Cholesterol Stimulation of Phosphatidyl Choline Formation

By LAURENCE O. PILGERAM, PH.D., AND DAVID M. GREENBERG, PH.D.

The conversion of ethanolamine to phosphatidyl choline was studied in liver slices from rats exposed to a high dietary intake of cholesterol in the presence of cholic acid. Cholesterol stimulated phosphatidyl choline formation per unit weight of liver tissue and increased the total liver mass thereby increasing further the total yield of phosphatidyl choline. These results are contrasted with the opposite effect of cholesterol on the liver phosphatides of the rabbit. A relationship between phosphatides and cholesterol metabolism and between species susceptibility to cholesterol induced atherosclerosis and phosphatide metabolism is pointed out.

The rat has been repeatedly demonstrated to be resistant to experimental atherogenesis\textsuperscript{1,2,3,4}. The reason for this phenomenon is unknown. Recent work by Lewis and Page\textsuperscript{5} indicates that species differences exist in the cholesterol-bearing lipoprotein molecules associated with atherosclerosis. Horlick \textit{et al.}\textsuperscript{6}, by utilizing the cholesterol tolerance technique, found that the disappearance time of intravenously injected cholesterol varied from 72 hours in the rabbit, to 24 hours in the chick, to 12 hours in the rat. Also, the feeding of cholesterol to the chick induces a high degree of hypercholesterolemia whereas proportional amounts fed to the rat have practically no effect\textsuperscript{7}. These results indicate a correlation between species ability to resist atherogenesis and the ability to metabolize cholesterol. The ability to metabolize cholesterol in a normal manner appears to be related in some unknown fashion to the phosphatides and to the proteins\textsuperscript{8,9} to which the phosphatides and cholesterol are bound. A survey of the literature indicates that there is a tendency for a reduced phospholipid phosphorus/total cholesterol ratio in atherosclerosis. However, since this ratio is a measure only of a heterogenous group of phosphatides and provides no information on the nature of the metabolic relationships between cholesterol and specific phosphatides it is obviously of importance to study in greater detail the nature of some of these relationships. This paper reports a study of the effect of dietary cholesterol upon the ability of rat liver to form phosphatidyl choline from ethanolamine.

**EXPERIMENTAL**

\textbf{Diets.} Young litter-mate male rats (Long-Evans) 34-38 days of age, and 85-95 gm. in weight, were fed diets containing cholesterol and/or cholic acid for periods of 14-21 days before sacrifice. The basic diet consisted of ground whole wheat (67.5 per cent), casein-tec. (15.0 per cent), skim milk powder (7.5 per cent), sodium chloride (0.75 per cent), calcium carbonate (1.5 per cent), melted fat (6.75 per cent), fish oil (Sardilene) (1.0 per cent), KI solution (0.9 \text{ mg} iodine per gm. diet). The approximate composition of the above diet is 24.5 per cent protein, 54.9 per cent carbohydrate, and 8.9 per cent fat. The basic diet was made up to contain 12 per cent crystalline cholesterol* and/or 1 per cent cholic acid*.

\textbf{Incubation Procedure.} The non-fasted rats were sacrificed by a sharp blow to the back of the head. The liver was immediately excised and placed in ice-cold 0.9 per cent sodium chloride. Liver slices, totalling 500 mg. in wet weight, prepared with a Stadie-Riggs microtome, were incubated aerobically at 37 C. for two hours. Incubation was carried out in 20 ml. pyrex beakers in a Dubnoff metabolic incubator\textsuperscript{a}. The shaking rate was maintained between 85-90 cycles per minute. The incubation was terminated by the addition of 2 ml. of a 3:1 ethyl alcohol-ethyl ether mixture and raising the temperature of the incubator bath to 75-80 C. while shaking.

\textbf{Substrates.} Five ml. of Krebs-Ringer phosphate solution, made as described by Umbreit \textit{et al.}\textsuperscript{9}, with the exception that the CaCl\textsubscript{2} was 0.0016 M, were

\* Purchased from Eastman Organic Chemicals, Rochester, N. Y.
† Wet weight equals the weight of the tissue slice after gentle blotting on filter paper.
CHOLESTEROL AND PHOSPHATIDYL CHOLINE FORMATION

used as the incubation supporting media. Substrates included ethanolamine-1, 2-C\textsuperscript{14}, methionine, ethanolamine, and C\textsuperscript{14}H\textsubscript{3}-methionine.

