Lipid Composition and Metabolism of Thromboatherosclerotic Lesions Produced by Continued Endothelial Damage in Normal Rabbits

By Allan J. Day, Frank P. Bell, Sean Moore, and Robert Friedman

ABSTRACT

Thromboatherosclerotic and fibrous lesions were produced by endothelial damage with polyethylene catheters inserted into the aortas in rabbits on a normal diet. Two weeks after insertion of the catheters, the concentration of both free cholesterol and cholesteryl ester in the thromboatherosclerotic lesions was significantly greater than that in the adjacent normal intima. A further increase in the concentration of free cholesterol and particularly of cholesteryl ester occurred during the remainder of the 4-month study period. Gas-liquid chromatography indicated that the raised thromboatherosclerotic lesions contained more cholesteryl oleate and less cholesteryl linoleate than did either the normal intima or the fibrous lesions. The incorporation of [1-14C]oleic acid into combined lipid in the aortas incubated in vitro showed that, by 2 weeks, two to three times more oleic acid had been incorporated into cholesteryl ester in the thromboatherosclerotic raised lesions than in the normal intima. A similar increase was demonstrated at 2 and 4 months. When [32P]phosphate was used as a precursor, incorporation into lecithin was higher and incorporation into phosphatidyl inositol was lower in the raised lesions than they were in the normal intima. Fibrous lesions did not differ significantly from the adjacent normal intima in their incorporation of either of these precursors into lipid. Therefore, the accumulation of cholesteryl ester in the thromboatherosclerotic lesions resulted in part from synthesis in the arterial wall, which was stimulated by the prominent platelet components of the lesions.

KEY WORDS atherogenesis arterial metabolism phospholipid cholesterol arterial injury platelet thrombi cholesteryl ester

The role of elevated serum lipids as an etiological factor in the development of atherosclerotic vascular disease has been studied extensively. In comparison, the roles of injury or mural platelet thrombi as agents primarily responsible for lipid accumulation in the early atherosclerotic lesion have received less attention. Most of the experimental techniques in which injury or thromboemboli are used to produce experimental atherosclerosis cause fibrous lesions (1–5), and elevation of serum lipid levels is normally necessary to unmask the effect of injury or thromboemboli on lipid accumulation in the lesion (3, 6–9). Hand and Chandler (10) have demonstrated, however, that autologous platelet thrombi injected into normocholesterolemic rabbits give rise to lipiddContaining lesions. The suggestion that platelet lipid contributes directly to the lipid accumulation of the early atherosclerotic lesions, however, is untenable, because the lipid composition of both platelets and organized platelet thrombi differs markedly from that of the atherosclerotic lesion (11, 12). More probably, platelet uptake by arterial wall cells (macrophages and smooth muscle cells) is associated with stimulated lipid synthesis in the resulting lesion.

Lipid-containing atherosclerotic lesions have recently been produced in rabbits and rats on normal diets following acute transverse injury to the aortic endothelium (13, 14). Thrombotic lesions produced in situ by chronic endothelial damage with polyethylene catheters implanted in the aorta of rabbits on normal diets have also been shown to develop into lesions resembling fatty streak (15) and fibrofatty (15, 16) lesions in man. The morphology of these lesions has recently been reported (15), and histochemical evidence has been obtained that agrees with the conclusion that stimulation of lipid synthesis by smooth muscle cells and macrophages accounts for some of the accumulated lipid. The
suggestion that the lipid accumulation in these lesions produced by catheter-induced injury is associated with the stimulation, possibly by the platelet thrombi which are formed, of lipid synthesis in the smooth muscle cells was investigated in the present paper. The lipid composition and the lipid synthesis of different lesions evolving from platelet thrombi or small fibrous lesions produced by continued endothelial damage in rabbits on normal diets are also presented.

Methods

New Zealand white rabbits (2.5-3.5 kg) were fed Purina rabbit chow throughout the experiment. Polyethylene catheters (1.2 mm, o.d.) were inserted into the aorta under sodium pentobarbital anesthesia through an incision in the femoral artery. The catheters were passed into the aorta to approximately the midthoracic region and sutured in place by a stay stitch around the femoral artery. After 2 weeks, 2 months, or 4 months, the rabbits were killed with sodium pentobarbital, and the whole aorta from the heart to the iliac bifurcation was removed with the catheter still in situ. Adventitial connective tissue was removed, and the aorta was opened longitudinally to display the lesions produced. Two types of lesions (fibrous and raised) were present. The fibrous lesions were flat and pearly in appearance corresponding to similar lesions described by Moore (15). These lesions were present at all of the time intervals studied, although at 2 weeks their presence was limited. Raised lesions were pendulous and, in some cases, appeared as large organized mural thrombi on the endothelium. In other cases, they were invasive and enclosed the catheter. These raised lesions corresponded to the raised and tunnel lesions described by Moore (15). Macroscopically normal intima between the lesions and its subjacent media were used for comparison. The two types of lesions and the normal intima were separately dissected, together with the underlying media. Careful dissection with cilia forceps permitted the separation of the intima-media segments corresponding to the two types of lesions and the normal intima-media.

