Influence of a Diet High in Unsaturated Fat upon Composition of Arterial Tissue and Atheromata in Man

By Seymour Dayton, M.D., Sam Hashimoto, M.S., and Morton Lee Pearce, M.D.

A NUMBER of modifications of the usual American-European diet have been found effective in depressing serum cholesterol levels in man, leading to hope that such modifications might retard the development of atherosclerosis and its complications. Clinical recommendations based on these considerations have for the most part emphasized either reduction in total fat intake or rigorous substitution of highly unsaturated for saturated fat.

A clinical trial of the latter type of diet was begun in this Center in 1959, with the primary objective of evaluating its possible effectiveness in preventing complications of atherosclerosis. The experimental diet resulted in an early drop in serum cholesterol levels, which have remained depressed throughout the study. Over-all difference between serum cholesterol concentrations of the experimental subjects and those of control subjects has been about 14 per cent of the baseline level throughout the period of observation. Mean body weight has fallen about 2 per cent in the control group and risen about 2 per cent in the experimental group.

In the course of the study, which is still in progress, a number of subjects of experimental and control groups have come to autopsy.

Following evaluation of the extent of atherosclerosis by gross inspection, arterial tissue has been analyzed in some detail with regard to component lipids. Results of the chemical analyses are described herein and related to type of diet. Relationships between atheroma lipids and antemortem serum lipids of the same subjects have also been studied.

Methods

Subjects

The participants in the study are elderly men living in a Veterans Administration Domiciliary unit. Upon entering the study each man was assigned, by use of a table of random numbers, either to the control group or to the experimental group. The control group was placed on a conventional diet containing 40 per cent fat calories. This diet differs only in minor respects from the regular institutional diet, which all subjects had been eating for 4 months or more prior to the study. The experimental group was placed on a diet also containing 40 per cent fat calories, but embodying major substitution of vegetable oil for the saturated fats of the natural foods. The subjects and experimental conditions are described in greater detail in earlier publications.

Since men living in the Domiciliary are free to leave during the day, it is not possible to enforce total adherence to the study diets. Presence or absence of each participant is therefore recorded at every meal.* The present report includes material from men with varying adherence rates. The 15 subjects of the control group had adherence rates from 43 to 93 per cent (mean, 76 per cent);

*Independent evidence that this gives a realistic appraisal of adherence is provided by data on composition of subcutaneous fat. By the end of 5 years, mean linoleic acid content had risen to 32 per cent of adipose tissue fatty acid in men of the experimental group with a mean adherence rate of 92 per cent, from a starting value of 11 per cent. The linoleic acid content of dietary fat (fig. 1) is 38 per cent.
Abundance of fatty acids in fat of experimental and control diets, determined by analysis of extracted fat. The fatty acids are identified by two numbers, designating chain length and the number of double bonds. Results are means of seven analyses, each representing a 4- to 9-week pool, weighted as to length of collection period. Reprinted with permission of the New England Journal of Medicine.1

The range for the 11 experimental subjects was 53 to 99 per cent, with a mean of 75 per cent.* Time on the study diet ranged from 279 to 1,536 days (mean, 891 days) for the control group, and 495 to 1,351 days (mean, 964 days) for the experimental group. Mean age on entering the study, for subjects of the present report, was 67 years in the control group and 69 in the experimental group.

Diets

Diets and methods of diet analysis have been described previously.1,2 Details of diet composition are summarized in table 1 and figure 1.

Tissues

Because it has been the routine practice in this institution to embalm bodies prior to autopsy, most of the tissues analyzed had been in contact with embalming fluid, generally for less than 1 week, prior to extraction. It was therefore necessary to evaluate the effects of embalming fluid and storage on the compounds being assayed. To do this, unembalmed atheromatous aortic tissue was obtained within 24 hours after death. The tissue was reduced to a fine mince, which was thoroughly mixed. Part of the mince was analyzed fresh and the remainder stored as a suspen-

*The numbers of cases in this report bear no relationship to the relative mortality rates of the two groups.

