The Origin of Aortic Phospholipid in Rabbit Atheromatosis

By D. B. Zilversmit, Ph.D., Moris L. Shore, B.A. and R. F. Ackerman, M.D.

Radioactive phosphorus (P32) was injected into normal and cholesterol-fed rabbits. A marked derangement of aortic phospholipid metabolism was found in the cholesterol-fed animals. The phospholipid found in the atheromatous aortas appeared to have been synthesized by the aorta itself rather than to have been deposited there from the plasma.

THE etiology of atheromatosis has been explored from different points of view. Blood cholesterol has been investigated intensively and found elevated in a portion of the patients and in all experimental animals developing this condition. Plasma phospholipids are usually elevated when the cholesterol level is high. Some workers, however, have related the increased cholesterol-phospholipid ratio to the development of atheroma. More recently attention has been focused on "physical hyperlipemia," that is, the presence of abnormal lipoproteins and exaggerated chylomicronemia in the plasma of atherosclerotics.

The increased cholesterol and phospholipid content of the atheromatous aorta was noted long ago. More recently Buck and Rossiter studied the fatty materials of plaques in fresh human aortas and reported that phospholipids, and, in particular, sphingomyelins constituted an important part of the plaque lipids. Chernick and co-workers have reported that the normal rat aorta is capable of synthesizing fatty acids and phospholipids in vitro but further relation of this observation to the mechanism of atherogenesis was not made.

In the present investigation we have attempted to evaluate the role of plasma and aorta phospholipids in rabbit atheromatosis. Analysis of specific activity data obtained from phospholipid molecules labeled with radioactive phosphorus revealed a major disturbance in the phospholipid metabolism of the rabbit aorta when atheromata are present.

METHODS

White New Zealand rabbits were fed 100 Gm. of Purina Rabbit Chow per day. In the experimental animals, the Purina diet was supplemented with 1 Gm. of cholesterol and 2.8 Gm. of Hamko vegetable fat per day for a period of five months. The total fat content of the experimental diet amounted to 5.3 per cent, as compared to 2.5 per cent for the normal diet. At the end of the five-month period 0.5 millicuries of radioactive phosphate (P32) was administered intravenously to control and experimental animals. Blood samples were taken from each animal two, four and six hours after injection. At the six-hour interval the animals were sacrificed and the liver and thoracic aorta were removed for analysis. Plasma and tissues were analyzed for phospholipid P32 and P31 by extracting twice for two hours with ethanol at 60 C. The tissue residue was subjected to an overnight Soxhlet extraction with ethyl ether, and subsequently the alcohol-ether extract was taken almost to dryness in vacuo under a carbon dioxide atmosphere. The phospholipids were re-extracted with petroleum ether, and P32 and P31 were determined in this extract. Where acid-soluble inorganic and organic phosphates were determined, separate tissue aliquots were homogenized in 5 cc. of 20 per cent trichloracetic acid. After centrifugation, the supernatant liquid was removed and the inorganic and organic acid soluble phosphates were separated by precipitation with magnesium. Aortic cholesterol was determined by the method of Sperry and Webb.

RESULTS

The analytic data are given in table 1. Liver phospholipid concentrations in the experimental animals were not elevated significantly above control levels, but plasma and aortic

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* Kindly donated in part by Merck and Company.

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phospholipid concentrations were increased 400 per cent and 100 per cent, respectively. The total amount of phospholipid in the whole thoracic aorta was actually about four times greater in the cholesterol-fed animals than in the control animals. Although the total aortic cholesterol was increased by an even greater amount, the data emphasize that phospho-
lipids constitute a significant portion of the plaque. If the entire increase in aortic lipid was due to the presence of fatty plaques, phospholipids account for 14 per cent of the plaque lipid.*

The P$^{32}$ data in table 1 are the means of specific activities, which are calculated as the percentage of the administered P$^{32}$ in the phospholipid fraction per milligram of phospho-
lipid phosphorus. This quantity is a relative measure of the percentage renewal of phospho-
lipid molecules provided the cholesterol feeding does not alter the rate of incorporation of P$^{32}$ into the phospholipid precursors. Apparently, cholesterol feeding did not affect these precursors, for the specific activities of the inorganic and organic acid-soluble phos-
phates of the liver and aorta are essentially the same in the experimental and control animals.

