The Fate and Fibrogenic Potential of Subintimal Implants of Crystalline Lipid in the Canine Aorta

Quantitative Histological and Autoradiographic Studies

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SUMMARY To determine the histological reaction to, and the rate of absorption of, different pure crystalline lipids from the aortic subintimal space, cholesterol (Ch), cholesteryl palmitate (ChP), glyceryl tripalmitate (TP), palmitic acid (PA), and dipalmitoyl lecithin (DPL) were implanted in the inner media of the thoracic aorta of 37 dogs. Tiny crystalline rods were fabricated averaging 0.29 mm in diameter and 2.0 mm in length. Twenty such rods were placed with a needle inserter 113 ± 52 (SD) μm beneath the exposed endothelial surface during two separate operations in each dog. In seven dogs, the lipids were 14C-labeled. The dogs were killed at intervals from 3 days to 20 months postoperatively and the implantation sites removed for quantitative histological and autoradiographic measurements. Ch, ChP, and TP were predicted to persist for 73, 50, and 2.4 years, respectively. PA and DPL were absorbed in 4 months. When compared to PA, DPL, and results of certain other control studies, Ch and ChP caused a significantly greater local fibrotic reaction and a marked fibromuscular proliferation of the overlying intima. Fibrotic encapsulation of the Ch and ChP implants was associated with a progressive decrease in their local medial and intimal tissue concentrations. When cholesterol was mixed with DPL, its absorption rate was increased 20-fold, and the anticipated local fibrogenic reaction, but not the intimal proliferative response, was abolished. Autoradiographic determinations of the distribution of tissue lipid concentrations support the concept that lipid transport from an implanted source occurs by the process of diffusion in the orthogonally anisotropic arterial media. Migration of phagocytic cells plays no significant role as a vehicle for lipid removal. The extraordinarily slow rate of absorption and the stimulus to connective tissue proliferation seen with crystalline cholesterol and its esters in this model are documented quantitatively. These observations provide one explanation for their observed persistence and their association with fibrosis and smooth muscle proliferation in the atherosclerotic plaque.

THE ASSOCIATION among intimal lipid deposits in various states of aggregation, intimal collagen production, and smooth muscle proliferation is common to most models of atherosclerosis. The establishment of pathogenic relationships among these interacting components of the plaque continues to be one of the major investigational goals of studies of this disease.1-7

Abnormal transport of lipids or of various lipid-bearing substances in arterial tissue is thought to account for their progressive accumulation in atherogenesis. Intimal lipids appear in lipoprotein form, as confluent droplets, and eventually as crystalline deposits. The latter are largely composed of cholesterol and certain of its esters.3

The crystalline configuration, referred to as the "cholesterol cleft," is one of high sterol density and is highly water insoluble. Much of the lipid in an advanced atherosclerotic plaque is found in crystalline form.9, 10 Regression of atherosclerosis would require, among other things, a net efflux of these crystalline lipids from the plaque. Therefore, the mechanics of transport of crystalline lipid in the arterial wall is of fundamental importance to our understanding of atherosclerosis and its regression. One approach to investigation of mural lipid transport and accumulation has been to add labeled lipid molecules directly or indirectly to the plasma and to record their subsequent appearance in the vessel wall.11-13 A second, little-explored approach is to place lipids in the arterial wall and to observe their rate and mode of disappearance and the histological reaction provoked.14, 15 This approach allows one to control the kind, amount, and physical state of the particular intramural lipid of interest for quantitative studies of transport processes. Byers and Friedman16 demonstrated that cholesteryl acetate in crystalline form provoked "plaque" formation in the rabbit aorta and persisted for 6 weeks without apparent absorption. Conversely, mural injections of hyperlipidemic serum disappeared rapidly and failed to provoke a histological reaction. Abdulla et al.18 studied the response of rat subcutaneous connective tissue to various implanted crystalline and liquid lipids. They concluded that the sequence: cholesterol, cholesteryl palmitate, saturated tri-
glyceride (tristearin), saturated free fatty acid (stearic acid, 18:0), and lecithin reflected the order of increasing rate of reabsorption and decreasing sclerogenic activity, as observed over a 6-week period.

The purpose of this investigation was to determine, in a quantitative fashion, the rates and mechanisms of reabsorption of certain crystalline lipids implanted subintimally in the canine aorta. A second goal was to characterize the histological responses of the arterial wall to the physical and chemical presence of these substances. Attention was focused on aspects of potential relevance to atherogensis and its possible reversal.

