The Synthesis of C\textsuperscript{14}-Lipids in Rabbit Atheromatous Lesions\textsuperscript{*}

H. A. I. Newman, Esther L. McCandless,† and D. B. Zilversmit‡

From the Department of Physiology, University of Tennessee, Memphis, Tennessee

(Received for publication, October 28, 1960)

Studies of the accumulation of phospholipids in the aortic intima of cholesterol-fed rabbits have been reported previously from this laboratory (1-4). After P\textsubscript{32}-phosphate injection, it was found that the phospholipid specific activity was higher in aortic intima than in plasma, and that this difference in specific activity was accentuated in eviscerated animals. Although this study gave evidence for the synthesis of the phosphate moiety of phospholipid in the aorta, the site at which the carbon skeleton of the molecule was synthesized still had to be established. In the present study rabbits were treated by injection with sodium acetate-\textsuperscript{14}C to measure the incorporation of this label into the phospholipids and other lipids of plasma and intima.

**EXPERIMENTAL PROCEDURE**

Eight-week-old New Zealand albino rabbits were fed for 4 months a diet of Purina rabbit chow containing 1 g of cholesterol\textsuperscript{1} suspended in 2.6 g of Konsal\textsuperscript{2} per 100 g of diet. Under sodium pentobarbital anesthesia these animals were then either sham-operated upon or the intestinal tract, spleen, and kidneys were removed and the liver isolated by ligation of portal vein and hepatic artery as described previously (5). In all animals a tracheal cannula was inserted and CO\textsubscript{2} collected in 10\% NaOH. A catheter was inserted into the internal jugular vein for blood sampling and in the eviscerated animals for administration of glucose at \%hour intervals (5). Eviscerated animals were kept warm by application of external heat. Approximately 1 mc of sodium acetate-\textsuperscript{14}C\textsuperscript{4} was injected into the ear vein. Blood samples, taken at intervals during the experimental period in syringes wetted with 2.5\% Mepesulfate,\textsuperscript{3} were centrifuged at 1000 \times g for 15 minutes. At the end of the experiment the animals were killed by an intracardiac injection of air. The liver and thoracic aorta were removed and the aortic intima was separated from media and adventitia.

Tissues were minced and ground with sand in methanol. Tissues and plasma were soaked in 20 volumes of 2:1 (volume per volume) chloroform-methanol\textsuperscript{1} overnight, and all samples were washed according to a slightly modified method of Folch et al. (6). The washed samples, containing less than 1 mg of lipid P\textsubscript{2}, were then placed on columns (15 \times 25 mm) containing 1 g of nonactivated silicic acid-Super-Cel\textsuperscript{5} (1:1), and the glyceride, cholesterol, and cholesterol ester were eluted with 20 ml of chloroform. The subsequent addition of 20 ml of methanol eluted the phospholipids. Fractionation into noncholine-containing phospholipid, lecithin, sphingomyelin, and lysolecithin was achieved by the method of Newman et al.\textsuperscript{6} Silicic acid\textsuperscript{4} (4 g), freshly activated by heating at 120-130\textdegree C for 48 hours, was mixed with chloroform and transferred to a 10- \times 100-mm column with a 250-ml solvent reservoir. The silicic acid was packed with sufficient pressure from dried air (approximately 1 pound per sq. in.) to maintain a flow rate of 0.5 ml per minute. The phospholipid sample in about 10 ml of chloroform was added, and the pressure increased gradually to 4 to 5 pounds to maintain the flow rate at 0.5 ml per minute. The eluates from the column were automatically collected through a volumetric siphon in 5-ml fractions. Loads from 78.6 to 740 \mu g of phospholipid phosphorus have been used with satisfactory results. When the sample had completely entered the column, it was washed with an additional 30 ml of chloroform which eluted most of the neutral lipids. Approximately 40 to 50 ml of 20\% methanol in chloroform (volume per volume) was then added to the column to elute the noncholine-containing phospholipids. The choline-containing phospholipids, which were left on the column, were separated by adding a series of solvents as follows: 125 to 140 ml of 40\% methanol in chloroform (volume per volume) eluted lecithin; 50 to 80 ml of 60\% methanol in chloroform (volume per volume) eluted sphingomyelin; finally, 50 ml of methanol eluted lysolecithin.