The data in table 1 indicate that the specific activity of liver phospholipids was not greatly affected by the cholesterol feeding. In con-
trast, the amount of radioactive phospholipid per cubic centimeter of plasma (specific activity times concentration) was five times as large in the cholesterol-fed animals as in the controls. Since all plasma phospholipids are presumably derived from the liver, it must be

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**Table 1.** Lipids and Acid Soluble Phosphates in Aorta, Liver, and Plasma

<table>
<thead>
<tr>
<th></th>
<th>Number of Animals</th>
<th>Mean ± S.E.*</th>
<th>Per cent Increase Over Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cont.</td>
<td>Chol.-Fed</td>
<td>Control</td>
</tr>
<tr>
<td>Rabbit Wt. (Kg.)</td>
<td>6</td>
<td>9</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>Whole Thoracic Aorta (Gm.)</td>
<td>6</td>
<td>9</td>
<td>0.55 ± 0.09</td>
</tr>
<tr>
<td>Per cent Phospholipid§</td>
<td>Aorta</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Lipid per Whole Thoracic Aorta (mg.)</td>
<td>Total Lipid</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Phospholipid</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Inorganic P. Specific Activity × 10^4‡</td>
<td>Liver</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Aorta</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Organic Acid Sol. P. Specific Activity × 10^4 ‡</td>
<td>Liver</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Aorta</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Phospholipid Specific Activity × 10^4 ‡</td>
<td>Liver</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Aorta</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Per cent of Inj. P$^{32}$ in Phospholipid of Entire Thoracic Aorta × 10^4</td>
<td>6</td>
<td>9</td>
<td>0.673 ± 0.131</td>
</tr>
</tbody>
</table>

* S.E. = Standard error.
‡ Specific activity expressed as the percentage of the injected P$^{32}$ per milligram of phosphorus.
§ Phospholipid P × 25, per 100 Gm. fresh tissue.

* This may not be entirely correct since some increase in adventitial fat may occur in the cholesterol-fed animals.
concluded that in the cholesterol-fed rabbits the exchange between liver and plasma phospholipids is accelerated. This observation does not confirm the findings of Perlman and co-workers, who observed that the administration of cholesterol to rats depressed the appearance of radioactive phospholipids in liver and plasma.

In the aortas of the experimental animals an even more pronounced stimulation of phospholipid synthesis was observed (bottom row table 1). Comparison of the specific activity data does not adequately express the great increase in the turnover of these phospholipids since the amount of the phospholipids in the thoracic portion of atheromatous aortas was four times as great as in the normal aortas. But the percentage of the injected $^{32}$P present in the phospholipids of the entire aorta indicates an increase in phospholipid synthesis of perhaps five to six fold. The comparison of the specific activities of the phospholipids in the aorta, plasma, and liver in table 2 shows that the specific activity of these lipids in the aorta of a few of the high cholesterol animals is even greater than the specific activity of the liver, which is normally the most active phospholipid synthesizing tissue in the body. In all instances the specific activity of the phospholipids in the aorta is much higher than the average or terminal specific activity of plasma phospholipids. This observation strongly suggests that the major portion of the phospholipids in the atheromatous plaque is synthesized in situ rather than being derived from plasma phospholipids or plasma lipoproteins.

One might object that an alternate explanation of the data is possible. Our results by themselves might be compatible with deposition of plasma phospholipids if the aorta were able to remove selectively one plasma phospholipid component having a specific activity not only considerably higher than that of the total plasma phospholipids but also exceeding the specific activity of the combined aortic phospholipid. It would be very difficult to rule out this possibility completely, since there might conceivably be present in plasma a particular lipoprotein of very high specific activity. However, the studies of Turner and co-workers indicate that the range of phospholipid specific activities in the lipoproteins of human plasma is rather narrow. In addition, our own data on the specific activity of plasma lecithin, cephalin and sphingomyelin of atherosclerotic rabbits have indicated that none of these fractions have sufficient $^{32}$P to account for the high specific activity of aortic phospholipids on the basis of deposition.