**Methods**

**Lipids**

The following series of representative lipids was selected because all are crystalline at body temperature and occur in relatively high concentration in normal and in pathological mammalian processes: cholesterol (Ch), cholesteryl palmitate (ChP), glyceryl tripalmitate (TP), palmitic acid (PA), and dipalmitoyl lecithin (DPL). The lipids were obtained commercially in highly purified form as crystalline powder. These substances were fabricated into tiny rods by packing the powder into and extruding it through a drawn glass die of inner diameter between 0.27 and 0.31 mm. The exact dimensions of each rod were determined microscopically and recorded. The average dimensions were 0.29 mm in diameter by 2.0 mm long. These lipid rods were fragile and crumbled easily. Their cross-sectional appearance on light microscopy was similar to that of atherosclerotic cholesterol deposits in packing density and random orientation of the crystalline spicules. The cylindrical surface of the rods was relatively smooth, thus presenting less surface area than the naturally occurring lipid deposit. Purified canine serum albumin rods were fabricated in similar fashion.

**Inserter**

Figure 1a shows a tiny lipid rod (lower left) and the 2-cm long machined stainless steel device used to implant it beneath the arterial intima. The three components are shown in their separate and assembled (bottom) forms.

**Figure 1**  
(a) Assembled and disassembled view of stainless steel needle inserter. From top to bottom are "inner trochar," "hollow needle shaft," "outer sleeve," and assembled view of inserter with lipid rod in the tip.  
(b) Magnified view of needle tip and lipid rod.  
(c) Magnified (2.5x) view of aortic intimal surface into which 10 insertions had just been placed. Intercostal orifices are seen.  
(d) Average dimensions of arterial thickness and implant depth, determined from postmortem measurements.
The lipid rod is slid into the tip of the hollow shaft of the inserter (see 5-fold magnification in Figure 1b). The inner trochar is then slid into place behind the lipid and locked into the transverse groove in the outer sleeve, as seen at the lower right. These assembled components, all prepared in sterile fashion, are then ready for use at surgery.

Surgery

Twenty-seven male and 10 female dogs with normal lipid determinations underwent two separate left lateral thoracotomies in the 4th and 6th intercostal spaces during anesthesia with intravenous pentobarbital sodium, 25-30 mg/kg. The two operations were separated by at least 6 weeks and often much longer. At each operation, three pairs of aortic intercostal arteries were carefully dissected and ligated to prevent retrograde bleeding into the opened aortic segment. This segment then was occluded proximally and distally, and the intimal surface was exposed through a 2- to 3-cm longitudinal ventral aortotomy. Silk retaining sutures were used to aid the exposure. Blood was flushed gently away with Ringer's lactate solution, which was then absorbed with cotton gauze sponges. Care was taken to keep the endothelial surface constantly covered with a film of Ringer's solution and to avoid any physical contact with the intima except at the points of insertion. To implant the lipid, the shaft of the inserter was held firmly in a hemostat, and its sharpened tip was advanced in a cephalad direction as superficially as possible in a plane of dissection generally 5 to 20 elastic lamellae beneath, and parallel to, the endothelial surface. Once the needle tip was adequately advanced under the intimal (3-4 mm), the outer sleeve which was coupled to the inner trochar was firmly grasped with a forceps and held stationary while the hemostat holding the inserter shaft was slid backward to remove the protective metal trochar and the lipid was compressed and crumbled at the site of intimal penetration by the inserter. Each implantation site was cut out in a 2- X 5-mm block of aortic tissue and suspended in 25% gelatin in distilled water at 37°C and cooled for 4 hours at 4°C to allow the system to gel. The solid gelatin surrounding the tissue was cut into blocks in proper orientation and fixed in 10% formalin for at least 72 hours before histological sectioning.

Sequential 10-μm frozen sections were cut perpendicular to the long axis of the lipid rod, along its entire length. Every 22nd section was mounted for microscopic measurements; there usually were 6 to 10 mounted sections from each implant. The section at the midpoint of the implant was stained with hematoxylin and oil red O, and examined for histological changes. Figure 2 shows representative stained sections as seen with light microscopy. The following microscopic measurements were made on each section, assuming the implant fit an elliptical shape: major axis (a); minor axis (b); cross-sectional area (πab/4). Percent remaining lipid was estimated by averaging the lipid cross-sectional area over all sections of a given implant, and comparing that to the pre-implantation area. Alternatively, in a smaller number of cases in which the lipid was compressed and had crumbled at insertion, residual volume was estimated by a numerical integration method and compared with the pre-implant volume. Depth of insertion (distance from endothelium to implant surface) was measured in microns.