Paper chromatography (7) of the fractions from the columns run against standards revealed that the fraction eluted with 20\% methanol had the \( R_F \) of phosphatidylethanolamine and -serine; the second peak of the fraction eluting with 40\% methanol gave one spot with the \( R_F \) of lecithin; the fraction eluted with 60\% methanol had the \( R_F \) of sphingomyelin with a small contaminant having the \( R_F \) of lecithin; and the fraction eluted with methanol had the \( R_F \) of lysolecithin.

Phospholipid phosphorus was determined by the method of King (8) or on small samples by the procedure of Chen et al. (9). The cholesterol ester (Fraction I) was separated from neutral lipids (Fraction II) by the chromatographic technique of Van Handel (10). The column separations were monitored by testing the eluates by the paper chromatographic technique.

\textsuperscript{*} Mallinckrodt silicic acid, 100 mesh, suitable for chromatographic analysis by the method of Ramsey and Patterson, Johns-Manville Hyflo Super-Cel.

\textsuperscript{†} Manuscript in preparation.
of Dieckert and Reiser (11), except that the solvent used for developing the paper chromatogram was 10% chloroform in petroleum ether, b.p. 60-70° (Skellysolve B). The two fractions were saponified at 60-80° for 60 minutes with 1 ml of 2.5% KOH in isobutanol per 10 mg of total lipid. The substitution of isobutanol for ethanol improved the saponification, probably by increasing the solubility of the cholesterol esters. The saponification mixture was acidified with glacial acetic acid and evaporated in a water bath with the continuous addition of petroleum ether until all isobutanol had evaporated. The acidification before the removal of isobutanol was required to prevent the degradation of cholesterol. To convert the fatty acids to soaps, the residual petroleum ether was removed in a water bath, and the dried samples were mixed with 10 ml of 2.5% NaOH in 25% ethanol and heated to 45° for 10 minutes. The nonsaponifiable fraction was extracted with petroleum ether. After this separation the sterols were precipitated with digitonin according to Sperry and Webb (12). However, for large samples the petroleum ether was evaporated and the residue redissolved in 95% ethanol to a concentration of 1 mg cholesterol per ml. This was acidified with 2 drops of 10% acetic acid per ml, and cholesterol digitonides were formed by the addition of an equal volume of 1.25% digitonin in absolute ethanol. After vigorous shaking and standing overnight, the samples were washed according to Sperry and Webb (12) and split by the Schoenheimer-Dam procedure (13). Aliquots were taken for radioactive measurement and for fatty acid determination by the method of Bragdon (15).

Lipid-04 was measured with a Packard liquid scintillation spectrometer, with an efficiency of 53%. Aqueous Na₂C₁₈O₄ was measured in a Micromil window gas flow counter by pipetting 1-ml aliquots from the sodium hydroxide traps into planchets and counting the samples immediately.

**RESULTS**

Table I shows the increase in phospholipid and triglyceride specific activities in the plasma of eviscerated and noneviscerated rabbits. Most of the plasma lipid specific activities increased gradually during the 5-hour experimental period, but the plasma phospholipid and triglyceride specific activities of eviscerated rabbit number 4 reached a maximum early in the experiment. The phospholipid specific activities in noneviscerated animals reached values of 314 and 103 at 5 hours. These values were higher than those for the eviscerated rabbits. The concentrations and specific activities of phospholipid and glyceride in intima of eviscerated and noneviscerated rabbits are also found in Table I. Phospholipid specific activities represent the radioactivity found in the whole molecule per μmole of phosphorus, and glyceride specific activities are given as the radioactivity in the fatty acid portion of the molecule per μmole of fatty acid.

In eviscerated rabbit number 1, which survived for only 2.5 hours, intima to plasma phospholipid specific activity ratio was 74. In other animals which lived for 5 hours, the terminal intima to plasma phospholipid specific activity ratios were 11 and 38 in the eviscerates, and 1.3 and 4.2 in the noneviscerates.

The phospholipid fractions of terminal plasma and intima were separated into noncholine-containing phospholipid, lecithin, sphingomyelin, and lysolecithin as shown by representative curves (Fig. 1). A comparison of terminal plasma and intimal specific activities in one of the eviscerated animals (Table II) shows that all intimal phospholipid fractions had considerably higher specific activities than did the corresponding terminal plasma samples. In the noneviscerated animal the specific activities of only the noncholine-containing phospholipids and lecithin were higher in intima than plasma.