Full details of the morphology of the two types of lesions studied have been reported previously (15). The fibrous lesions did not stain for lipid; they were normally covered with endothelium and contained appreciable numbers of smooth muscle cells. The raised lesions contained platelet fibrin thrombi and showed lipid staining as early as 2 days after insertion of the catheters. Most of this lipid was present intracellularly in macrophages and foam cells. Some of these lipophage cells showed smooth muscle cell characteristics by electron microscopy; fat was prominent in medial smooth muscle cells at 2 weeks. At later time intervals, extracellular lipid became more prominent with foam cells surrounding the lipid pool.

CHEMICAL ANALYTICAL STUDIES

Because the tissues contained relatively small amounts of lipid, it was necessary to pool material from 3-5 rabbits for examination in batches. Twelve rabbits (three batches) were examined at 2 weeks, 19 or, for some analyses, 21 rabbits (four or six batches) were studied at 2 months, and 6 rabbits (two batches) were studied at 4 months. The combined normal intima, fibrous lesions, or raised lesions in the batches at each time interval were homogenized and extracted with a chloroform-methanol solution (2:1 v/v), and the resulting lipid extract was washed as described by Folch et al. (17). The protein precipitate present after extraction with the chloroform-methanol solution was separated for determination of dry defatted weight and DNA content (18). The lipid extract was reserved for determination of its free cholesterol, cholesteryl ester, and lipid phosphorus content. Because of the small quantity of cholesteryl ester present in the extracts, the following procedure, which is essentially the procedure described by Day and Wahlqvist (19), was adopted. An internal standard containing a known amount of cholesteryl heptadecanoate and a known tracer amount of 14C-labeled cholesterol was added to the lipid extract. The cholesteryl heptadecanoate provided a basis for quantification of the cholesteryl esters which were measured as methyl esters by gas-liquid chromatography following initial separation of the cholesteryl esters by thin-layer chromatography. Recovery of free cholesterol was monitored by the labeled cholesterol. No internal standard for phospholipid was added.

After the addition of the internal standard, the cholesterol, phospholipid, and cholesteryl esters in the lipid extract were separated by thin-layer chromatography on silica gel G using a N-hexane-diethyl ether-acetic acid solvent system (146:50:4 v/v/v). Spots were visualized by spraying with 0.2% dichlorofluorescein. Cholesteryl ester was scraped directly into ampules and methyl-esterified with 5% H2SO4 in methanol; its fatty acid composition and content were determined by gas-liquid chromatography as described in the following section. Free cholesterol was eluted with chloroform; a sample was counted to monitor recovery, and another sample was assayed by gas-liquid chromatography. Phospholipid was eluted by the method of Arvidson (20); a sample of the eluate was used for determination of lipid phosphorus (21) and another sample was methyl-esterified with 5% H2SO4 in methanol for determination of its fatty acid composition by gas-liquid chromatography.

GAS-LIQUID CHROMATOGRAPHY

Methyl esters were separated on 15% diethylene-glycol-succinate columns using a gas chromatograph (Hewlett Packard 402) at a column temperature of 170°C. Separation of standards KA and KD (Applied Science Laboratories) showed a percent composition comparison with an error of less than 10% for minor compo-
CHEMICAL ANALYSIS