Phosphatidyl Choline Isolation Procedure. Following denaturation of the incubations, the incubation flasks were covered with parafilm and stored in the deep freeze until ready for extraction. Extraction of the phosphatides, hydrolysis, and column chromatographic separation of the phosphatide constituents on the cation exchange resin Dowex-50 (250-500 mesh) were carried out as described previously\textsuperscript{a}, with the exception that the phosphatides were not precipitated with acetone. The lipids of the liver slices were extracted with a 3:1 ethyl alcohol-ethyl ether mixture. This extract was evaporated to dryness and then extracted with petroleum ether. Three to ten ml. unit volumes of the petroleum ether extract were transferred directly to digestion flasks, evaporated to dryness, and then digested for 9 hours in 35 ml. of 3.6 per cent hydrochloric acid solution.

The reineckate salt derivative of choline was prepared by taking up the dried choline chloride from polyethylene collecting planchets in a minimum volume of water and then adding to the aqueous choline 5 times its volume of a 2 per cent reinecke salt-methanol solution; choline reineckate was allowed to precipitate quantitatively by storing in the deep freeze for 6 hours. The crystals were then plated on 4.25 cm. Whatman No. 42 filter paper by conventional procedures and counted. Counting was performed by means of a gas flow counter\textsuperscript{f} utilizing helium saturated with 100 per cent ethyl alcohol at the temperature of an ice-water bath.

RESULTS AND DISCUSSION

The data presented in table 1 demonstrate that dietary cholesterol increases the yield of phosphatidyl choline in the in vitro rat liver system. Cholic acid is necessary for the reaction to occur. In the absence of cholic acid the reaction to dietary cholesterol was quite variable with about as many negative as positive results. The role of cholic acid may be one of increasing the absorption of cholesterol from the rat gastro-intestinal tract. This is indicated by the work of Swell et al.\textsuperscript{11}, where it was found that presence of 1 per cent cholate in the diet caused a progressive increase in the concentration of blood cholesterol which continued up to three weeks. In our experiments, cholic acid was observed to increase the yield of phosphatidyl choline in the absence of cholesterol addition to the basic diet. This effect may be due to an increased absorption of the normal dietary cholesterol since the magnitude of the increase was not as great as when cholic acid and cholesterol were fed together.

The variability of male rats of the same strain but of different litters in the capacity to form phosphatidyl choline was considerable. However, when litter mates of the same sex were used, reproducible results with a variability of \(\pm 35\) per 1000 counts per 100 seconds were obtained. The values reported in Table 1 represent the averages taken from seven groups of litter mates.

The livers of the animals receiving cholic acid or cholesterol alone were generally macroscopically normal, but, livers obtained from the animals receiving both cholesterol and cholic acid were invariably enlarged and very fatty in consistency with a faded out yellowish-tan appearance. Consequently, when the yield of phosphatidyl choline is calculated on the basis of the total liver mass the results become much more significant with a 64.5 per cent increase.

The increase in the yield of phosphatidyl choline obtained in the presence of labeled methionine as well as in the presence of labeled ethanolamine indicates that the effect of cholesterol is upon the main chemical reaction rather than upon the metabolic pool of a single compound such as ethanolamine.

With but few exceptions there was loss of weight among the animals on the cholic acid and cholesterol diet. However, in those cases where there was no loss of weight, or the loss was negligible, the yield of phosphatidyl choline was still high as compared to the average of the controls, thus indicating that the effect can not be ascribed to the animal losing weight. Nor can the effect of cholesterol and cholic acid be ascribed to the total amount of dietary intake since there was no significant difference in this respect between the control and the experimental groups.

The results indicate that an increase in the yield of liver phosphatidyl choline accompanies...
TABLE 1.—The Effect of Cholesterol and Cholic Acid upon Phosphatidyl Choline Formation

<table>
<thead>
<tr>
<th>Dietary Regimen</th>
<th>No. of Expt. Results</th>
<th>Average Liver Wt. Gm.</th>
<th>Condition of Liver</th>
<th>Initial Average Wt. of Animals Gm.</th>
<th>Final Average Wt. of Animals Gm.</th>
<th>Total Counts in Phosphatidyl Choline per 100 Seconds per 500 Mg. of Tissue</th>
<th>Total Liver Count* per 100 Seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>Cholic Acid</td>
<td>Basic Diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>8†</td>
<td>8.4</td>
<td>Normal</td>
<td>80</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3†</td>
<td>10.6</td>
<td>Normal</td>
<td>90</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>8†</td>
<td>9.3</td>
<td>Fatty</td>
<td>89</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2†</td>
<td>11.8</td>
<td>Fatty</td>
<td>88</td>
</tr>
</tbody>
</table>

* Total liver count = (Liver weight in mgm/500) × (Phosphatidyl choline count per 100 seconds).

