 per cent aqueous acetic acid as the movable phases, and the distribution of radioactivity in each eluate was determined.

In order to study the relationship between bile flow and cholesterol metabolism, chicks were kept on a poultry ration for 5 weeks and were then divided into 4 groups of 5 birds each. One group served as a control. The hepatic and cystic ducts in another group of chicks were ligated and the cystic or the cystic and hepatic ducts in 2 others were cannulated with polyethylene tubing (size PE 60). The bile was collected in centrifuge tubes which were taped to the individual chicks with adhesive tape. The birds were kept on the same diet for a total period of 154 hours, then bled via heart puncture, and the livers removed. Two ml. of blood were withdrawn from each bird before and 48 hours after surgery for the determination of serum cholesterol. Total serum cholesterol was analyzed according to the method of Sperry and Webb, and total liver cholesterol was analyzed as described previously. The isolation and the analysis for low density serum lipoproteins were carried out according to the ultracentrifugal flotation method of Gofman.

Results

The results indicated that the level of dietary protein influenced the rate at which the radioactive carbon of the injected cholesterol-4-C₁⁴ was excreted (fig. 1). Chicks kept on a high protein diet excreted an average of 9.41 per cent of the injected cholesterol-4-C₁⁴ into bile, while those on a low protein diet excreted an average of 6.87 per cent of radioactivity. This difference was statistically significant at the 1 per cent level. A dietary source of cholesterol did not appear to alter the ratio of cholesterol-4-C₁⁴ excretion in birds kept on a high protein diet as compared with those on a low protein diet.

A similar effect was noted in bile duct cannulated rats (fig. 2). However, rats which had been kept on a high protein diet excreted an average of 45.7 per cent of the injected cholesterol-4-C₁⁴, while those on a low protein diet excreted an average of 29.7 per cent of radioactivity during the 72 hour period. These values were statistically significant at the 1 per cent level. It is well known that hypercholesteremia and subsequent atherosclerosis can be
Figure 2
Excretion of cholesterol C\textsuperscript{14} in bile duct cannulated rats. Each curve represents an average value of 3 rats. Average cumulative percentage and the standard error of the mean in high protein and low protein groups after 72 hours was 45.70 ± 0.71 per cent and 29.67 ± 0.72 per cent, respectively.

produced more easily in chicks than in rats. The present results may partially explain this species difference, as rats excreted cholesterol-4-C\textsuperscript{14} approximately 5 times faster than chicks.

Fractionation of the constituents in chick bile indicated that only 6.7 to 7.4 per cent of biliary radioactivity remained in the unsaponifiable material; the major portion of the radioactivity was found in the bile acids which had been removed from the aqueous phase by extraction with ethyl ether (table 2). No biliary radioactivity was retained in the Skellysolve F-extractable fatty acids or in the aqueous phase. The main metabolites of cholesterol-4-C\textsuperscript{14} were found to be present as dihydroxycholanic acids (chenodesoxycholic acid and desoxycholic acid) and cholic acid. Furthermore, chicks which had been kept on a low protein diet incorporated less cholesterol-4-C\textsuperscript{14} into cholic acid than those on a high protein diet, both in the presence and absence of dietary cholesterol.

It has been shown\textsuperscript{4} that the serum cholesterol level in chicks kept on a low protein diet was significantly elevated as compared with those on a high protein diet, and that the presence of dietary cholesterol also significantly increased the serum cholesterol level at both high and low protein levels. However, the liver cholesterol level in birds kept on a low or high protein diet was not significantly increased, unless the birds had been fed dietary cholesterol. As the liver is the main site for the conversion of cholesterol into bile acids, and as liver cholesterol is considered to exist in equilibrium with serum cholesterol, the level of liver or serum cholesterol may have some effect on the rate of cholesterol-4-C\textsuperscript{14} catabolism because of the dilution effect of liver cholesterol. However, the present results indicated that the liver cholesterol level did not seem to influence the rate of cholesterol-4-C\textsuperscript{14} catabolism, as shown by the following observations. (1) A dietary source of cholesterol significantly elevated the serum and liver cholesterol levels, but it did not lower the rate of excretion of cholesterol-4-C\textsuperscript{14} at either high or low dietary protein levels. (2) In the absence of dietary cholesterol, the birds kept on the high protein diet excreted more cholesterol-4-C\textsuperscript{14} as bile acids than those on the low protein diet, in spite of a similar liver cholesterol content. It is possible that all of the liver cholesterol was not in a readily available form for cholesterol catabolism. It therefore appeared necessary to investigate how serum β-lipoproteins and serum and liver cholesterol are related to the catabolism of cholesterol.