Streptomycin and penicillin (50 µg and 50 units/ml, respectively) were also added to the incubation medium. After incubation, the aortas were removed and washed thoroughly in 0.9% sodium chloride solution; the normal intima and the fibrous and raised lesions were separated and extracted separately with a chloroform-methanol solution as previously described. Extracts were washed as described by Folch et al. (17), and the total cholesterol content and the counts/min of both 14C and 32P were determined. A sample of the lipid extract was then separated into phospholipid, cholesterol, fatty acid, triglyceride, and cholesteryl ester by thin-layer chromatography using the method of Skipski et al. (23). Individual lipid spots were identified by spraying with 0.2% dichlorofluorescein and counted directly following scraping into counting vials using the dioxane-water scintillator described by Snyder (22). The incorporation of both [1-14C]oleic acid and [32P]phosphate into individual phospholipids was followed by separation of the lipid extract into individual phospholipids by thin-layer chromatography using the method of Skipski et al. (23). Phospholipid spots were identified with 0.2% dichlorofluorescein and counted directly with the dioxane-water scintillator (22) after they had been scraped into counting vials. Counting was done with a liquid scintillation counter and double-labeled techniques to separate 14C and 32P. Quenching and 32P decay were monitored and corrected for where necessary. All data were expressed as counts per minute of the respective lipid 14C or 32P incorporated per microgram of DNA in the respective portion of the artery—normal intima, fibrous lesion, or raised lesion.

CHEMICAL ANALYSIS

Lipid phosphorous was determined by the method of Bartlett (21). Cholesterol was determined in samples of the lipid extracts or in the eluates by the automated procedure of Block et al. (24) using an autoanalyzer (Technicon). DNA was determined by the method of Dische (18).

STATISTICAL ANALYSIS

Analyses of variance were carried out on both analytical and metabolic data from the three groups—normal intima, fibrous lesions, and raised lesions—at each time interval. Statistical significance was calculated by Student’s t-test.

Results

The concentration of lipids in the normal aorta and in the fibrous and raised lesions at the three time intervals studied is shown in Table 1. The free cholesterol concentration of the fibrous lesion was only marginally increased over that of the normal intima. The free cholesterol content of the raised lesion, however, was considerably increased over both the corresponding normal intima and the corresponding fibrous lesion (P < 0.01 at both 2 weeks and 2 months). Cholesteryl ester showed the most marked increase of all the lipids in the raised lesions. At 2 weeks, 114 µg cholesteryl ester/mg DNA was present compared with 17 µg/mg DNA in the adjacent normal intima. The cholesteryl ester concentration of the raised lesions rose to 538 µg/mg DNA and 597 µg/mg DNA at 2 months and 4 months, respectively. These values were significantly different compared with the cholesteryl ester concentration of either normal intima or fibrous lesions (P < 0.01. The marked rise in the cholesteryl ester content in the raised lesions accounted for an elevation in the percent of cholesteryl ester in the aorta from 2.8% in the normal intima to 8.3, 24.5, and 28.6% in the raised lesion at 2 weeks, 2 months, and 4 months, respectively. Changes in the cholesteryl ester content of the fibrous lesions were much smaller; the levels did not differ significantly from those in the adjacent normal intima.

The increased amount of cholesteryl ester present in the normal intima at 2 months (64.5 µg/mg DNA) requires some explanation. The amount of cholesteryl ester present in the normal artery is extremely small; therefore, small amounts of cholesteryl ester could account for marked changes in cholesteryl ester concentration. It is possible that, although the apparently normal artery studied was macroscopically free of fatty deposits, microscopic fat-containing lesions could have been present. Moore (15) has reported that flat fatty lesions are seen microscopically in similar material considered normal by gross examination. Despite

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The proportion of cholesteryl oleate had increased in the raised lesion at 2 weeks compared with that in the normal intima, and this effect became marked by 2 months when cholesteryl oleate had increased and cholesteryl linoleate had decreased in relation to their values in the normal intima. However, the cholesteryl ester fatty acid composition of the fibrous lesions at 2 months was almost identical with that of the normal intima. This change from cholesteryl linoleate to cholesteryl oleate in the raised lesion is demonstrated by the 18:1/18:2 ratio at 2 months which changed from 1.57 and 1.56 in the normal intima and the fibrous lesions, respectively, to 2.51 in the raised lesion. Only two batches were studied at 4 months and an even further elevation in the proportion of this relatively high content of cholesteryl ester in the macroscopically normal intima at 2 months, the raised lesions from the same aortas showed considerably higher levels of cholesteryl ester.

The lipid phosphorous concentration of the raised lesion was slightly less than that of the corresponding normal intima or fibrous lesion, but this difference was only statistically significant at 2 months ($P < 0.05$).