Table 1

Composition of Diets

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total daily calories*</td>
<td>2,400</td>
<td>2,425</td>
</tr>
<tr>
<td>Protein, Gm./day*</td>
<td>91</td>
<td>92</td>
</tr>
<tr>
<td>Fat, Gm./day*</td>
<td>108</td>
<td>106</td>
</tr>
<tr>
<td>Fat calories, % of total*</td>
<td>41</td>
<td>39</td>
</tr>
<tr>
<td>Iodine value of fat*</td>
<td>53</td>
<td>101</td>
</tr>
<tr>
<td>Cholesterol, mg./day†</td>
<td>721</td>
<td>365</td>
</tr>
<tr>
<td>α-sitosterol, mg./day†</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>β-sitosterol, mg./day†</td>
<td>16</td>
<td>187</td>
</tr>
<tr>
<td>α-tocopherol, mg./day‡</td>
<td>2.4</td>
<td>22.6</td>
</tr>
</tbody>
</table>

*Mean of 216 1-week pooled collections.
†Mean of seven analyses, each representing a 4- to 9-week pool, weighted as to length of collection period.
‡Mean of three 1-week pools.

The FFA fractions thus show a relationship at −20 C. for varying periods prior to analysis, which was carried out by the procedures described below. Table 2 shows results of silicic acid fractionation as well as the fatty acid content of the sterol ester fraction. Apart from some small fluctuations in analytical values, attributable to the difficulties of sampling minced tissue, there were no significant changes for 8 weeks. Fatty acids of triglycerides and phosphatides, not shown in the table, were similarly stable. Since tissue concentrations of embalming compounds in aortas from embalmed bodies must have been a great deal lower than in this experiment, one may be quite confident that embalming and subsequent storage of tissues did not produce changes affecting these analyses. Because not all bodies were embalmed and because embalming might have been incomplete, a similar experiment was done with minced tissue suspended in saline. Here, too, there was no change during storage for 8 weeks.

Degree of hydrolysis of lipids during storage was evaluated in a separate experiment, in which aliquots of diced aortic tissue were extracted with acidified isopropanol-heptane, and the extracted free fatty acid (FFA) was titrated with sodium hydroxide. Samples extracted immediately contained 3.28 µEq. of FFA per gram of fat-free tissue. After 1 week in embalming fluid at −20 C. the FFA content had increased by 58 per cent, and after 1 week in saline at −20 C. it had increased by 74 per cent. The FFA fractions thus include significant contributions from hydrolysis.

†Composition of the embalming fluid is sodium carbonate monohydrate 16 Gm., sodium borate 53 Gm., formaldehyde (37 per cent) 200 ml., diethylene glycol 118 ml., eosin Y 0.16 Gm., Aquaremove Special 5.3 ml., Igepon 1.7 ml., water to make 1 liter.
including an uncertain amount of hydrolysis prior to autopsy. However, FFA of atheroma samples averaged only 2 per cent of total lipid, indicating that hydrolysis would have had no important influence on the data for major esterified lipid fractions.

Coronary atheroma was freed from underlying media and adventitia. The coronary atheroma sample for each subject consisted of a pool of all atheromatous material that could be dissected from all the coronary arteries in this manner. Aortas were stripped free from adherent fat and adventitia, then divided lengthwise into two parts of roughly equal size. One half of each sample was used, without further dissection, for quantitation of major lipids and of calcium in whole aorta. The other half was used as a source of isolated atheroma, which were analyzed separately. Aortic atheromata were dissected through a plane just beneath the lesions and then trimmed carefully to remove all surrounding uninvolved tissue. Aortic lesions were sorted into two pools of tissue, one consisting of uncomplicated and the other of complicated atheromata. The latter included all lesions displaying gross calcification, ulceration, thrombosis, or aneurysm formation. Tissues were stored at −20°C until extracted.

Serum samples from fasting blood, obtained from all subjects at 4-month intervals, were placed into ampoules, gassed with pre-purified nitrogen, and sealed. Foaming during gassing was controlled by addition of Polyglycol (Dow Chemical Co.). Sera were stored at −20°C.

**Chemical Procedures**

Tissues were dried to constant weight *in vacuo* at 60°C and reduced to a fine mince for lipid extraction. Serum and tissue lipids were extracted by the Folch procedure. Cholesterol, triglyceride, and lipid phosphorus were determined colorimetrically on the extract of each undissected half-aorta. Lipids of serum samples and atheromata were fractionated by column chromatography on silicic acid. Hydrocarbon was eluted with petroleum ether. Sterol esters, triglycerides, and free sterol were eluted with, respectively, 1, 12, and 40 per cent diethyl ether in petroleum ether, monoglycerides (?) with chloroform, and phosphatides with methanol. Free fatty acids were separated from the triglyceride fraction either by thin-layer chromatography on silica gel G, using

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{Stability of Aortic Lipids during Storage in Embalming Fluid} \\
\hline
\text{Time, weeks:} & 0 & 8 & 13 & 38.2 \\
\text{Hydrocarbon} & 30.8 & 26.9 & 30.0 & 2.9 \\
\text{Sterol ester} & 12 & 14 & 19 & 1.1 \\
\text{Triglyceride} & 6.1 & 25.7 & 32.0 & 4.2 \\
\text{Phospholipid} & 0 & 0.3 & 0.7 & 0.6 \\
\hline
\end{array}
\]