With the single stained section, the following additional determinations were made: depth of insertion, in number of lamellae; thickness of aortic wall, in millimeters; thick-
FIGURE 2. Examples of different histological sections selected as typical responses. Scale: implant diameter in f is 0.30 mm; b and f are at the same magnification. All others are magnified 2.5× further. a: Normal endothelial surface. b: Cholesteryl palmitate implant at 11 days showing proliferation of immature intimal myocytes. c: Magnified view of right side of b. d: Cholesteryl palmitate at 2.5 months showing persistence of lipid, maturation of intimal proliferative response, and onset of local fibrosis. The arrows mark the internal elastic lamina. e: Cholesterol at 5 months showing reduction of intimal cellularity and increased intimal fibrosis, and marked oriented fibrosis around the implant. f: Cholesterol at 10 months viewed with partially crossed polarization. This accentuates the orientation of the fibrosis, which extends into the adjacent interlamellar spaces. A return to normal intimal thickness is demonstrated. g: Palmitic acid at 3 months, showing reduction in lipid mass, mild thickening and increase in density of endothelial monolayer, growth of medial myocytes/macrophages into the lipid.
ness of intima, in cell layers and in microns at a point overlying the implant and at a point remote from it. Tissue reaction was rated on a scale of 0 to 4+ for the occurrence of local cellular proliferation and local fibrous reaction, the latter with polarized light. Phagocytic activity was assessed by the presence of oil red O positive cells surrounding the implant.

Quantitative Autoradiography

Seven of the 37 dogs received implants of $^{14}$C-labeled lipids, and 20 albumin-$^{125}$I implants were placed at four operations in two dogs. The following purified $\beta$-emitting lipids (Amersham/Searle) were mixed with their "cold" isotopes in proportions needed to achieve a specific activity of either 1.0, 2.0, or 10.0 $\mu$Ci/mg lipid: cholesterol [4-$\alpha$-$^{14}$C], cholesteryl (palmitate[1-$^{14}$C]), glyceryl tri-(palmitate[1-$^{14}$C]), and palmitic acid [1-$^{14}$C]. Albumin was labeled with covalently bound $^{125}$I by a modification of the McFarlane method. These radiolipids and albumin were formed into rods, implanted, and harvested exactly as described above. Histological processing was done as above except that tissues for autoradiography were exposed to formalin for only 24 hours initially and again for 48 hours after gelatin embedding; the sections were cut 5 $\mu$m thick and were gently freed of the disk of implanted lipid by brushing the tissue section while in the water bath with a fine artist's brush just prior to mounting on a glass slide. The latter was done to prevent the overlapping halo of intense autoradiographic activity that normally occurs around the pure radiolipid in the implant, which tended to mask the activity of the radiolipid which had actually diffused into the tissues. Preliminary studies with comparable specimens demonstrated that grain count density was linear with exposure time and specific activity, and increased with section thickness (if less than 7 $\mu$m). These studies established the validity of the expression:

\[
\% \text{ lipid content} = \frac{(N - B) S_o t_o}{(N_o - B_o) S_t} \times 100
\]

where:

- $N$ = number of grains counted/72 $\mu^2$ at a given point in the tissue.
- $N_o$ = number of grains counted/72 $\mu^2$ over the implanted radio-lipid.
- $B, B_o =$ background count density
- $t, t_o =$ the respective exposure times for the implanted and standardizing specimens.
- $S, S_o =$ the specific activity of the radiolipid in the implanted specimen and the standardizing specimen. $S_o$ was 1.0 $\mu$Ci/mg. $S$ was either 1.0, 2.0, or 10.0 $\mu$Ci/mg.

"% lipid content" is a measure of local tissue lipid derived from the implant expressed as a percentage of its possible maximum, i.e., solid crystalline lipid. Local tissue lipid concentration, in mg/ml, was determined from the known density ($\rho$) of these solids by the formula:

\[
\text{local lipid concentration} = \left(\% \text{ lipid content}\right) \cdot (10\rho).
\]

All grain counts as in Figure 5b were converted to "% lipid content," using Equation 1. The two values at mirror image points in the full plane of the specimen were averaged to provide data for the half-plane contour maps of Figure 5, c-f. All four radiolipids were studied at implant durations of 3 days, 1 month, and 3 months. Contours of constant % lipid content were drawn by connecting all points of a given lipid content by a smooth contour line. The approximate locus of all points on a given contour was determined by spatial interpolation among existing data points.