The fatty acid composition of the cholesteryl esters present in the normal intima and in the fibrous and raised lesions is shown in Table 2. In view of the small amount of material present, data was not available for the fibrous lesions at 2 weeks. The proportion of cholesteryl oleate had increased in the raised lesion at 2 weeks compared with that in the normal intima, and this effect became marked by 2 months when cholesteryl oleate had increased and cholesteryl linoleate had decreased in relation to their values in the normal intima. However, the cholesteryl ester fatty acid composition of the fibrous lesions at 2 months was almost identical with that of the normal intima. This change from cholesteryl linoleate to cholesteryl oleate in the raised lesion is demonstrated by the 18:1/18:2 ratio at 2 months which changed from 1.57 and 1.56 in the normal intima and the fibrous lesions, respectively, to 2.51 in the raised lesion. Only two batches were studied at 4 months and an even further elevation in the proportion of

### Table 2
Per cent Distribution of Cholesteryl Ester Fatty Acids of Normal Aorta and Fibrous and Raised Lesions

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Normal</th>
<th>Raised</th>
<th>Normal</th>
<th>Fibrous</th>
<th>Raised</th>
<th>Normal</th>
<th>Fibrous</th>
<th>Raised</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0 (Palmitic)</td>
<td>13.8 ± 0.8</td>
<td>16.8 ± 0.8</td>
<td>15.3 ± 0.9</td>
<td>14.7 ± 0.5</td>
<td>15.9 ± 0.8</td>
<td>18.3</td>
<td>15.1</td>
<td>16.9</td>
</tr>
<tr>
<td>16:1 (Palmitoleic)</td>
<td>9.4 ± 0.3</td>
<td>3.5 ± 0.3</td>
<td>7.0 ± 1.5</td>
<td>4.2 ± 0.2</td>
<td>3.2 ± 0.1</td>
<td>11.6</td>
<td>4.7</td>
<td>3.4</td>
</tr>
<tr>
<td>18:0 (Stearic)</td>
<td>6.4 ± 0.6</td>
<td>7.3 ± 0.6</td>
<td>5.3 ± 0.2</td>
<td>5.8 ± 0.5</td>
<td>5.6 ± 0.3</td>
<td>8.7</td>
<td>5.1</td>
<td>5.2</td>
</tr>
<tr>
<td>18:1 (Oleic)</td>
<td>39.8 ± 2.2</td>
<td>46.4 ± 2.2</td>
<td>39.6 ± 1.1</td>
<td>41.1 ± 0.4</td>
<td>48.9 ± 1.1</td>
<td>32.3</td>
<td>38.6</td>
<td>55.5</td>
</tr>
<tr>
<td>18:2 (Linoleic)</td>
<td>21.5 ± 2.6</td>
<td>22.6 ± 2.6</td>
<td>25.6 ± 1.9</td>
<td>26.7 ± 2.0</td>
<td>19.8 ± 0.9</td>
<td>19.3</td>
<td>32.1</td>
<td>14.7</td>
</tr>
<tr>
<td>20:4 (Arachidonic)</td>
<td>9.1 ± 0.7</td>
<td>3.3 ± 0.7</td>
<td>7.2 ± 1.8</td>
<td>7.7 ± 1.8</td>
<td>6.7 ± 1.0</td>
<td>8.3</td>
<td>4.6</td>
<td>4.3</td>
</tr>
<tr>
<td>18:1/18:2</td>
<td>1.84 ± 0.3</td>
<td>2.13 ± 0.3</td>
<td>1.57 ± 0.09</td>
<td>1.56 ± 0.11</td>
<td>2.51 ± 0.16</td>
<td>1.71</td>
<td>1.23</td>
<td>3.81</td>
</tr>
</tbody>
</table>

Numbers in the same row with the same superscripts are significantly different at the following levels: a-a $P < 0.05$, b-b $P < 0.01$, c-c $P < 0.001$, d-d $P < 0.001$, and e-e $P < 0.05$.

*Data are means of two batches (eight rabbits).
†Data are means ± SE of three batches (12 rabbits).
‡Data are means ± SE of four batches (19 rabbits).
§Data are means ± SE of six batches (36 rabbits).
¶Data are means ± SE of two batches (six rabbits).
TABLE 3
Percent Distribution of Phospholipid Fatty Acids of Normal Aorta and Fibrous and Raised Lesions