*Free fatty acids were separated but not quantitated in this experiment.
†Fatty acids are identified by chain length and number of double bonds.

\[J. T. Baker silicic acid was used after removal of fine particles by suspension in methanol. The silicic acid was slurred into 2-3 volumes of methanol. The mixture was allowed to stand 20 minutes and the methanol was siphoned off. After three such treatments, the sedimented silicic acid consisted mostly of 80-100 mesh material.*
as developing solvent 83 per cent petroleum ether (B.P. 60-90 C.), 16 per cent diethyl ether, and 1 per cent acetic acid,7 or by column chromatography on Florisil.8 Sterol esters and phosphatides were saponified overnight at room temperature with 2 per cent sodium hydroxide in 95 per cent ethanol. Benzene was added when necessary to aid in solution of these lipids. After saponification, an equal volume of water was added, and nonsaponifiable material was extracted with petroleum ether. The aqueous ethanol solution was acidified with hydrochloric acid, and fatty acids were extracted with petroleum ether. Fatty acids and triglycerides were methylated with boron trifluoride methanol.9 Methyl esters were analyzed on a Barber-Coleman Model-10 gas-liquid chromatograph equipped with an ionization chamber detector, with tritium foil as the radiation source. Stationary phase was 12 per cent ethylene glycol succinate polyester and the carrier gas was argon. A single sample size was used, suitable for the major components, and current amplification was increased 10-fold for components emerging after methyl arachidonate. No effort was made to quantitate trace peaks or components that did not appear on the single chromatogram. Further information concerning chromatographic conditions and reproducibility of results has been published.10 Reproducibility of the gas-liquid chromatographic (GLC) determination of linoleic acid, not reported in the reference just cited, was evaluated by replicate analysis of a mixture of methyl esters on 11 separate days. Mean and standard deviation for linoleic acid were 34.1 ± 1.5 per cent of the total fatty acid.

For calcium determination, dried, lipid-free aortic pieces were ground with a mortar and pestle. One to five grams of this material were digested by wet combustion with nitric and perchloric acids.11 Calcium was precipitated as the oxalate at pH 3.5,12 and converted to the carbonate in a muffle furnace at 475-500 C. Calcium was determined by titration with EDTA using Calcon, 1-(2-hydroxy-1-naphthylazo)-2-naphthol-4-sulfonic acid, as an indicator.13

Results

Except as noted, results of all analyses showed no significant correlation with adherence or with period of time on the study. For this reason, data for each diet group are pooled and presented, for the most part, as means and standard deviations.

Results of analyses of the undissected half-aortas are shown in table 3. Variances of all components were large, in keeping with what might be expected in subjects exhibiting different degrees of atherosclerosis. There was no difference between the two diet groups with regard to total lipid and the major lipid fractions. The two-fold difference in mean aortic calcium levels was not statistically significant (0.05<p<0.10 by both t test and rank sum test).

Concentrations of major lipid components in isolated atheromata are shown in table 4. These figures are derived from gravimetric data on the eluted fractions from silicic acid chromatography. Since isolated atheroma more nearly reflects a single tissue type than does intact aorta (table 3), variances are smaller. Phosphatide consistently accounts for about one fourth of atheroma lipid. Sterol ester fractions are slightly larger than free sterol. Since these are gravimetric data and thus include the fatty acid portion (about 40 per cent) of the sterol ester, there is more unesterified than esterified sterol in all types of lesion.

Aortic atheroma, whether complicated or uncomplicated, contains much less triglyceride than does coronary atheroma. Aortic plaques are richer than coronary lesions in cholesterol ester and, less consistently, in unesterified cholesterol. Composition of aortic atheroma is strikingly independent of whether the lesions are uncomplicated or complicated.

There are no significant differences between the control and experimental groups with regard to concentrations of major fractions as

<p>| Table 3 |
| Lipid and Calcium Concentrations of Intact Half-Aortas |</p>
<table>
<thead>
<tr>
<th>Concentration, per cent of dry aortic weight</th>
<th>Control (14 cases)</th>
<th>Experimental (10 cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipid</td>
<td>9.4 ± 3.4</td>
<td>8.8 ± 4.1</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>4.0 ± 1.2</td>
<td>3.6 ± 2.4</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>1.4 ± 0.7</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td>Phosphatide</td>
<td>1.9 ± 0.7</td>
<td>1.7 ± 0.8</td>
</tr>
<tr>
<td>Calcium</td>
<td>6.4 ± 5.0*</td>
<td>3.1 ± 2.3†</td>
</tr>
</tbody>
</table>

Data are given as mean ± standard deviation.
*Eleven cases. Includes one sample with aneurysm, containing 7.8 per cent calcium.
†Nine cases. Includes one sample with aneurysm, containing 1.4 per cent calcium.