Table I shows the intimal triglyceride specific activities in the eviscerated and sham-operated rabbits. In the former, the specific activity of the triglyceride was from 3.4 to 10 times that of the terminal plasma. In the latter, the plasma specific activity exceeded that of the intima. The accentuation of the difference in specific activities of intimal and plasma lipids in eviscerated-nephrectomized rabbits was due to suppression of synthesis of plasma phospholipids and triglycerides by removal of the vena. To ascertain whether the triglyceride was pure or actually a mixture of mono-, di-, and triglyceride fatty acids, the neutral lipid fractions from plasma and aortic intimas of other rabbits fed cholesterol were chromatographed on silicic acid.
**Fig. 1.** Representative silicic acid column fractionation of terminal plasma (left) and intima (right) phospholipids of cholesterol-fed rabbits: 20% methanol elutes noncholine-containing phospholipids; 40% methanol elutes lecithin in plasma, and in intima elutes first an unidentified peak, then lecithin; 60% methanol elutes sphingomyelin; and 100% methanol elutes lysolecithin.

**Table II**

Specific activities of phospholipid fractions from rabbits treated by injection with Na acetate-1-C\textsuperscript{14}

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Tissue</th>
<th>Exper. period</th>
<th>Free cholesterol\textsuperscript{a}</th>
<th>Cholesterol ester cholesterol\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Concentration</td>
<td>Specific activity*</td>
</tr>
<tr>
<td>4 (eviscerated)</td>
<td>Intima</td>
<td>0.75</td>
<td>19.5</td>
<td>e</td>
</tr>
<tr>
<td></td>
<td>Terminal plasma</td>
<td>0.75</td>
<td>9.4</td>
<td>e</td>
</tr>
<tr>
<td>5 (noneviscerated)</td>
<td>Intima</td>
<td>5</td>
<td>81.0</td>
<td>1.67</td>
</tr>
<tr>
<td></td>
<td>Terminal plasma</td>
<td>5</td>
<td>6.50</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Intima</td>
<td>5</td>
<td>54.7</td>
<td>0.919</td>
</tr>
<tr>
<td></td>
<td>Terminal plasma</td>
<td>5</td>
<td>5.50</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>Intima</td>
<td>5</td>
<td>44.7</td>
<td>0.636</td>
</tr>
<tr>
<td></td>
<td>Terminal plasma</td>
<td>5</td>
<td>3.82</td>
<td>3.62</td>
</tr>
<tr>
<td></td>
<td>Intima</td>
<td>5</td>
<td>71.0</td>
<td>0.132</td>
</tr>
<tr>
<td></td>
<td>Terminal plasma</td>
<td>5</td>
<td>14.2</td>
<td>0.086</td>
</tr>
</tbody>
</table>

\textsuperscript{a} As counts per minute per \( \mu \)mole multiplied by suitable factors so that injected doses are equalized. Specific activities represent the radioactivity found in the whole molecule per \( \mu \)mole of phosphorus. Measurements were made 5 hours after acetate injection.

**Table III**

Specific activities of cholesterol and cholesterol ester in cholesterol fed rabbits after Na acetate-1-C\textsuperscript{14} injection

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Tissue</th>
<th>Exper. period</th>
<th>Free cholesterol\textsuperscript{a}</th>
<th>Cholesterol ester cholesterol\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Concentration</td>
<td>Specific activity*</td>
</tr>
<tr>
<td>1 Intima</td>
<td>0.75</td>
<td>19.5</td>
<td>e</td>
<td>24.4</td>
</tr>
<tr>
<td>2 Intima</td>
<td>0.75</td>
<td>9.4</td>
<td>e</td>
<td>10.4</td>
</tr>
<tr>
<td>4 Intima</td>
<td>5</td>
<td>81.0</td>
<td>1.67</td>
<td>120</td>
</tr>
<tr>
<td>5 Intima</td>
<td>5</td>
<td>6.50</td>
<td>4</td>
<td>0.50</td>
</tr>
<tr>
<td>3 Intima</td>
<td>5</td>
<td>54.7</td>
<td>0.919</td>
<td>100</td>
</tr>
<tr>
<td>5 Intima</td>
<td>5</td>
<td>5.50</td>
<td>1.58</td>
<td>20.5</td>
</tr>
<tr>
<td>5 Intima</td>
<td>5</td>
<td>44.7</td>
<td>0.636</td>
<td>75.6</td>
</tr>
<tr>
<td>5 Intima</td>
<td>5</td>
<td>3.82</td>
<td>3.62</td>
<td>32.4</td>
</tr>
<tr>
<td>5 Intima</td>
<td>5</td>
<td>71.0</td>
<td>0.132</td>
<td>67.2</td>
</tr>
<tr>
<td>5 Intima</td>
<td>5</td>
<td>14.2</td>
<td>0.086</td>
<td>35.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Free and esterified cholesterol specific activities are expressed as counts per minute in the cholesterol fraction per \( \mu \)mole. Specific activities multiplied by suitable factors so that injected doses are equalized.

