Fatty Acid Composition of Serum and Aortic Intimal Lipids in Rabbits Fed Low- and High-Cholesterol Diets

By DONALD B. ZILVERSMIT, PH.D., CHARLES C. SWEELEY, PH.D., AND HOWARD A. I. NEWMAN, PH.D.

The rabbit maintained on a high-cholesterol diet rapidly accumulates lipids in the aortic intima. The origin of these lipids has been much debated. On the one hand, evidence has been presented that aortic lipid plaques are residues from lipids originally present in serum, whereas other experiments have demonstrated the ability of the aorta of the rabbit to synthesize various lipid fractions. Isotopic studies in particular have contributed to our knowledge on the development of atheromatous lesions. Thus it has been shown that various phospholipids of the atheromatous aorta turn over more rapidly than those of the normal artery and that, of the phospholipids present at any one time, few if any of their constituents are derived from plasma. \(^1\) In the case of cholesterol, however, the situation seems to be different. Although a measurable turnover of cholesterol in the atheromatous lesion takes place, a considerable portion of the total cholesterol present in the lesion was once a plasma constituent. \(^2\) In order to throw further light on the origin of atheromatous lipids, we have compared the composition of different lipid fractions in the plasma to their counterparts in the atheromatous lesion. If lipids were deposited from plasma at random and subsequently did not undergo metabolic alterations in the aorta, then the composition of aortic lipid fractions should be identical to that of plasma. Differences in composition could, therefore, be interpreted as evidence against the deposition or filtration theory or as evidence that lipids, once deposited, undergo metabolic alterations in the arterial wall.

Methods

Albino New Zealand rabbits were maintained on Purina chow for about eight weeks and then divided into two groups. Half of them were continued for 16 additional weeks on the chow diet whereas the other half received daily 1 Gm. of cholesterol dissolved in 2.6 Gm. of Humko hydrogenated cottonseed oil* added to the diet. At the end of the period, all animals were bled by heart puncture into syringes wetted by 2 per cent Mepesulfate in saline and the entire thoracic aorta and portions of adipose tissue removed for analysis. The intima and adventitia were separated and ground up separately before extraction. Lipids from all tissues were extracted with chloroform-methanol and purified by an adaptation of the Folch procedure. \(^3\) Neutral lipids were separated from phospholipids on small activated silica acid columns. \(^4\) The neutral lipids were further fractionated by the method of Horning et al. \(^5\) whereas the phospholipids were separated according to Newman et al. \(^6\) as follows: 4 Gm. of Mallinekrodt 100 mesh silicic acid (suitable for chromatographic analysis), freshly activated by heating at 120 to 130 C. for 48 hours, was slurried with chloroform and then transferred to a column of the dimensions 10 X 100 mm. having a tapered tip and a glass-sealed 250-ml. reservoir. The silicic acid was packed with sufficient pressure from dried air (approximately 1 lb. per square inch) to maintain a flow rate of 0.5 ml. per minute. The phospholipid sample in chloroform was added, and the pressure increased gradually to 4 to 5 lbs. to maintain the 0.5-ml.-per-minute flow rate. Loads from 0.147 to 1.23 mg. phospholipid phosphorus were used. The eluates from the column were automatically collected through a volumetric siphon in 5-ml. fractions. When the sample had completely entered the column, it was washed with 30 ml. more chloroform, which eluted most of the neutral lipids. Approximately 40 to

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*Cholesterol USP grade was graciously donated by Merck Co., Rahway, New Jersey, and hydrogenated cottonseed oil by Humko Co., Memphis, Tennessee.

†Anticoagulant, Hoffmann La Roche.
Table 1
Per Cent Fatty Acids of Diet, Adipose Tissue, Plasma, and Intimal Triglycerides*

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<td>43.8</td>
<td>6.8</td>
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<tr>
<td>High cholesterol + fat chow</td>
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<td>18.1</td>
<td>1.2</td>
<td>6.2</td>
<td>43.1</td>
<td>26.9</td>
<td>3.9</td>
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<td>26.8</td>
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<td>30.6</td>
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<tr>
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<td>24.1</td>
<td>4.3</td>
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<td>28.1</td>
<td>6.0</td>
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<td>Plasma cholesterol-fed†</td>
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<td>23.1</td>
<td>2.7</td>
<td>6.6</td>
<td>37.9</td>
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<tr>
<td>Intima cholesterol-fed§</td>
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<td>29.4</td>
<td>4.4</td>
<td>12.4</td>
<td>29.6</td>
<td>17.1</td>
<td>2.1</td>
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</tbody>
</table>

*Little or no C_{19} acid was present in any of these samples.
†All values are means of three animals.
‡Pooled intima from six animals.
§Mean value of two intimas.
||One value was 11.4 and the other 22.9.