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Normal</th>
<th>Raised</th>
<th>Normal</th>
<th>Fibrous</th>
<th>Raised</th>
<th>Normal</th>
<th>Fibrous</th>
<th>Raised</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0 (Palmitic)</td>
<td>18.3</td>
<td>31.1 ± 2.7</td>
<td>19.6 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.2 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.9 ± 4.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.5</td>
<td>19.2</td>
<td>30.6</td>
</tr>
<tr>
<td>18:0 (Stearic)</td>
<td>20.3</td>
<td>20.7 ± 1.8</td>
<td>18.8 ± 0.2</td>
<td>19.8 ± 0.3</td>
<td>23.4 ± 2.9</td>
<td>22.7</td>
<td>22.0</td>
<td>19.5</td>
</tr>
<tr>
<td>18:1 (Oleic)</td>
<td>19.2</td>
<td>16.9 ± 2.0</td>
<td>16.8 ± 0.2</td>
<td>15.6 ± 0.2</td>
<td>15.3 ± 0.8</td>
<td>16.4</td>
<td>16.0</td>
<td>17.2</td>
</tr>
<tr>
<td>18:2 (Linoleic)</td>
<td>11.3</td>
<td>21.4 ± 1.8</td>
<td>11.2 ± 0.5</td>
<td>14.8 ± 0.8</td>
<td>15.6 ± 4.7</td>
<td>9.8</td>
<td>12.8</td>
<td>16.6</td>
</tr>
<tr>
<td>20:4 (Arachidonic)</td>
<td>31.0</td>
<td>9.8 ± 2.5</td>
<td>32.6 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.6 ± 0.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12.0 ± 3.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>29.6</td>
<td>30.1</td>
<td>16.0</td>
</tr>
</tbody>
</table>

Numbers, in the same row with the same superscripts are significantly different at the following levels: a–a P < 0.01, b–b P < 0.01, c–c P < 0.001, and d–d P < 0.001.
*Data are means of two batches (eight rabbits).
†Data are means ± SE of three batches (12 rabbits).
‡Data are means ± SE of four batches (19 rabbits).

Cholesteryl oleate and a further reduction in cholesteryl linoleate were present; therefore, the 18:1/18:2 ratio had altered from 1.71 in the normal intima (similar to that at 2 months) to 3.81 in the raised lesions.

The phospholipid fatty acid composition of the normal aorta and of the two types of lesions is shown in Table 3. Again, data for the fibrous lesion was not available at 2 weeks because of the paucity of material at this time. The phospholipid fatty acid composition of the fibrous lesion at the two time intervals studied was almost identical to that of the normal intima. The phospholipid fatty acid composition of the raised lesion, however, markedly contrasted with that of the normal intima. The phospholipid fatty acid composition of the raised lesion, however, markedly contrasted with that of the normal intima; elevated palmitic (16:0) and reduced arachidonic (20:4) acid were observed at all time intervals. This difference was clearly established in the rabbits studied after 2 weeks and persisted essentially unchanged to 4 months.

**METABOLIC EXPERIMENTS**

The incorporation of [1-14C]oleic acid into total lipid, phospholipid, triglyceride, and cholesteryl ester in the normal aorta and in the fibrous (2 and 4 months) and raised lesions is shown in Table 4. Since different incubation media were used at different time intervals, comparison between similar groups from different time intervals cannot be made. Comparison within each time interval, however, is valid, since identical incubation media were used for each of the aortas, and, of course, the two lesions and the normal intima were incubated in the same incubation media prior to their dissection. The figures for the raised lesion at 2 weeks include data from one aberrant rabbit that showed very high incorporation figures in relation to the content of DNA. The variation indicated by the standard errors for these figures was extremely high, and the corresponding means and standard errors for the three remaining rabbits are given in parentheses together with the total figures. The marked elevation in cholesterol concentration of the raised lesions which was demonstrated in the analytical studies is also shown in the metabolic series. The total cholesterol concentration of the raised lesions rose to 2.95 μg/μg DNA and 2.57 μg/μg DNA at 2 months and 4 months, respectively.

The incorporation of oleic acid into lipid was lower in the raised lesions than it was in the corresponding normal lesions at both 2 weeks and 2 months (Table 4). This finding may reflect the larger amount of inert thrombus present in the raised lesions. Incorporation into lipid in the normal intima and the fibrous and raised lesions was similar, however, by 4 months. The relative incorporation of [1-14C]oleic acid into phospholipid, triglyceride, and cholesteryl ester differed, however, in the raised and the fibrous lesions compared with the normal aorta. At each of the time intervals (2 weeks, 2 months, and 4 months), there was appreciable diversion of label to cholesteryl ester in the raised lesion. This feature can be seen by observing the incorporation of oleic acid into cholesteryl ester in relation to incorporation into phospholipid (Table 4). The ratio of cholesteryl ester to phospholipid incorporation was significantly higher for the raised lesion compared with that for the corresponding normal intima at 2 weeks (P < 0.001), at 2 months (P < 0.01), and at 4 months.
### TABLE 4
Incorporation of [1-14C] Oleic Acid into Combined Lipid in Normal Aorta and Fibrous and Raised Lesions (counts/min incorporated/μg DNA)