Results

Lipid Reabsorption

Figure 3 illustrates the time course of disappearance of the implants. The filled circles represent the area-averaging estimate; open circles represent the volume estimate based on a numerical integration method. Results by the two techniques did not differ. The numerical data, based on linear regression analysis of these points, are given in Table 2A. Cholesterol and cholesteryl palmitate were extremely persistent and had disappearance rates which were not significantly different from zero. Tripalmitin was gradually absorbed at a rate of approximately 3.5% per month. Palmitic acid and dipalmitoyl lecithin were relatively rapidly absorbed with disappearance rates of 27% and 26% per month, respectively. The lecithin-cholesterol mixture had a rapid phase of disappearance at 34% per month for the first 2 months, then slowed to...
Histological Changes

In general, implantation of these substances, as well as the control insertions, provoked a local cellular reaction, a local progressive fibro-proliferative response, and a cellular proliferation in the intima overlying the implant. The intensity and extent of these responses depended on the nature of the substance and the duration of implantation. Control studies included sham insertions (stabs) into the subintimal space, and implantation of comparably sized platinum rods.

In the first days after implantation, there was a transient small hemorrhagic and acute inflammatory response which was supplanted within 4–6 days by a mononuclear response of varying intensity. These mononuclear cells immediately surrounding the implant appeared to be immature medial smooth muscle cells. At the lipid-tissue interface with Ch, ChP, and TP, there were often a few apparent smooth muscle cells whose intracellular compartment contained oil red O-positive material, presumably ingested lipid. These cells were never seen at any distance from the implant. Although the response was variable, local medial myocyte proliferation was generally moderate in response to ChP, less so with Ch, and mild to moderate with TP, PA, and platinum. DPL and stab provoked little or no response, and albumin disappeared too rapidly to provoke more than local hemorrhagic and acute inflammatory changes, leaving a tiny residual scar.

With time, the cells around the implant, which were initially large, rounded, and mononuclear with dark-staining large nuclei and slightly basophilic cytoplasm, began to assume a more fibroblastic appearance as shown by comparison of Figure 2c, d, and e (11 days, 2.5 months, and 5 months, respectively). The extracellular matrix in the spaces surrounding the implant became filled with a dense oriented fibrous material identified as collagen by special staining and by electron microscopy.

The regions of greatest fibrosis were the lateral triangular spaces bounded by the implant surface and the two separated elastic lamellae (Fig. 2, d, e, f). Fiber orientation was circumferential around the implant, as demonstrated with polarization microscopy (Fig. 2f). A semiquantitative characterization of the peri-implant fibrous reaction used the cross-sectional area of the implants (A0 = 0.084 mm²) as a reference dimension. Thus the area of tissue surrounding the implant which was involved in fibrosis was expressed as a fraction of the standard implant area: 0: no fibrosis; 1+: fibrosis involving less than 20% A0 (characteristic of residual scar from a stab); 2+: surrounding fibrosis involving 20% to 50% A0; 3+: 50 to 100% A0 fibrotic; 4+: greater than 100% A0 fibrotic. All specimens were examined with light and polarization microscopy. As can be seen from Table 3, fibrosis occurred, in approximate order of decreasing intensity, with Ch, ChP, platinum, TP, and PA. DPL, albumin, Ch-DPL, and stab did not provoke observable fibrosis. When it occurred, significant fibrosis appeared within the first month, and the encapsulation process was largely complete by 4–5 months. For Ch and ChP, particularly in the older implants, the fibrosis was not limited to the lateral triangular spaces.
<table>
<thead>
<tr>
<th>Substance</th>
<th>Absorption rate (—b)* (% per mo.)</th>
<th>95% confidence range for —b (% per mo.)</th>
<th>Predicted absorption time*</th>
<th>Anisotropy index (Dx/Dr)</th>
<th>Maximum tissue lipid content at 25 μM from implant (mg/ml)</th>
<th>Maximum intimal lipid content—15 μM from endothelial surface (mg/ml)</th>
<th>Dx ± 1 SD (cm²/sec)</th>
<th>Dr ± 1 SD (cm²/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol M.W. 387</td>
<td>0.12</td>
<td>-0.47 to 0.70</td>
<td>73 years (&gt;12 years)</td>
<td>14.5</td>
<td>32</td>
<td>15</td>
<td>5.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Cholesteryl palmi-tate M.W. 625</td>
<td>0.17</td>
<td>-0.34 to 0.68</td>
<td>50 years (&gt;12 years)</td>
<td>12.2</td>
<td>1.3</td>
<td>0.5</td>
<td>0.5</td>
<td>0.22</td>
</tr>
<tr>
<td>Glyceryl tripalmi-tate M.W. 807</td>
<td>3.5</td>
<td>2.8 to 4.2</td>
<td>2.4 years</td>
<td>16.0‡</td>
<td>12</td>
<td>13</td>
<td>11</td>
<td>1.5</td>
</tr>
<tr>
<td>Palmitic acid M.W. 284</td>
<td>27</td>
<td>23 to 31</td>
<td>3.8 mo.</td>
<td>14.5</td>
<td>10</td>
<td>15</td>
<td>13</td>
<td>1.9</td>
</tr>
<tr>
<td>Cholesterol-lecithin mixture (1:1 by weight)</td>
<td>First 2 mo. 34</td>
<td>N.D</td>
<td>1.5 years</td>
<td>5.3</td>
<td>33</td>
<td>(7 days)</td>
<td>N.D</td>
<td>N.D</td>
</tr>
<tr>
<td>After 2 mo. 2.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.D. = not determined.
* Based on averaged absorption rate over 20 months.
† Based on averaged absorption rate over 20 months.
‡ Based on average of 3-day and 3-month data.
§ Possibly subject to formalin fixation artifact.
Table 3  Characterization of Time-Course and Extent of Fibrotic Response to Various Subintimal Substances