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Table 4

Major Lipid Components of Atheromata

<table>
<thead>
<tr>
<th>Diet group and site of atheroma</th>
<th>Number of cases</th>
<th>Concentration as per cent of total atheroma lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hydro-carbon†</td>
<td>Sterol ester</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary</td>
<td>10</td>
<td>1.5 ± 1.5</td>
</tr>
<tr>
<td>Aortic, uncomplicated</td>
<td>10</td>
<td>3.0 ± 3.7</td>
</tr>
<tr>
<td>Aorticc, complicated</td>
<td>8</td>
<td>1.4 ± 1.3</td>
</tr>
<tr>
<td>Experimental</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary</td>
<td>9</td>
<td>1.0 ± 1.6</td>
</tr>
<tr>
<td>Aortic, uncomplicated</td>
<td>8</td>
<td>0.5 ± 1.2</td>
</tr>
<tr>
<td>Aortic, complicated</td>
<td>7</td>
<td>0.5 ± 0.5</td>
</tr>
</tbody>
</table>

Data are shown as mean ± standard deviation.

* * p < 0.05
** p < 0.01
*** p < 0.001
† for difference between given value and that for coronary artery; p values determined on paired observations.14

†Standard deviations greater than the means in some instances reflect non-normal distribution of the data, owing to values of zero in several cases.

Two or more data from one subject omitted. See text (Results) for details.

In the case of the half-aorta samples, concentrations of individual fractions of atheroma lipid did not correlate with time in the study. As indicated by the footnote in table 4, one control sample was omitted from the tabulation of data from four other samples from this aorta were submitted to the laboratory. The highest phosphatide (31.5 per cent of total phosphatide) and half-aorta (24 per cent of total lipid) and the lowest phosphatide (16.4 and 7.7 per cent of total phosphatide) in the control subject and atherojacinated thrombus were covered by endothelial cells. The complicated aortic atheroma sample from this aorta were submitted to the laboratory for analysis of the atheroma sample from the control subject for further analyses. The control sample was submitted to the laboratory for analysis of the atheroma sample from the control subject for further analyses. The control sample was submitted to the laboratory for analysis of the atheroma sample from the control subject for further analyses.
Table 5

Fatty Acids in Sterol Ester Isolated from Atheromata

<table>
<thead>
<tr>
<th>Site of lesions</th>
<th>Diet</th>
<th>Number of subjects</th>
<th>16:0†</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>20:3</th>
<th>20:4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary</td>
<td>Control</td>
<td>9</td>
<td>15.5 ± 4.5</td>
<td>5.6 ± 1.0</td>
<td>2.1 ± 1.2</td>
<td>42.1 ± 5.4</td>
<td>27.8 ± 7.5</td>
<td>1.1 ± 1.0</td>
<td>4.8 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>9</td>
<td>15.3 ± 4.5</td>
<td>4.5 ± 1.8</td>
<td>1.9 ± 0.9</td>
<td>37.2 ± 5.6</td>
<td>35.6 ± 4.6*</td>
<td>1.1 ± 0.6</td>
<td>3.5 ± 1.4</td>
</tr>
<tr>
<td>Aortic, uncomplicated</td>
<td>Control</td>
<td>10</td>
<td>14.8 ± 2.3</td>
<td>4.7 ± 1.1</td>
<td>1.2 ± 0.7</td>
<td>35.4 ± 4.5</td>
<td>37.3 ± 4.2</td>
<td>0.6 ± 0.6</td>
<td>5.8 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>8</td>
<td>16.5 ± 3.1</td>
<td>4.7 ± 1.1</td>
<td>1.3 ± 0.6</td>
<td>32.7 ± 7.5</td>
<td>38.4 ± 9.3</td>
<td>1.3 ± 1.2</td>
<td>4.6 ± 2.2</td>
</tr>
<tr>
<td>Aortic, complicated</td>
<td>Control</td>
<td>10</td>
<td>16.1 ± 1.3</td>
<td>6.3 ± 1.9</td>
<td>3.2 ± 3.4</td>
<td>35.3 ± 2.6</td>
<td>31.9 ± 4.2</td>
<td>0.8 ± 1.0</td>
<td>6.0 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>9</td>
<td>16.5 ± 2.6</td>
<td>4.8 ± 1.2*</td>
<td>1.8 ± 0.9</td>
<td>31.2 ± 3.7*</td>
<td>40.8 ± 4.7***</td>
<td>0.5 ± 1.0</td>
<td>4.1 ± 2.5</td>
</tr>
</tbody>
</table>

Results are given as mean ± standard deviation.