**DISCUSSION**

The observation that the amount of phospholipid in the whole thoracic aorta of the cholesterol-fed rabbit is four times as great as in control animals confirms the findings of Weinhouse and Hirsch in the rabbit and of Buck and Rossiter in human autopsy material that phospholipids constitute an appreciable fraction of the newly developed atheromatous plaques. However, the studies of Turner and co-workers indicate that the range of phospholipid specific activities in the lipoproteins of human plasma is rather narrow. In addition, our own data on the specific activity of plasma lecithin, cephalin and sphingomyelin of atherosclerotic rabbits have indicated that none of these fractions have sufficient $^{32}$P to account for the high specific activity of aortic phospholipids on the basis of deposition.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Type</th>
<th>Liver Activity $\times 10^4$</th>
<th>Aorta Activity $\times 10^4$</th>
<th>Plasma Activity $\times 10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>N.</td>
<td>8.69</td>
<td>3.38</td>
<td>0.948</td>
</tr>
<tr>
<td>F</td>
<td>N.</td>
<td>9.50</td>
<td>4.70</td>
<td>1.60</td>
</tr>
<tr>
<td>P</td>
<td>N.</td>
<td>8.79</td>
<td>9.80</td>
<td>2.96</td>
</tr>
<tr>
<td>R</td>
<td>N.</td>
<td>6.30</td>
<td>4.14</td>
<td>1.26</td>
</tr>
<tr>
<td>T</td>
<td>N.</td>
<td>7.60</td>
<td>5.92</td>
<td>1.86</td>
</tr>
<tr>
<td>U</td>
<td>N.</td>
<td>7.43</td>
<td>7.94</td>
<td>1.70</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>8.05</td>
<td>5.98</td>
<td>1.72</td>
</tr>
<tr>
<td>S.E.</td>
<td></td>
<td>0.47</td>
<td>1.00</td>
<td>0.28</td>
</tr>
</tbody>
</table>

- See footnote accompanying table 1.

* Unpublished preliminary observations.
plaques. The similarity in composition of the early plaques to the lipid pattern of plasma has prompted the idea that the lipids in the plaque are derived from the plasma lipids. This theory has received support from the recent discovery that certain individuals with a tendency toward atherosclerosis show the presence of excessive amounts of abnormal lipoproteins in plasma. Even though no direct proof is available, the possibility that these abnormal lipoproteins might be the precursors of atheromatous deposits has been recognized. Our studies in the rabbit, however, give a different interpretation to the presence of large amounts of phospholipids in the atheromatous deposits. It appears that the atherosclerotic rabbit has acquired the ability to synthesize large amounts of phospholipids in the aorta. The observation that the specific activity of the phospholipids in the atheromatous plaques is much higher than the average specific activity of the plasma phospholipids over a period of six hours, excludes the possibility that an appreciable fraction of the plaque's phospholipids was derived from the lipoproteins in the bloodstream.

The relation of these observations to the process of atherogenesis in rabbits has suggested two theories: (1) The atheromatous plaque, that is, the cholesterol and phospholipid, are both derived from a local derangement of the lipid metabolism in the aorta. The abnormal blood lipoproteins are not the cause, but the result of a similar derangement of lipid metabolism in the liver. (2) The cholesterol in the plaque is derived from the blood plasma, but the phospholipids are synthesized by the aorta. This possibility appears to be supported by the findings of Biggs and Kritchovsky who observed that orally-administered labeled cholesterol is found in the plaque of rabbits whereas labeled water is not converted to cholesterol in the artery.

A possible role of plasma phospholipids in keeping cholesterol in solution has been postulated by many workers. One might similarly speculate that the accelerated phospholipid synthesis by the aorta is an attempt to solubilize the cholesterol in the atheromatous plaque, or a reflection of the intense activity of the granulomatous inflammation as it reacts to cholesterol.

Whatever interpretation is put on the data obtained in the cholesterol-fed rabbits, our findings indicate that these animals suffer from an alteration in the metabolism of the aorta. The mechanism of atherogenesis in man might be elucidated if a similar situation could be discovered in the human artery. Our preliminary studies on human arteries have indicated that radioactive phospholipids do accumulate there after $^{32}$P administration.

**Summary**

The incorporation of radioactive phosphorus ($^{32}$P) in the liver, plasma, and aorta phospholipid of cholesterol-fed and normal rabbits has been measured. Increased amounts of phospholipids were found in the plasma and aorta of the cholesterol-fed animals. Phospholipids in the atheromatous aortas were synthesized five times as fast as in the normal aortas. The major amount of the plaque's phospholipid appeared to be synthesized by the aorta, rather than derived from plasma. The bearing of these findings on atherogenesis is discussed.

**Acknowledgment**

The authors are indebted to Florence Blevins, Lovell Lawrence, and Frances Carpenter for their assistance with the analyses.

**Sumario Español**

La incorporación de fósforo radioactivo ($^{32}$P) en el higado, plasma y la fosfolípida de la aorta en conejos alimentados con colesterol ha sido determinada. Cantidad aumentadas de fosfolípinas fueron encontradas en el plasma y la aorta de los animales alimentados con colesterol. Las fosfolípinas en las aortas ateromatosas fue sintetizada cinco veces más rápidamente que en las aortas normales. La mayor porción de la fosfolípina en placas aparentemente fue sintetizada por la aorta en lugar de ser derivada del plasma. El significado de estos hallazgos en cuanto a la aterogenesi se discute.

**References**

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