<table>
<thead>
<tr>
<th>Substance</th>
<th>Total no. of implants (n)</th>
<th>Mean fibrosis rating (0-4+) ± 1 SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-1 mo.</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>39</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Cholesteryl palmitate</td>
<td>51</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Platinum</td>
<td>11</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Glyceryl tripalmitate</td>
<td>29</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>38</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Albumin</td>
<td>12</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Chol/DPL</td>
<td>19</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Stab</td>
<td>7</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>Dipalmitoyl lecithin</td>
<td>19</td>
<td>0.2 ± 0.2</td>
</tr>
</tbody>
</table>

I.D. = insufficient data.

areas; there was also replacement of the nearby interlamellar medial myocytes by oriented collagen (Fig. 2f).

The intima immediately overlying the implants often changed profoundly from the initially normal endothelial monolayer. About one-third of the 3-day specimens had endothelial denudation localized to the intimal segment overlying the implant; in none of these specimens did endothelial disruption occur at any points remote from the implant. At 1 week, the endothelial monolayer was reconstituted; however, these cells showed nuclear and cytoplasmic swelling with increased cytoplasmic basophilia. Whereas normal endothelial cells are almost devoid of visible cytoplasmic contents and have an average cell thickness of about 1.5 μm on light microscopy (Fig. 2a), the endothelial cells overlying the implants underwent demonstrable cytoplasmic thickening, with an increase in the nuclear population density along the intimal surface. These minimal intimal changes are demonstrated in Figure 2g.

For Ch and ChP, there was a striking intimal proliferation, producing multiple layers of apparent immature myocytes, resembling in several respects the proliferative lesion of early atherosclerosis. These changes were seen as early as 1 week; for example, the photomicrograph of Figure 2, b and c, shows an 11-day ChP implant. Initially, the cells had large rounded basophilic nuclei, lack of cellular orientation, and basophilic cytoplasm. By 2–3 months, the appearance had changed to that of an oriented fibromuscular plaque, as illustrated for cholesteryl palmitate at 2.5 months in Figure 2d. As fibrous encapsulation of the implant occurred (see Table 3) and the tissue and intimal ChP and Ch concentration fell (see Table 2B), the intimal proliferative response waned and the fibromuscular plaque became increasingly acellular and fibrous, as illustrated in Figure 2e, for a 5-month cholesterol implant. Apparent reabsorption of intimal collagen frequently resulted in a nearly normal intimal appearance in the late stages, as seen in Figure 2f, a 10-month cholesterol implant. A unique exception appeared to be cholesteryl palmitate for which a number of instances of lamellar reduplication (i.e., persistence of the proliferative response with synthesis of collagen and elastin) resulted in a persistent intimal thickening at 9–10 months, which was significant (P < 0.05) in comparison with the intima in the near vicinity of the implant.