* p < 0.05

** p < 0.001

† Occurrence of standard deviation greater than the mean value for some minor components is due to non-normal distribution of the individual values, generally because of values of zero in several subjects. Standard deviations for these values are included in the table in the interest of simplified data presentation, but these standard deviations are very crude indices of the dispersion of individual values.

The major influence of the experimental diet upon atheroma composition was an increase in mean linolate content of all fractions. This effect is seen best when paired observations fail to reveal significant differences.

The data in figure 3, for aortic lesions, are also scrutinized for paired values. The dominant fatty acids are palmitate (16:0), stearate (18:0), and oleate (18:1).

Here, there is a suggestion that coronary atheroma contains more oleate and less linolate than do aortic lesions, but analysis of the same individuals. These data, with statistical evaluation based on paired values, are summarized in figure 2. In atheroma triglyceride (table 6), oleate (18:1) accounts for nearly half of the fatty acid (18:1), and linolate (18:2) is quite small. The dominant fatty acids are palmitate (16:0), stearate (18:0), and oleate (18:1).
### Table 6

**Fatty Acids in Triglyceride Isolated from Atheromata**

<table>
<thead>
<tr>
<th>Site of lesions</th>
<th>Diet</th>
<th>Number of subjects</th>
<th>Abundance as per cent of total fatty acid in fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary</td>
<td>Control</td>
<td>11</td>
<td>14:0† 16:0 16:1 18:0 18:1 18:2</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>11</td>
<td>1.7 ± 1.3 22.6 ± 1.6 6.8 ± 1.9 6.2 ± 1.5 50.8 ± 4.4 9.9 ± 2.5</td>
</tr>
<tr>
<td>Aortic, uncomplicated</td>
<td>Control 10</td>
<td>10</td>
<td>1.0 ± 0.6 25.5 ± 3.2 4.8 ± 0.5 6.6 ± 1.9 48.9 ± 2.7 12.0 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>9</td>
<td>0.4 ± 0.9† 22.1 ± 5.1 3.6 ± 1.1** 7.2 ± 1.4 46.6 ± 3.9 18.7 ± 6.8**</td>
</tr>
<tr>
<td>Aortic, complicated</td>
<td>Control 10</td>
<td>10</td>
<td>1.9 ± 1.4 24.2 ± 3.0 5.2 ± 2.1 7.5 ± 1.6 47.6 ± 3.0 11.8 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>9</td>
<td>1.2 ± 1.5 23.0 ± 2.2 4.6 ± 1.6 8.3 ± 2.2 40.1 ± 4.6*** 20.4 ± 2.8***</td>
</tr>
</tbody>
</table>

Results are given as mean ± standard deviation.
* * p < 0.001 for difference between experimental and control values.
†Fatty acids are identified by two numerals, designating chain length and number of double bonds.

### Table 7

**Fatty Acids in Phosphatide Isolated from Atheromata**

<table>
<thead>
<tr>
<th>Site of lesions</th>
<th>Diet</th>
<th>Number of subjects</th>
<th>Abundance as per cent of total fatty acid in fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary</td>
<td>Control</td>
<td>11</td>
<td>16:0† 16:1 18:0 18:1 18:2 20:4</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>10</td>
<td>28.1 ± 5.7 2.2 ± 0.8 22.5 ± 2.7 28.6 ± 3.4 9.9 ± 2.3 7.2 ± 2.9</td>
</tr>
<tr>
<td>Aortic, uncomplicated</td>
<td>Control 10</td>
<td>10</td>
<td>32.7 ± 4.9 1.4 ± 1.1 21.1 ± 2.3 24.5 ± 2.4 13.0 ± 3.1 6.3 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>8</td>
<td>32.1 ± 6.3 1.0 ± 1.3† 23.4 ± 3.5 21.5 ± 2.2* 15.7 ± 4.1 4.9 ± 4.6</td>
</tr>
<tr>
<td>Aortic, complicated</td>
<td>Control 10</td>
<td>10</td>
<td>33.3 ± 5.5 1.4 ± 1.3 20.7 ± 4.6 28.6 ± 4.9 11.1 ± 3.9 4.3 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>9</td>
<td>30.3 ± 6.2 0.4 ± 0.6 22.6 ± 5.0 24.2 ± 3.7 15.1 ± 3.7* 3.7 ± 3.5</td>
</tr>
</tbody>
</table>