\textsuperscript{b} Less than 2 c.p.m. in total sample.

acid-impregnated paper prepared by the method of Dieckert and Reiser (11) with a solvent system of 250:1 (volume per volume) Skelly B (b.p. 60-70°)-methanol. In this system monoglyceride gave an \( R_F \) of 0.16, diglyceride and free fatty acid an \( R_F \) of 0.89, cholesterol an \( R_F \) of 0.88, whereas triglyceride and cholesterol ester were front-running. In plasma and intima samples only two spots could be discerned, one with the \( R_F \) of cholesterol and the other with the \( R_F \) of triglyceride. Therefore, the triglyceride in Fraction II was judged not to be contaminated with diglyceride, monoglyceride, cholesterol ester, or free fatty acid.

The specific activities of free and esterified cholesterol found in Table III are expressed as counts per minute in the cholesterol fraction per \( \mu \)mole of cholesterol. Since both fractions were carried only through the digitonide step for purification, the radioactivity given may be higher than that found in pure cholesterol, because of contamination with high counting companions. Nevertheless, the total radioactivities in both cholesterol fractions were much lower than in the triglyceride and less than 1% of that in the phospholipids. In contrast to the high ratios of intima to plasma phospholipid specific activities in the eviscerates, similar ratios for cholesterol and cholesterol ester were less than 1. From these data one cannot conclude that intimal cholesterol was derived from synthesis. Neither can synthesis be denied on the basis of these experiments, since the low specific...
activity may be the result of a low rate of incorporation into the preformed cholesterol pool of the aortic intima.

The eviscerated animals expired as CO₂ 19.7 and 20.4% of the injected dose in the 5-hour experimental period, and the sham-operated animals expired 32.2 and 22.2% of the injected dose as CO₂ in the same interval. According to these findings there was little difference in the magnitude of the acetate pool sizes in the eviscerated and sham-operated animals.

**DISCUSSION**

When Chernick et al. (16) incubated rat aorta with randomly labeled C¹⁴-acetate, C¹⁴-labeled fatty acids could be isolated, but too little label was found in the digitonin-precipitable material to permit the accurate determination of radioactivity. In contrast, others have shown that, in *vitro*, normal (17, 18) and “sclerotic” (17) arterial tissues of rabbits convert C¹⁴-labeled acetate to nonsaponifiable lipid and fatty acid to the same extent. This indicates metabolic activity in the aorta of the normal as well as of the cholesterol-fed rabbit, but the nonsaponifiable lipid had only one-third of the total lipid C¹⁴. Cholesterol synthesis *in vitro* was also observed by Azarnoff (19) who found that cholesterol purified through the dibromide exhibited radioactivity when C¹⁴-labeled acetate was incubated with normal rabbit aorta. However, Werthessen and Schwenk (20) have shown with pig liver that great increases in cholesterol synthesis occurred when the liver was cut or pounded with a brush. This casts considerable doubt upon the estimation of rates of cholesterol biosynthesis in the intact animal from data derived from experiments *in vitro*.

Our data concerning cholesterol synthesis *in vivo* in the rabbit aorta show that a very small proportion of the C¹⁴-acetate is incorporated into digitonin-precipitable material. In a series of experiments in which Biggs and Kritchevsky (21) compared aortic cholesterol in rabbits fed either tritium-labeled cholesterol or tritium-enriched water, they observed that the rabbit atherosclerotic aorta derives most of its plaque cholesterol from plasma rather than from endogenous synthesis. Experiments in our laboratory in which cholesterol-4-C¹⁴ was fed to rabbits have confirmed these findings. Schwenk and Stevens (22) and Dury and Swell (23) similarly showed entrance of labeled plasma cholesterol into rabbit aorta.