The results indicated that cannulation of the bile duct caused a significant decrease in serum cholesterol levels, but no significant change was observed in liver cholesterol levels.

Table 2
Percentage of Distribution of Injected Cholesterol-4-C\textsuperscript{14} Radioactivity in Bile Constituents

<table>
<thead>
<tr>
<th>Diet</th>
<th>Total bile acids</th>
<th>Dihydroxycholanic acids</th>
<th>Cholic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>High protein</td>
<td>93.1</td>
<td>55.5</td>
<td>17.9</td>
</tr>
<tr>
<td>Low protein</td>
<td>92.9</td>
<td>54.9</td>
<td>10.2</td>
</tr>
<tr>
<td>High protein plus cholesterol</td>
<td>93.3</td>
<td>51.2</td>
<td>20.1</td>
</tr>
<tr>
<td>Low protein plus cholesterol</td>
<td>92.6</td>
<td>54.9</td>
<td>10.9</td>
</tr>
</tbody>
</table>

*Pooled samples of 5 chicks in each group.
Table 3
Serum and Liver Cholesterol Levels (mg. per cent) in Chicks

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum cholesterol</th>
<th>Liver cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>108±10*</td>
<td>113±5</td>
</tr>
<tr>
<td>Ligated†</td>
<td>114±4</td>
<td>245±9</td>
</tr>
<tr>
<td>Cannulated‡</td>
<td>113±7</td>
<td>107±10</td>
</tr>
<tr>
<td>Cannulated§</td>
<td>112±5</td>
<td>91±6</td>
</tr>
</tbody>
</table>

*Mean ± standard error of mean.
†Ligated hepatic and cystic ducts.
‡Cannulated cystic duct.
§Cannulated hepatic and cystic ducts.

On the other hand, ligation of the bile duct significantly increased both serum and liver cholesterol levels, but the elevating effect on serum cholesterol was more pronounced than on liver cholesterol levels. The most striking change due to bile duct cannulation or ligation was observed in the serum β-lipoproteins. The β-lipoprotein levels of bile duct cannulated chicks were 3 times lower and those of bile duct ligated chicks 3 times higher than those of normal chicks (fig. 3). The β-lipoprotein pattern of the bile duct ligated chicks became more heterogeneous, and an increase in the lipoprotein level was observed in the low Sf range (normal range) but not in the high Sf range (above Sf 20), where the increases in β-lipoproteins due to hyperlipemia were observed. These results seemed to indicate that serum cholesterol, especially the cholesterol in the low Sf β-lipoproteins transported into the liver, rather than the cholesterol in the liver may be the primary source of catabolic cholesterol. In other words, when liver cholesterol is converted into the soluble lipoprotein form, it may be catabolized at a faster rate than cholesterol which is less readily available.

Discussion

Although no definite explanation of the effect of dietary protein on cholesterol catabolism can be given at the present time, a low dietary protein level may impair directly or indirectly the enzymatic systems involved in cholesterol catabolism due to a combination of the following five possibilities: (1) A low protein diet may partially impair some enzyme system involved in cholesterol catabolism. (2) A low protein diet may inhibit the production of some coenzymes or cofactors necessary for cholesterol catabolism. (3) If cholesterol is assumed to be mobilized for catabolism in the form of lipoproteins, a low protein diet may produce less protein necessary for mobilization, which may result in lowered cholesterol catabolism. (4) Low protein may produce less taurine which is necessary to conjugate free bile acids. (5) As the metabolism of essential fatty acids seemed to be correlated with cholesterol catabolism in vivo, a low protein diet may impair the metabolism of essential fatty acids in liver, which may indirectly decrease the rate of cholesterol catabolism.

It is well known that rats are extremely resistant to experimental atherosclerosis. The results obtained in the present study showed that rats excreted the C14 of injected cholesterol-4-C14 approximately 5 times faster than chicks and may partially explain the species difference in susceptibility to hypercholesteremia and subsequent atherosclerosis. As rats do not have gall bladders, it is possible that the rate of circulation of bile acids may be faster in rats than in chicks. Rats seem to have a greater potential capacity for cholesterol catabolism so they can replace immediately the bile acids lost in the feces. This greater capacity may be responsible for the
species difference in the rate of catabolism of cholesterol-4-C\textsuperscript{14}, even when the bile ducts were cannulated. As cholesterol is considered to exist in dynamic equilibrium between the rate of synthesis and degradation, differences in catabolism under normal physiologic conditions, the rate of biosynthesis, and the turnover of tissue cholesterol must be compared in order to clarify the species difference in experimental atherosclerosis.