Substrates:
† Ethanolamine-1,2-C\(_1\)\(_4\)(3.8 × 10\(^{-4}\) M and 236,000 ct. per 100 sec.); Methionine (7.5 × 10\(^{-4}\) M).
† C\(_3\)H\(_7\)-Methionine (3.0 × 10\(^{-4}\) M and 311,000 ct. per 100 sec.); Ethanolamine (3.8 × 10\(^{-4}\) M).

the ingestion and absorption of dietary cholesterol in the rat. The data may be interpreted as indicating an increased rate of formation of phosphatidyl choline or as a retardation of the normal catabolism of the phosphatide. Present knowledge favors the former point of view. It has been reported that the administration of cholesterol to rabbits caused a decrease in the phosphatides of the liver\(^{12}\). This finding is of interest from the standpoint of the present study. The results may be interpreted as indicating that the metabolism of cholesterol requires phosphatides. The decrease observed in the rabbit liver is understandable if the mechanism for the formation of the phosphatide is limited and can not keep up with the demand placed upon it by excess amounts of cholesterol. This is in contrast with the results reported here for the activity of the phosphatidyl choline synthesis system in the rat liver* and thus brings to light a possible species difference in the metabolism of phosphatides which may be correlated with the difference in susceptibility to experimental cholesterol induced atherosclerosis displayed by the rat and the rabbit. If, as it appears, phosphatide synthesis is necessary for normal cholesterol metabolism, then the results reported here on the rat and the rabbit would explain, in part, why the disappearance time of intravenously injected cholesterol in the rabbit is six times longer than in the rat\(^{4}\), and why the rabbit is easily susceptible to cholesterol induced atherosclerosis.

The conclusion that phosphatidyl choline is necessary for cholesterol metabolism is also supported by observations from this laboratory showing that the chick and the guinea pig, which are comparatively susceptible to cholesterol induced atherosclerosis, possess a much lower capacity for the formation of phosphatidyl choline compared to the rat which is notably resistant\(^{14}\).

**SUMMARY**

Employing an in vivo system derived from the livers of rats which had been fed a diet containing cholesterol and cholic acid, it has been possible to demonstrate that cholesterol and cholic acid stimulate the formation of phosphatidyl choline from ethanolamine-1,2-C\(_1\)\(_4\) and methionine or C\(_3\)H\(_7\)-methionine and ethanolamine.

It is suggested that phosphatidyl choline is necessary for normal cholesterol metabolism and that the difference in species susceptibility to cholesterol induced atherosclerosis may be correlated with differences in their capacity for phosphatidyl choline formation.

**ACKNOWLEDGMENTS**

It is a pleasure to acknowledge the assistance rendered by Mr. Akira Nakao and Mr. Paul Griffith.
Choline and Myocardial Necrosis

The basic causes of myocardial necrosis remain an enigma. It is, therefore, of interest to note that dietary factors, such as triglycerides occurring in lard and beef fat, can cause myocardial necrosis in choline-deficient rats; further, that it can be prevented by administration of lipotropic agents.

The sequence of histological changes, deduced from examination of myocardial lesions at all stages of development, appears to be a) an initial infiltration of cardiac tissue with fat, b) rupture of all membranes with release and phagocytosis of fat globules, and c) advanced necrosis with few or no remnants of fat. While concomitant renal and hepatic lesions may conceivably constitute contributory factors, it appears that choline and its precursors are required by rats to maintain a normal state of the myocardium.

The basic processes by which lipotropic action affects metabolism of cells or their membranes unfortunately remain unknown. The relevance of such observations on rats to human myocardial necrosis also remains to be determined. However, a possible approach to solution of this problem is suggested by these important observations.

Cholesterol Stimulation of Phosphatidyl Choline Formation
LAURENCE O. PILGERAM and DAVID M. GREENBERG

doi: 10.1161/01.RES.3.1.47

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1955 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/3/1/47

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/