Synthesis *in vitro* of P³²-labeled phospholipids in normal rat aorta was first demonstrated by Chernick et al. (16). Our own experiments in intact rabbits confirmed the ability of the normal artery to synthesize phospholipids from P³²-phosphate and indicated the possible role of arterial phospholipid synthesis in the development of lipid lesions in the aortas of cholesterol-fed rabbits. In agreement with the C¹⁴-acetate experiments reported in this paper, we observed that evisceration accentuated the difference in specific activity of P³²-labeled phospholipids between artery and plasma. In the P³² experiments the removal of liver, intestines, and kidneys from the circulation lowered the incorporation of P³² into plasma phospholipids to nearly one-tenth that observed in the sham-operated animals, yet the incorporation of P³² into intimal phospholipids of the two groups of animals did not differ (3). These experiments clearly indicate that the phosphate moiety of the intimal phospholipid originated in the atheroma but do not, of course, prove that the carbon skeleton of the phospholipids had a similar origin. The experiments in the present paper lend evidence toward the concept that phospholipid fatty acids are also synthesized by the arterial wall.

After the injection of C¹⁴-acetate, C¹⁴ could be incorporated into arterial phospholipids in one of three ways: (a) the transfer of intact labeled phospholipids from plasma to aorta, (b) the interesterification of fatty acids between plasma and aortic phospholipids, and (c) the incorporation of C¹⁴-acetate into aortic phospholipid fatty acids by the metabolic activity of the arterial wall.

By these three hypotheses one could explain a specific activity of lipid in the artery that is higher than the specific activity of the corresponding lipid in the terminal plasma in two ways: (a) the arterial lipids are synthesized *in situ*, or (b) the arterial lipids are derived from plasma and the specific activity of the plasma lipid reaches a maximum before the termination of the experiment. The second alternative was ruled out in all animals except rabbit number 4 in which early plasma phospholipid and triglyceride samples exhibited higher specific activities than the corresponding 5-hour terminal samples. In this animal the ratio of the terminal arterial lipid specific activity to the maximal plasma lipid specific activity might be of greater value in excluding the plasma lipid as a source of arterial lipid. This ratio is 20 for the total phospholipid and 1.6 for the triglyceride. These ratios are of the same order of magnitude as the ratios of the specific activities of terminal arterial lipids to terminal plasma lipids in the other two eviscerated rabbits (numbers 1 and 2) which are 74 and 11 for the phospholipids and 3.4 and 3.8 for the triglycerides. It appears, therefore, that both phospholipids and triglycerides of the atheromatous aorta are largely synthesized *in situ*.

**SUMMARY**

1. Phospholipid specific activities in the aortic intima of eviscerated, cholesterol-fed rabbits treated by injection with acetate-1-C¹⁴ were higher than those in plasma.
2. In comparing the specific activities of individual plasma and intimal phospholipid classes, viz. noncholine-containing phospholipids, lecithin, sphingomyelin, and lysolecithin, it was found that all intimal phospholipids had higher specific activities than the corresponding terminal plasma phospholipids.
3. Intimal triglyceride specific activities of eviscerated rabbits exceeded those of the terminal plasma samples, but in the sham-operated animals the plasma specific activities were higher than those of the intima.
4. The degree of incorporation of label into free cholesterol in the cholesterol-fed rabbit intima was less than 1% of that going into phospholipid. An even lower incorporation was observed in the ester cholesterol.
5. Apparently phospholipids and triglycerides of atheromatous lesions are synthesized *in situ*, although the data furnish no proof that cholesterol is derived from local synthesis.

**Acknowledgment**—We wish to thank Mrs. Suzanne Rhea, Mrs. Jo Northrop, and Mrs. Peggy Barnes for their technical assistance. Our thanks are also extended to Mrs. Anne Boals for her assistance in surgery.

**REFERENCES**

The Synthesis of C\textsuperscript{14}-Lipids in Rabbit Atheromatous Lesions
H. A. I. Newman, Esther L. McCandless and D. B. Zilversmit


Access the most updated version of this article at
http://www.jbc.org/content/236/5/1264.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/236/5/1264.citation.full.html#ref-list-1