The above intimal proliferative changes are summarized in the averaged data of Figure 4, showing the
temporal progression of intimal thickening. TP and PA appeared to provoke a mild intimal thickening, but this was not statistically significant when compared to the surrounding intima (horizontal dashed line). The proliferative response was particularly augmented with the Ch and ChP implants, for which the intima was thicker than the surrounding normal intima at highly significant levels. Albumin, DPL, platinum, and stab (all not plotted) did not provoke observable thickening. The Ch and ChP thickening was significantly greater than that for PA, DPL, albumin, platinum, and stab \( P < 0.05 \) during the first 3 months, but not significantly greater than TP \( P < 0.10 \). The mixture of Ch-DPL, 1:1 by weight (not plotted), behaved essentially like cholesterol, with a mean intimal thickness of 4.5 ± 2.8 (SD) \( \mu \)m at 0 to 1 month, 7.3 ± 5.3 at 1 to 2.5 months, 7.8 ± 5.3 at 2.5 to 4.5 months, and 3.7 ± 1.2 beyond 4.5 months.

**Autoradiography**

Figure 5a shows an autoradiographic specimen; Figure 5b shows the basic grain count data with points of equal grain density connected by contour lines. Data in this format were reduced to "% lipid content" using Equation 1. Figure 5, c–f, shows the results of these measurements for Ch, ChP, TP, and PA for three different implantation periods. Similar studies, not shown, were completed for albumin at 3 days and Ch-DPL at 7 days.

Cells looking like mural smooth muscle cells near the lipid surface were occasionally shown to be laden with lipid by this method. Because cells such as these, seen as

![autoradiographic data](image-url)
clusters of increased grain density (e.g., for ChP at 1 month in Figure 5a (see arrow) were relatively rare and were never seen at a distance greater than 100 μm from the implant, it is unlikely that migration of these phagocytic cells plays a significant role in lipid removal. They may, however, participate in lipid mobilization, e.g., emulsification, at the tissue interface and thus play an indirect role in the transport process.

The contour plots of Figure 5, b-f, provide some new insight into the mechanics of mural "free lipid" transport. It can be shown that the generally elliptical shape of the concentration contours is consistent with the notion that lipid moves by a diffusion mechanism in a highly anisotropic medium. For Fick diffusion, the Fourier "heat" equation can be solved for the concentration of a species in space and time. For a point source in an infinite two-dimensional anisotropic medium, solutions of constant concentration are ellipses whose major-to-minor axis ratio \((a_x/br)\) equals the square root of the corresponding directional diffusion coefficient ratio: \((D_8/Dr)^{1/2}\). Concentration increases radially. When boundary and source configurations are more complex, this simple relationship may become distorted. To evaluate this possibility, a finite element method of digital computation was employed to generate solutions for two-dimensional anisotropic diffusion which approximately fit the experimental boundary and source configurations. The coordinate system was oriented along the principal axes of structural anisotropy of the arterial wall with \(x\), the circumferential, and \(r\), the radial axis. The ratio of directional diffusion coefficients, \(D_8/D_r\), was independently varied. For each value of this ratio, a family of iso-concentration contours similar to those of Figure 5, c-f, was generated. For each family of contours, an average value of \(a_x/br\) was determined by direct measurement. The value, \(a_x/br\), was defined as the distance from the implant surface to the lateral tip of a given contour; \(b\), was the radial distance from the implant to the point on the same contour nearest the adventitial surface. Thus over the range of boundary, source, and contour configurations observed experimentally, a computer-predicted graphic relationship between \(a_x/br\) and \(D_8/D_r\) was obtained:

\[
(D_8/D_r)^{1/2} = 1.25 \left(\frac{a_x}{br}\right) - 0.55; r = 0.998
\]  

Using this expression, the diffusion coefficient ratios \((D_8/D_r)\) were estimated by measuring the elliptical axis ratios \((a_x/br)\), as defined above, from the contours of Figure 5, c-f. The values of \(D_8/D_r\) thus determined for Ch, ChP, TP, and PA ranged between 12.2 and 16.0, as shown in Table 2B. Simply stated, this means that it is 12 to 16 times easier for free lipid to move circumferentially than to move radially, perpendicular to the oriented structures of the arterial wall. This is consistent with the obvious structural anisotropy of the wall easily seen on routine histological section.

A combination of the data on absorption rates, diffusion ratios, and concentration contours allows direct estimation of values for \(D_8\) and \(D_r\). Briefly, a quasi-steady state of two-dimensional anisotropic Fick diffusion was assumed for this system, with the coordinates defined as above. The computations involved a numerical summation around the implant of the predicted local lipid efflux. A linear (Fick) relationship was assumed between efflux and concentration gradient along each of the principal axes. Total efflux across a surface surrounding the lipid was equated to the measured rate of lipid loss, -b, of Table 2A. This equation could be solved for \(D_8\) and \(D_r\). In practice, the band of area between two of the iso-concentration contours of Figure 5, c-f, was divided in half by another contour line, \(s\), midway between the two, and then divided, pie-fashion into approximately 10 (i = 1 through 10) segments of arc length, \(s_i\), along \(s\). The local concentration gradient, \(G_i\), perpendicular to \(s_i\), was estimated as the concentration difference between the two original contours, divided by the distance separating them. The angle \(\theta\) between the local gradient vector and the vertical axis of the system was determined. The rate of loss of area in the lipid implant, \(b\), was taken from Table 2A. Autoradiographic specimens were selected for which individual b-values closely approximated the general average. The ratio \(D_8/D_r\) was determined as described above for the 3-day implant, which was least likely to be modified by fibrosis. The value of \(D_r\) was then computed using the following expression:

\[
b = \sum_i G_i s_i (D_8/D_r) \sin^2 \theta + \cos^2 \theta. \quad (4)
\]

The value of \(D_8\) was then computed from the known ratio of diffusion coefficients. \(D_8\) and \(D_r\) were calculated using two different concentration contour bands for both the 3-day and the 1-month implant specimens. These four values were averaged to determine diffusion coefficients for each of the four lipids studied. These are reported in Table 2 and are to be compared with \(D(20^\circ\text{C}, H_2O) = 6 \times 10^{-7} \text{cm}^2/\text{sec}\) for the free diffusion of albumin.

A selective barrier to efflux appeared to exist at the blood interface for all lipid species. The apparent intersection of the isoconcentration contours with the endothelial surface is seen in many of the examples of Figure 5, b-f. This actually reflects a crowding together of many of the contours overlying the implant into a narrow band in the endothelial zone near the blood interface, defining a region of high local concentration gradient across that zone. This implies a localized barrier to radial efflux at or near the blood interface. The resolution of the technique, as it was employed in these studies, was not sufficient to define further the anatomy of this barrier zone. While this occurred with all lipid species studied, it was more pronounced with palmitic acid. It is unlikely that this represents an artifact of postmortem diffusion during formalin fixation. The background counts in the gelatin immediately overlying the endothelium were as low as elsewhere in the background (see Fig. 5b). Furthermore, preliminary studies with short- and long-term exposure to formalin excluded significant postmortem diffusion of any of the implanted lipid species. Formalin did wash out radiolabeled albumin to a measurable extent, and for this reason, its autoradiographic diffusion constants are not reported.

There are a number of practical and conceptual prob-
lems in the quantitative autoradiographic analysis which require a more adequate discussion. They center largely on the computation of $D_1$ and $D_r$. First, to assume a linear concentration gradient between two contours in Figure 5, $c-f$, is a relatively gross approximation, particularly since the increments in concentration were large. The actual radial decrease in concentration clearly is not a linear function, yet the extent to which these increments could be further reduced for these calculations was limited by the precision with which the locus of a given concentration contour could be determined. Second, the two-dimensional assumption introduces error at distances greater than two implant diameters from the lipid surface, since the implants were only about seven times longer than their diameters. As the diffusion pattern becomes three-dimensional at increasing distances from the implant, the concentration decreases more rapidly than one would predict from a two-dimensional analysis. To minimize this effect, calculations were based on contours lying within two implant diameters of the lipid surface, and sections for autoradiographic analysis were cut from the midpoint of the implant. In spite of these precautions, the $D_1$ and $D_r$ values determined from the outer contours were consistently larger than those from the inner ones. Third, the steady state assumption is of borderline validity for Ch and ChP, since their net efflux, $b$, in addition to being relatively uncertain for these species, probably varied considerably as the fibrous encapsulation progressed. Since the $b$-value used was the 20-month average rate, estimates of $D_1$ and $D_r$, using Equation 4, are likely to be somewhat low. It would have been more appropriate to use instantaneous $b$-values for calculations with data from the 3-day and 1-month specimens; however, the methods used lack sufficient resolution to do this.

Discussion

The sequence: cholesterol, cholesteryl palmitate, glyceryl tripalmitate, palmitic acid, and dipalmitoyl lecithin generally reflects the order of increasing rate of lipid reabsorption, decreasing sclerogenic potential, and decreasing magnitude of the associated intimal cellular proliferative reaction. These species were chosen as representative crystalline members of the different lipid classes.