The present study also indicated that the liver cholesterol level was not the primary factor influencing the rate of cholesterol catabolism. Although the distribution of lipids in rat liver cell fractions has been studied recently,\textsuperscript{12,13} it is not known how the cholesterol combines with tissue protein or with any other constituents of the liver, or how it can be mobilized for catabolism by the enzyme systems known to be present in the mitochondria of the liver cell.\textsuperscript{14} Cholesterol in some of the liver cell fractions may not be readily available for catabolism. Furthermore, it is known that feeding cholesterol to animals causes a cholesterol-type fatty liver, and that a large increase of esterified cholesterol is concentrated in the floating layer as lipid globules, while only a small increase in the other cellular components is observed.\textsuperscript{15} It is questionable if the cholesterol esters in the floating layer are readily available for catabolism.

In the present study, a 25 per cent decrease in the serum cholesterol level and a 65 per cent decrease in the \( \beta \)-lipoprotein levels was noted in bile duct cannulated chicks. It is known that serum cholesterol exists in equilibrium with liver cholesterol, but our results seem to indicate that serum cholesterol, especially in the form of low Sf \( \beta \)-lipoproteins, rapidly restores an equilibrium with liver cholesterol, while liver cholesterol is being converted to bile acids. A study is in progress in our laboratory to clarify whether \( \beta \)-lipoproteins or some other activated form of cholesterol is the direct precursor for cholesterol catabolism in vivo.

Under normal physiologic conditions, bile acids are subject to enterohepatic circulation, and those not absorbed from the intestinal lumen would be excreted as fecal bile acids. Furthermore, intestinal bacteria are known to convert bile acids to various metabolic products, some of which are not absorbed from the intestinal lumen but are excreted in the feces, thus influencing the rate of loss of bile acids. As bile duct cannulation does not allow an animal to reutilize bile acids, cannulation could be considered to cause a profound change in the rate of cholesterol catabolism. Thus, the present results dealt with the comparison of the maximum capacity of the liver to convert cholesterol-4-C\textsuperscript{14} to bile acids under different dietary conditions. However, because of the difficulty in controlling the level of circulating bile acids and the growth of intestinal organisms under normal physiologic conditions, the results obtained by many workers on the fecal output of bile acids and sterols derived from cholesterol under various dietary conditions are contradictory.

It is difficult to increase the rate of cholesterol catabolism since the enterohepatic circulation of bile acids serves as an efficient means of conserving liver cholesterol. Therefore, if the amounts of endogenous or exogenous cholesterol in an animal exceed the maximum amounts of cholesterol which can be utilized in catabolism via the bile acids, the excessive cholesterol accumulates in the liver and serum, causing a cholesterol-type fatty liver, hypercholesteremia, and subsequent atherosclerosis.

Gall bladder disease is known to elevate serum cholesterol and \( \beta \)-lipoprotein levels and is often associated with atherosclerosis.\textsuperscript{10-18} This may be a result of a greatly depressed cholesterol catabolism with subsequent increase of serum cholesterol. In the metabolism of cholesterol, catabolism may be the rate-limiting reaction. Although catabolism may be inhibited by certain diseases, it cannot easily be accelerated above its normal rate. Therefore, atherosclerosis may be influenced by the following measures: (1) intake of exogenous cholesterol; (2) biosynthesis of endogenous cholesterol; (3) mobilization of tissue cholesterol; (4) cholesterol catabolism; (5) factors
influencing the stability of serum lipoproteins; and (6) factors influencing the denaturation of the lipoproteins in the tissue cells of the intima.

Summary

The level of dietary protein influenced the rate of cholesterol-4-C\(^{14}\) catabolism in bile duct cannulated chicks and rats. Animals which had been kept on a high protein diet for 5 weeks excreted cholesterol-4-C\(^{14}\) as bile acids at a faster rate than those on a low protein diet. The presence of dietary cholesterol did not change the rate of cholesterol-4-C\(^{14}\) catabolism at either high or low dietary protein levels. A species difference in the rate of cholesterol catabolism was also noted. Bile duct cannulated chicks excreted an average of only 8 per cent, while bile duct cannulated rats excreted as much as 46 per cent of injected cholesterol-4-C\(^{14}\) in a 72 hour period. It was shown that cannulation of the bile ducts caused a significant decrease in the serum cholesterol and serum \(\beta\)-lipoprotein levels in chicks. Furthermore, the liver cholesterol level did not seem to influence the rate of cholesterol catabolism. Serum cholesterol, especially in the form of low Sf \(\beta\)-lipoproteins, appeared to be used rapidly for the conversion of cholesterol to bile acids.

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Metabolism of Cholesterol-4-C\textsuperscript{14} in Bile Duct Cannulated Chicks and Rats
TOSHIRO NISHIDA, AKIRA UENO and FRED A. KUMMEROW

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