<table>
<thead>
<tr>
<th></th>
<th>2 Weeks</th>
<th></th>
<th>2 Months</th>
<th></th>
<th>4 Months</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Raised</td>
<td>Normal</td>
<td>Fibrous</td>
<td>Raised</td>
<td>Normal</td>
</tr>
<tr>
<td>Total lipid</td>
<td>89.3 ±8.5</td>
<td>89.3 ±34.2</td>
<td>228 ±26.9</td>
<td>287 ±68.6</td>
<td>140 ±12.5</td>
<td>89.0 ±11.0</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>48.3 ±5.3c</td>
<td>30.8 ±14.6</td>
<td>112 ±13.4</td>
<td>130 ±28.4c</td>
<td>45.4 ±6.0</td>
<td>46.0 ±3.25</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>28.5 ±3.5</td>
<td>23.7 ±7.2</td>
<td>69.2 ±12.4</td>
<td>100 ±32.7</td>
<td>43.2 ±3.4</td>
<td>24.2 ±7.87</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>2.95 ±0.52</td>
<td>7.45 ±3.2</td>
<td>18.1 ±3.1</td>
<td>23.2 ±5.8</td>
<td>13.6 ±1.9</td>
<td>1.86 ±0.47</td>
</tr>
<tr>
<td>Ratio of cholesteryl ester to phospholipid</td>
<td>0.060 ±0.005</td>
<td>0.254 ±0.011</td>
<td>0.169 ±0.030</td>
<td>0.177 ±0.026</td>
<td>0.301 ±0.008</td>
<td>0.039 ±0.009</td>
</tr>
<tr>
<td>Total cholesterol concentration (μg/μg DNA)</td>
<td>0.627 ±0.037</td>
<td>1.341 ±0.394</td>
<td>(0.952)</td>
<td>0.940 ±0.128</td>
<td>1.34 ±0.184f</td>
<td>2.95 ±0.329f</td>
</tr>
</tbody>
</table>

The data obtained after 2 weeks and 4 months were from four rabbits (means ± SE). Numbers in parentheses are means ± SE for three rabbits. The data obtained after 2 months were from five rabbits. Numbers in the same row with the same superscripts are significantly different at the following levels: a-a P < 0.05, b-b P < 0.05, c-c P < 0.01, d-d P < 0.01, e-e P < 0.001, and f-f P < 0.001.

### TABLE 5
Incorporation of [1-14C] Oleic Acid into Individual Phospholipids in Normal Aorta and Fibrous and Raised Lesions (counts/min incorporated/μg DNA)

<table>
<thead>
<tr>
<th></th>
<th>2 Weeks</th>
<th></th>
<th>2 Months</th>
<th></th>
<th>4 Months</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Raised</td>
<td>Normal</td>
<td>Fibrous</td>
<td>Raised</td>
<td>Normal</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>0.29 ± 0.59a</td>
<td>0.18 ± 0.06</td>
<td>(0.10 ± 0.01)a</td>
<td>2.06 ± 0.59e, d</td>
<td>0.44 ± 0.09a</td>
<td>0.12 ± 0.06f</td>
</tr>
<tr>
<td>Lecithin</td>
<td>31.7 ± 3.50a</td>
<td>23.1 ± 10.9</td>
<td>(12.5 ± 3.5)a</td>
<td>79.6 ± 9.3a</td>
<td>90.6 ± 18.2b</td>
<td>32.5 ± 4.1ab</td>
</tr>
<tr>
<td>Phosphatidyl inositol</td>
<td>8.3 ± 0.9c</td>
<td>3.6 ± 1.75</td>
<td>(1.86 ± 0.37)c</td>
<td>13.1 ± 2.3a</td>
<td>21.7 ± 6.3b</td>
<td>5.14 ± 0.75bc</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>7.8 ± 0.92c</td>
<td>3.7 ± 1.8</td>
<td>(2.0 ± 0.3)c</td>
<td>16.8 ± 2.1a</td>
<td>21.1 ± 3.63c</td>
<td>7.42 ± 1.19ac</td>
</tr>
</tbody>
</table>