Results are given as mean ± standard deviation.
* p < 0.05 for difference between experimental and control values.
†Fatty acids are identified by two numerals, designating chain length and number of double bonds.
†Occurrence of standard deviation greater than the mean value for some minor components is due to non-normal distribution of the individual values, generally because of values of zero in several subjects. Standard deviations for these values are included in the table in the interest of simplified data presentation, but these standard deviations are very crude indices of the dispersion of individual values. P values have not been calculated for these acids.
terol ester linoleate, and of serum triglyceride linoleate against atheroma triglyceride linoleate, for the three different types of atheroma. Correlation coefficients, shown in figure 4, are positive in all instances, and in all but one of the comparisons (cholesterol ester in uncomplicated aortic atheroma) the correlations are statistically significant. In the case of phosphatide (not shown in figure 4), corresponding correlation coefficients ranged from +0.46 to +0.59, but with \( p > 0.05 \) in each case.

**Discussion**

The analytical data of greatest interest in a study of this type are those which might yield evidence that atherosclerosis had progressed at different rates in the two groups of participants. From this point of view, total lipid concentration of intact arterial tissue (table 3) is theoretically of considerable importance. Unfortunately, the likelihood of getting useful affirmative information from this source is extremely small. Because the total lipid content of an individual's aorta on entry into the study is unknown, one is limited to a simple comparison of the terminal values for the control and experimental subjects. Because variances in the terminal total lipid concentrations are very large (table 3), statistical significance can be established only if the means of the two groups differ greatly or if data are available on very large numbers of subjects. The comparison is further hampered by the fact that data are derived not from a random sample of each dietary group but rather from men who died during the period of follow-up, a fact which would almost certainly bias the values in both groups of subjects. For all these reasons, it seems unlikely that prolonged accumulation of data such as those in table 3 will provide substantial evidence concerning possible effectiveness of the experimental diet.

The values for total aortic lipid and for the major subfractions reported in table 3 are comparable to Böttcher's data on "Stage II" atherosclerotic aorta and to the figures reported by Mead and Gouze for aortas of grades 1 through 4. The analyses of dissected atheroma by silicic acid chromatography (table 4) show strikingly little difference between complicated and uncomplicated aortic lesions. Comparison with published data on comparable material reveals a larger percentage of phosphatide than reported by Smith for aortic atheroma at various stages or by Luddy et al. The observation that coronary atheroma contains more triglyceride and less sterol than does aortic atheroma con-

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**Figure 2**

Comparison of coronary and aortic atheromata with regard to content of cholesteryl olate (18:1) and linoleate (18:2). P values were calculated by t test applied to paired observations; the asterisks refer to comparison of the two adjacent values. In no instance was there a significant difference between uncomplicated and complicated aortic atheroma.
Figure 3
Comparison of atheromata of control and experimental subjects in regard to linoleic acid content of various lipid fractions (left, uncomplicated aortic atheroma; center, complicated aortic atheroma; right, coronary atheroma). C.E., cholesterol ester; T.G., triglyceride; P.L., phosphatide. *P < 0.05, **p < 0.01, ***p < 0.001—for comparison of control and experimental groups (by t test).

Figure 4
Relationship of atheroma cholesterol ester linoleic acid and triglyceride linoleic acid to the linoleic acid concentration in corresponding fractions of the last antemortem serum samples in subjects on experimental diet.
firms a similar finding by Böttcher et al.\textsuperscript{19} obtained on less extensively dissected tissue. The metabolic basis for this difference is not at all apparent from available information. A number of possibilities can be proposed, including differential uptake of different lipid classes from plasma, differences in amounts of sterol or triglyceride contributed by local synthesis within the arterial wall, or quantitative differences in mechanisms for disposing of sterol and triglyceride.

Fatty acid compositions of cholesterol ester and triglyceride found in atheromata of control subjects of the present study were in general similar to data reported by previous investigators.\textsuperscript{16, 19–21} However, we found only traces of compounds with 22 and 24 carbon atoms in phosphatide, in contrast to the material of Böttcher et al.,\textsuperscript{19} which contained about 15 per cent of these higher acids in "Stage II/III" lesions from aortas and coronary arteries. Like the latter authors, we found a small free fatty acid fraction in atheroma; but as indicated under Methods, a significant part of this was due to postmortem hydrolysis of esterified lipid.