Table 2
Per Cent Fatty Acids of Plasma and Intimal Cholesterol Esters in Cholesterol-Fed Rabbits

<table>
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<td>0.9</td>
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<td>4.5</td>
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<td>44.2</td>
<td>55.0</td>
<td>23.0</td>
<td>16.1</td>
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</tbody>
</table>

*Less than 0.5 per cent.

50 ml. of 20 per cent methanol in chloroform (v/v) 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 per cent methanol in chloroform (v/v) eluted lecithin; 50 to 80 ml. of 60 per cent methanol in chloroform (v/v) eluted sphingomyelin; finally, 50 ml. of methanol eluted lysolecithin.

Fatty acids were determined by gas-liquid chromatography. Methyl esters from each lipid fraction were obtained by transmethylation in methanol-concentrated sulfuric acid (95/5, v/v) at 75 C. for 12 hours in sealed tubes. The esters were extracted from the reaction mixtures with redistilled hexane. Analyses were made on a 6 ft. by 4 mm. glass tube packed with ethylene glycol-succinic acid polyester on Chromosorb W—50 to 100 mesh, 16 per cent (w/w) of polyester. The column was maintained at 185 C. with an inlet pressure of 22 to 25 pounds per square inch of argon. The retention time for methyl stearate was 10 minutes, and the separation factor for methyl oleate and methyl stearate was 1.18. The column load was usually 25 to 50 Rg. of mixed methyl esters. The gas-liquid partition chromatography instrument was equipped with an argon ionization detector. Area calculations were based on measurements of peak height and width at half height. The results, which are reported in the tables, are in weight per cent, obtained by a normalization calculation from the component areas.

Results
In order to obtain sufficient lipid for the various fractionations, intimas from six normal rabbits were pooled before extraction, but intimas from cholesterol-fed animals were analyzed separately. All plasma and adipose tissue samples were also analyzed separately. Although the fatty acid composition of the diet of the cholesterol-fed animals differed from that of the controls by the addition of 2.6 per cent hydrogenated cottonseed oil, no remarkable differences appeared in the fatty acid composition of plasma or adipose tissue triglycerides (table 1). The fatty acid composition of adipose tissue was very similar to the fatty acid composition of the cholesterol- and hydrogenated-fat-supplemented diet. The high percentage of linoleic (18-2) acid of the unsupplemented chow was
Figure 1

Chromatographic separation of plasma phospholipids in normal (left) and cholesterol-fed (right) rabbit; diagonal lines = lecithin analyzed by gas-liquid chromatography (GLC); vertical lines = sphingomyelin analyzed by GLC; horizontal lines = lysolecithin analyzed by GLC.

not reflected in a corresponding enrichment of that acid in adipose tissue. It should be noted, however, that although the total fat intake of the two groups of animals differed, the total intake of linoleic acid was approximately the same. A comparison of fatty acids in plasma and arterial triglycerides in the normal or cholesterol-fed animals also failed to show any consistent differences. Nor did the medial and adventitial triglyceride fatty acid pattern differ significantly from that in the intima or plasma.

The cholesterol esters of plasma and intima showed more striking differences. Although analyses of the pooled normal intima were made, too little cholesterol ester was present to give reliable results. However, it is clear from table 2 that the cholesterol esters accumulating in the atheromatous intima contained primarily oleic (18:1), palmitic (16:0), and linoleic (18:2) acid. A comparison of cholesterol esters in the plasma and intima of cholesterol-fed animals reveals a remarkable similarity in composition, except for the presence of about 3 per cent polyunsaturated 18-carbon acids in the atheromatous intima, whereas none were found in the plasma esters. Smaller but consistent differences were noted in the oleic (18:1) and linoleic (18:2) acid fractions. A typical distribution of phospholipids in plasma and aorta of normal and cholesterol-fed rabbits is shown in figures 1 and 2. In confirmation of previous results, the choline-containing phospholipids in the atheromatous intimas increased much more than the noncholine types. In two of the three aortas from cholesterol-fed animals the separation of lecithin and sphingomyelin was made difficult by the appearance of a considerable fraction which consisted of a mixture of these two lipids (see fig. 2, right). Gas chromatographic analyses are reported on material obtained from the lecithin, sphingomyelin, and lysolecithin fractions as indicated.
in figures 1 and 2. In other animals the lecithin and lysolecithin were found to contain less than 1 per cent impurity by paper chromatography\(^a\) whereas the sphingomyelin fraction contained up to 10 per cent lecithin. Fatty acid pattern of the noncholine fractions are not reported, since this fraction was relatively small and appeared to be contaminated with some glyceride. Cholesterol feeding appeared to have little or no effect on the lecithin fatty acids of plasma or intima (table 3). In both normal and cholesterol-fed animals, however, the relative content of linoleic (18-2) acid in plasma far exceeded that in the intima, whereas the palmitoleic (16-1) was consistently less in the former. In the atheromatous intima the elution pattern showed a pronounced tailing of the lecithin fraction. Fatty acids from this portion of the chromatogram are not included in table 3. Although the plasma samples of the cholesterol-fed animals showed appreciable amounts of arachidonic (20-4), behenic (22-0), tricosanoic (23-0), lignoceric (24-0), and particularly nervonic (24-1) acids in this portion of the eluate, the intimal lesions showed practically no such acids. This portion of the eluate also showed a relatively high palmitoleic (16-1) acid content of artery compared to that of plasma.