The data obtained after 2 weeks and 4 months were from four rabbits (means ± SE). The numbers in parentheses are means ± SE of three rabbits. The data obtained after 2 months were from five rabbits. Numbers in the same row with the same superscripts are significantly different at the following levels: a-a P < 0.05, b-b P < 0.05, c-c P < 0.01, and d-d P < 0.01.
Some comment is necessary regarding the difference between the incorporation into cholesteryl ester and phospholipid in the normal intima at 2 months compared with that at 2 weeks and 4 months. As was indicated for the corresponding analytical data, the amount of cholesteryl ester present in the normal intima at 2 months was higher than that present at either 2 weeks or 4 months. This higher amount was reflected in the higher relative incorporation into cholesteryl ester in the normal intima at 2 months compared with that at 2 weeks and 4 months and might have been due to some microscopic lesion present in the macroscopically normal intima at this stage. However, the increased relative incorporation into cholesteryl ester in the raised lesion was still marked at each of the time intervals.

The incorporation of \(\left[1-^{14}C\right]\)oleic acid into individual phospholipids in the three portions of the aortas is shown in Table 5. When one takes into account the reduced amount incorporated into total phospholipid in the raised lesions at 2 weeks and 2 months (Table 4), it is apparent that there was no marked change in the relative incorporation of oleic acid into the individual phospholipids of either the raised or the fibrous lesions from that in the normal aorta. The possible exception is the incorporation into sphingomyelin: a marked reduction in \(\left[1-^{14}C\right]\)oleic acid incorporation was a feature of the raised lesion at all time intervals. However, incorporation into sphingomyelin was very low in all of the areas studied and at each time interval.

Some comment is necessary regarding the incorporation into sphingomyelin of \(\left[1-^{14}C\right]\)oleic acid. The incorporation of \(\left[32P\right]\)phosphate into phospholipids by the different portions of the aorta is shown in Table 6. The reduced incorporation into phospholipid in the raised lesion from the rabbits studied at 2 weeks and 2 months seen in the \(\left[1-^{14}C\right]\)oleic acid incorporation studies was also demonstrated for the \(\left[32P\right]\)phosphate incorporation. In the normal intima the two major phospholipids were lecithin and phosphatidyl ethanolamine, and at both 2 weeks and 4 months, incorporation of \(\left[1-^{14}C\right]\)oleic acid into individual phospholipids was very low in all of the areas studied and at each time interval.
macroscopically normal intima were phosphatidyl inositol and lecithin, but they were present in approximately equal amounts with a ratio of phosphatidyl inositol to lecithin incorporation of 0.960. Data for the fibrous lesion at 2 months and 4 months were essentially the same as those for the normal intima with marked incorporation into phosphatidyl inositol and with ratios of phosphatidyl inositol to lecithin incorporation similar to those for the corresponding normal intima. The incorporation of $[^{32}P]$phosphate into phosphatidyl inositol in the raised lesion, however, was appreciably less than that for the corresponding normal intima or fibrous lesion. Although labeling of this component was appreciable, that of lecithin was much higher and accounted for about two-thirds of the $[^{32}P]$phosphate incorporated into phospholipid in the raised lesion. The reduced incorporation into phosphatidyl inositol is indicated by the ratios of phosphatidyl inositol to lecithin incorporation given in Table 6. At each time interval, this ratio was significantly less than that for the corresponding normal intima.

**Discussion**

The concentration of both free cholesterol and cholesteryl ester increased appreciably in the raised lesion compared with that in the corresponding normal intima at each of the time intervals studied. The relative increase in the concentration of cholesteryl ester, however, far exceeded that of free cholesterol. Two weeks after insertion of the catheters, cholesteryl ester concentration had increased to six to seven times that of the corresponding normal intima, and by 4 months it had risen to over thirty times that of the normal intima; at this time the cholesteryl ester accounted for almost 29% of the total cholesterol present. The cholesteryl esters that accumulated in the raised lesions contained more cholesteryl oleate and less cholesteryl linoleate than did those in the corresponding normal intima. Bondjers and Björkerud (25) have also reported the accumulation of cholesteryl oleate in lesions developed in rabbits on a normal diet following mechanical trauma. The accumulation of cholesteryl oleate within lipophage cells in human fatty steak lesions is also well recognized (26). In the lesions produced in this study, the progressive elevation of cholesteryl oleate suggests that preferential esterification of cholesterol with oleic acid occurs within the lipid-containing cells of the raised lesion. The different composition of the small amount of cholesteryl esters in the normal intima compared with that of cholesteryl esters accumulating in the raised lesion supports this possibility. Entry and retention of cholesteryl esters from the plasma cannot, therefore, explain the compositional changes. In the normal intima, little cholesterol esterification occurs, and levels of cholesterol-esterifying enzymes are low (27, 28). However, cholesterol-esterifying activity is stimulated in the atherosclerotic lesion (27-29). In cholesterol-fed animals, this stimulation is related to increased entry of cholesterol from the blood. Other etiological agents, however, may also stimulate cholesterol-esterifying enzymes. In the present experiments, serum lipid levels were low, but other etiological factors, namely endothelial injury and platelet mural thrombi were present. The metabolic studies performed in the present experiments suggest that these agents are associated with the stimulation of cholesterol esterification in the arterial wall. The raised lesions had a higher capacity to incorporate fatty acid into cholesteryl ester than did the corresponding normal intima. Direct confirmation of increased cholesterol-esterifying enzyme activity in cell-free preparations of raised lesions is precluded, however, by the relatively small amount of material available.