Since animal tissues are not known to synthesize linoleic acid (18:2), the presence of increased amounts of this compound in atheromata of men on the experimental diet very

\textbf{Figure 5}  

\textit{Ratio of linoleic acid in atheroma lipid fractions to linoleic acid in corresponding fractions of the last antemortem serum samples, plotted against time on experimental diet. C.E., cholesterol ester; T.G., triglyceride; P.L., phosphatide.}
likely represents the result of exchange of lipids between atheroma and plasma. As in the case of experiments involving use of isotopically labeled lipids, it is difficult to be entirely certain that rising linoleic acid concentration is a result of a physiologic process as opposed to a passive physicochemical exchange between tissue lipid and the lipoprotein lipid of plasma. An alternative possibility—simple accretion of new, unsaturated atheroma on top of old—might have contributed to the increment in linoleic acid. However, it seems unlikely that this is a major reason for the rise in 18:2. To account for the increase in triglyceride linoleate by this mechanism, one would have to postulate that atheroma lipid mass had increased by 55 to 80 per cent in less than 3 years. A rise of this magnitude would (if limited to the group on experimental diet) be apparent in the total lipid data of table 3. An estimate of the mean rate of increase in aortic lipid in "normal" men at this age, based on measurements of cholesterol concentration, is 6 per cent per year. Additional evidence that large-scale accretion of lipid did not occur is provided by the fact (see below and fig. 5) that there was no progressive rise in ratio of atheroma linoleic acid to plasma linoleic acid over the period from 495 days to 1,351 days. It is striking that the changes in complicated aorta atheroma were at least as great as in the smaller and presumably younger lesions. This does not imply, however, that the changes occurred at the same rate in both types of atheroma.

Linoleic acid concentrations of atheroma triglyceride and cholesterol esters in the experimental subjects were clearly related to the corresponding linoleic acid concentrations of the last antemortem sera (fig. 4). Considering that the single serum sample may have been unrepresentative of the situation over the entire period of the study, the correlations appear satisfactorily clear, but they do not necessarily indicate a linear relationship. Phosphatide also shows a positive correlation, but not at a statistically significant level. Atheroma linoleic acid showed surprisingly little correlation with the individual's record of adherence or with time on the experimental diet prior to death. (This observation is in contrast to the situation in adipose tissue, in which linoleic acid concentrations were still rising at 5 years.*) Lack of correlation with time on the diet appears (see below and fig. 5) to be due simply to the fact that the entire rise in linoleic acid concentration occurred prior to the death of the first man in this group at 495 days (except for cholesterol ester in uncomplicated aortic atheroma). A number of factors very likely contribute to the lack of correlation with adherence: the over-all adherence figure may not be representative of the few months immediately before death, which would have the greatest influence on atheroma composition; nonadherence might in part reflect meals missed completely rather than deviation from the diet; the relationship, if any, would be obscured by differences in linoleic acid concentrations of both serum and atheroma lipids at the start of the study (cf. variances in control data of tables 5 to 7); and there is appreciable experimental error in the linoleic acid determinations.

Plots of linoleic acid concentrations of atheroma lipid fractions against time in the study for the experimental group show no consistent patterns, presumably because of considerable variations in initial values. Plots of the ratios of linoleic acid content in atheroma lipid fractions to the concentrations in corresponding serum fractions are a good deal more informative and are shown in figure 5.

The ratios for the experimental subjects show no rise over the period from 495 to 1,351 days, with the possible exception of cholesterol ester during the very first interval. The data of Gunning et al. indicate that serum cholesterol ester would have reached a new, constant content of linoleic acid during the first month of the study. The same is true of erythrocyte phosphatide and therefore, presumably, of plasma phosphatide as well. Thus the relatively constant ratio implies a constant concentration in the cholesterol ester and phosphatide of atheromata. If one views the

*Unpublished data.