The sphingomyelin fraction of both plasma and aortic intima showed an increase in concentration similar to or greater than that observed for lecithin. The fatty acids of plasma sphingomyelin of cholesterol-fed animals did not differ too much from those of control animals (table 4) except that there seemed to be a significantly higher percentage of palmitoleic (16-1) acid in the control animals. The intima of the cholesterol-fed animals appeared to have an elevated percentage of palmitoleic acid and particularly of nervonic (24-1) acid which was not found in normal intima. Conversely, the percentage of arachidic (20-0) and lignoceric (24-0) acids decreased below that of the normal intima. A comparison of intimal and plasma sphingomyelin in the normal animals revealed a

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**Figure 2**

Chromatographic separation of intimal phospholipids in normal (left) and cholesterol-fed (right) rabbit; diagonal lines = lecithin analyzed by GLC; vertical lines = sphingomyelin analyzed by GLC; horizontal lines = lysolecithin analyzed by GLC.

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\(^a\) ZILVERSMIT, SWEELEY, NEWMAN

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### Table 3

**Per Cent Fatty Acids of Plasma and Intimal Lecithins**

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*Less than 0.5 per cent.

### Table 4

**Per Cent Fatty Acids of Plasma and Intimal Sphingomyelins**

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*Less than 0.5 per cent.
higher content of lignoceric (24-0) acid in
the intima, whereas in the cholesterol-fed
animal the intima contained much less pal-
mitic (18-0) acid and more laurie (12-0),
myristic (14-0), palmitoleic (16-0), oleic
(18-1), and lignoceric (24-0) acid than the
corresponding plasma. The amount of lyso-
lecithin phosphorus found in plasma and
aorta of normal or cholesterol-fed animals
varied from 10 to 20 per cent of the total
lipid phosphorus. This relatively high lyso-
lecithin content of plasma parallels that
observed in the plasma of the rat6 and is
somewhat higher than that in the plasma of
man.11 The analysis of lysolecithin fatty acids
(table 5) showed that the normal intima
contained a smaller percentage of linoleic
(18-2) acid and a higher percentage of lino-
lenic (18-3) acid than normal plasma, whereas
the intima of the cholesterol-fed animal con-
tained relatively more laurie (12-0), myristic
(14-0), palmitoleic (16-1), and linolenic
(18-3) acid and less linoleic (18-2) acid than
the corresponding plasmas.

**Discussion**

Previous studies in our laboratory have dem-
strated that the phospholipids of atheroma-
tous lesions in the aorta of the cholesterol-fed
rabbit turn over rapidly.1 Studies with P32
and C14-acetate have indicated that the radio-
active phospholipids present in the arterial
lesions of these rabbits are predominantly
synthesized in situ.12 13 The pronounced
differences in fatty acid composition of
arterial intima and plasma reported in this
paper also support the concept that plaque
phospholipids are not merely random deposits
of plasma phospholipids. The case for cho-
lesterol appears to be somewhat different:
in the intact cholesterol-fed rabbit the cho-
lesterol synthesis by the artery makes little
or no contribution to the cholesterol of the
lesion.2 This observation is not necessarily in
conflict with the finding that fragments of
rabbit artery in vitro are capable of convert-
ing C14-acetate to sterol.14 15 If such con-
version occurs in the intact cholesterol-fed
rabbit it merely represents an insignificant
proportion of the total cholesterol flux into.
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