No information about the origin of the free cholesterol or of the cholesterol component of the cholesteryl ester in the lesions studied is available from the present work. It is possible that this cholesterol came from the plasma in the rabbits on normal diets, although their plasma cholesterol levels were around 50 mg/100 ml. The origin of this cholesterol, however, needs to be investigated further.

Morphological details of the lesions produced in the present study have been reported previously (15). At the early stage of development of the raised lesion, platelet and fibrin thrombi were prominent and much of the lipid was present intracellularly in the macrophages and foam cells. By 2 weeks, significant numbers of smooth muscle cells had also appeared. By 2 months and 4 months, a certain amount of the lipid was extracellular but, even at these time intervals, cellular infiltration was marked; foam cells and smooth muscle cells were prominent. There is abundant evidence for the active involvement of foam cells and smooth muscle cells in lipid synthesis in the developing atherosclerotic lesion (28, 30-32); it appears likely that, in the lesions described in the present paper, these cells with their contained cholesterol-esterifying enzymes (33) are the direct source for...
the cholesteryl ester which accumulates in the lesion. Direct evidence is not available at present to suggest that the development of these cells or their cholesteryl-esterifying enzyme activity is directly stimulated by injury or by platelet thrombi, but the evidence from the present work makes such a conclusion probable.

The total phospholipid concentration in the raised lesion was similar to that in the normal intima. The fatty acid composition of the phospholipid, however, was markedly different in the raised lesion from that in the normal intima because the palmitic acid content was lower than in the normal intima. The high relative incorporation of \[^{32}P\] phosphate into phosphatidyl inositol in the raised lesion is striking and, therefore, this raised lesion resembles metabolically that of the atherosclerotic lesion in the cholesterol-fed rabbit, a shift from phosphatidyl inositol to phosphatidyl choline synthesis occurs (34), and this shift persists in cells prepared in tissue culture from aortas of cholesterol-fed rabbits (36). The significance of the changing pattern of phospholipid synthesis in the developing atherosclerotic lesion is not clear. However, the similarity of the raised lesions described in the present paper to this characteristic is striking and, therefore, this raised lesion resembles metabolically that of the atherosclerotic lesion in the cholesterol-fed rabbit.

The marked changes in chemical composition and in metabolism of the raised lesion described with the composition and metabolism of the fibrous lesions. The fibrous lesions did, however, show some cholesteryl ester accumulation after 2 months. This observation agrees with the morphological findings previously described (15). As previously mentioned, the apparently normal intima at 2 months showed an elevated cholesteryl ester concentration compared with that of the normal intima at 2 weeks and 4 months. Moore (15) has indicated that at 2 months microscopic lesions similar to those present in the fibrous lesions may be present in macroscopically normal intima, and their presence may well account for some of the anomalous findings reported for the normal intima at 2 months with respect to its composition and metabolism. Although there was evidence for some accumulation of cholesteryl ester in the fibrous lesions, its fatty acid composition was essentially the same as that of the corresponding normal intima, and the incorporation of \[^{1-14}C\] oleic acid into cholesteryl ester and of \[^{32}P\] phosphate into phospholipid was similar at 2 months when maximal cholesteryl ester accumulation occurred in this lesion. No evidence for the accumulation of this lipid by metabolism in the fibrous lesion is forthcoming.

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References


Lipid Composition and Metabolism of Thromboatherosclerotic Lesions Produced by Continued Endothelial Damage in Normal Rabbits
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