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enhanced linoleic acid concentrations in atheromata of the experimental group as due largely to exchange with serum, this constant level suggests that the various fatty acid pools would have turned over several times during the first 2 years. While we cannot estimate half-times from these data, it appears that they are probably less than 1 year. A longer figure was estimated by Farquhar et al.26 in an abstract describing atheroma analyses in five men on corn oil formulas. Their data apparently indicated atheroma lipid turnover rates comparable to those of stored adipose tissue fat which, as reported by Hirsch et al.,27 has a half-time of 1 to 2 years.* Since there exists no direct information concerning fatty acid kinetics of individual lipid fractions in human atheromata, it seems worthwhile to seek some kinetic inferences in the data of the present study. Figure 5 offers some clues. In all fractions, linoleic acid concentration stabilizes at a level below the corresponding serum level. One might postulate preferential uptake of other fatty acids and their esters, or particularly rapid degradation or "excretion" of linoleic acid by the atheroma. However, a number of facts appear to suggest that we are observing mainly the results of dilution by locally synthesized acids. Some dilution of this type must occur, since fatty acid synthesis occurs in atheromata28 and since fatty acid synthesized in atheromata would be linoleic-free. Furthermore, the ratios seen in figure 5 for cholesterol ester, triglyceride, and phosphatide are rather similar, suggesting that these ratios probably originate from a process common to all fractions. If we assume that dilution by fatty acids synthesized within the artery wall represents the major reason for the difference between linoleic acid concentrations of atheroma and plasma, then the ratio of the concentration in plasma to that in atheroma, when both are at plateau levels, constitutes an estimate of the fraction of atheroma fatty acyl moieties derived from plasma.* It thus appears (fig. 5) that plasma is the source of 70 to 90 per cent of the cholesterol ester fatty acid in the control group and about 65 per cent in the experimental group. It also appears that the percentage is higher in uncomplicated aortic plaques of the control subjects than in any other group of lesions, and that the experimental diet induced a small but significant decrease, particularly in uncomplicated plaques;† in the percentage of cholesterol ester fatty acid derived from plasma. It is possible that these observations are related to the fact, reported from this laboratory, that normal rat aorta takes up labeled cholesterol from plasma more rapidly in animals on diets containing saturated fat (coconut oil) than in animals on unsaturated fat (safflower oil).30 However, these animal experiments involved tracing cholesterol, and the results do not necessarily apply to the fatty acyl groups of the cholesterol esters.

Percentage of atheroma triglyceride fatty acid derived from plasma appears similar to that for cholesterol ester in the experimental

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*A similar figure is estimated from analyses of adipose tissue obtained in the present study (unpublished data).

† Even omitting the two values in the experimental group at 495 and 666 days, p < 0.01 by t test.
group (fig. 5). Despite poor agreement among the triglyceride values for the control subjects, the ratio appears closer to unity than in the experimental group. The ratio for phosphatide fatty acid is somewhat lower, about 0.5 for both groups. Interpretation of this value is more difficult than in the case of cholesterol ester and triglyceride, since phosphatide is a more distinctly heterogeneous group of compounds for which assumptions of indiscriminate uptake from plasma and indiscriminate disposal are of dubious validity. Böttcher and van Gent\(^{31}\) have reported that atheroma phosphatide contains considerable sphingomyelin, which is almost free of linoleic acid. This fraction, whether derived from plasma or from local synthesis, could account for occurrence of a lower linoleic acid content in atheroma phosphatide as compared with plasma phosphatide.

**Summary**

Detailed chemical analyses have been carried out on aorta, and on coronary and aortic atheromata, of men who died during a study of prolonged use of diets rich in unsaturated fat and of control subjects. Concentrations of total aortic lipid and of total aortic calcium were not significantly different for the two groups of subjects. Concentrations of cholesterol and cholesterol esters, triglyceride, and phosphatide showed no difference between the two groups in analyses of aorta, uncomplicated aortic atheromata, complicated aortic atheromata, and coronary atheromata. Coronary atheroma lipid contained substantially more triglyceride than did aortic atheroma lipid, whether derived from complicated or uncomplicated plaques.

Atheroma triglyceride in experimental subjects contained more linoleic acid than in control subjects, in all types of plaques. Lesser but significant increases in linoleic acid were seen in cholesterol ester and phosphatide of coronary atheromata and complicated aortic atheromata. In the case of uncomplicated aortic atheroma, changes in linoleic acid of cholesterol ester and phosphatide were smaller and not significant. In both groups of subjects, coronary atheroma contained more cholesteryl oleate and less linoleate than did aortic atheroma.

Linoleic acid contents of atheroma lipid fractions were positively correlated with the linoleic acid figures for corresponding fractions of the most recent antemortem serum samples. Ratio of linoleic acid in an atheroma lipid fraction to the linoleic acid content of the corresponding serum fraction was, in most cases, constant after the time of the first sample, obtained after 495 days on experimental diet. It is postulated that this ratio constitutes an estimate of the fraction of atheroma fatty acid derived from plasma. In cholesterol ester and triglyceride, the ratio was about 0.65 in experimental subjects, higher in subjects on the control diet. The ratio for phosphatide was about 0.5 in both groups of subjects.

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