In this model, the predicted time for disappearance of cholesterol and its esters may approximate a human lifetime, and is certainly greater than 12 years. Triglycerides are more rapidly absorbed, with an implant life of 2.4 years. Fatty acids and phospholipids have similar rapid disappearance rates, with implant lives of approximately 15 weeks. Migration of phagocytic cells does not appear to make a substantial contribution to lipid transport. This is different from the situation in rat subcutaneous tissue which perhaps explains the more rapid removal of lipids from that space. The phagocytic cells in the arterial wall appear to be smooth muscle cells, or "myogenic foam cells," whose transport capacity is felt to be considerably less than the tissue macrophages of reticuloendothelial origin that appear in subcutaneous tissue in response to implanted lipid. Autoradiographic analyses, done in a quantitative fashion, suggest that the lipids are transported by molecular migration in a concentration gradient. Some mechanisms of transport which are consistent with the diffusion model are: (1) lipid binding to a soluble protein carrier(s) with subsequent anisotropic diffusion in the arterial interstitial fluid; (2) lipid incorporation into the membranous component of the arterial smooth muscle cells with migration of the labeled species by molecular exchange; (3) binding of the lipid to fixed connective tissue proteins with migration of the labeled species by molecular exchange. While there were usually fewer exposed silver grains in the emulsion overlying the elastin, the histological sections (5 μm) and the emulsions were not sufficiently thin to localize the grains definitively to any particular cellular or connective tissue structure or compartment.

Whatever the mechanism of reabsorption of crystalline lipid from the arterial wall, the data from this model reveal it to be a discouragingly slow process. Yet there are encouraging reports of substantial removal of mural cholesterol during 6 to 18-month "regression diets" in animals with experimentally induced atheroma formation. The difference between these reports and our data probably reflects the different states from which the cholesterol is being reabsorbed. In early experimental atherosclerosis, lipid tends to be dispersed in multiple intra- and extracellular droplets which present a very large surface and which are in a less (thermodynamically) stable form and therefore are more easily solubilized. Our results would suggest that, once an advanced fibrous plaque develops in which a substantial amount of cholesterol is in crystalline aggregate, its regression would be virtually impossible within a practical time frame unless some solubilizing technique were employed. This is consistent with one reported failure of significant cholesterol reduction to reverse angiographically advanced human coronary atherosclerosis, whereas diet-induced regression has been demonstrated in a less advanced peripheral form of the disease.

The rapid reabsorption of cholesterol when it is solvent-mixed equally by weight with dipalmitoyl lecithin lends support to the concept that phospholipid might be used to solubilize mural cholesterol. Although this model is highly artificial and therapeutic implications are not intended, these observations are consistent with epidemiological data suggesting a protective effect of serum HDL against atherogenesis. The "solubilizing capacity" of serum HDL (cholesterol + triglyceride) of human HDL is approximately three times greater than LDL and seven times greater than VLDL. It is thus a more hydrophilic molecule and is richer in substances that have been used to solubilize lipid in the laboratory.

The collagen content of atherosclerotic arteries is increased, as is arterial collagen production in association with atherogenic diets. Leary advanced the notion that crystalline cholesterol and particularly its esters were "irritants" and thus fibrogenic in the intima. Our observations on canine aorta (see Fig. 2 and Table 3) support this concept and agree with those of Abdulla et al. These investigators found that subcutaneous implants of chole-
terol and its esters provoked a marked increase in local fibroblast activity and collagen production. Because our chemically inert platinum rods provoked a moderate fibrogenesis (although less than that seen with Ch and ChP), and because the Ch-DPL mixture was not fibrogenic, one has difficulty interpreting the increased fibrosis seen with Ch and ChP as a chemically specific response. The physical state is clearly important. Fibrosis may, in this case, represent a "foreign body reaction" to some structural property of these crystalline aggregates.

Intimal collagen production has potentially beneficial and detrimental aspects. On the detrimental side, collagen makes a major contribution to the mass of the atherosclerotic plaque.\textsuperscript{36} It also appears to encapsulate and trap deposits of crystalline lipid within the plaque. Figure 2f supports this concept. Hyalinization and local oriented fibrosis progressively surrounded the implants and became well established at 2 months and nearly complete at 4-5 months. During this period, the concentration of labeled Ch and ChP in the artery around the implant progressively fell by as much as 6-fold. Concomitantly, the Ch and ChP concentration in the intima fell by 10-fold (See Table 2B). It is not difficult to conceive that a dense fibrous matrix surrounding the lipid deposit would trap it in the wall and thus reduce its efflux by diffusion. A beneficial aspect of fibrous encapsulation lies in this same reduction of local tissue concentration of diffusible species. As discussed below, the intimal proliferative response appears to be related to the presence of diffused Ch and ChP in the intima. Indeed, as shown in Figure 6, there is an inverse relationship between the magnitude of intimal thickening and the extent of fibrous encapsulation for all Ch implants examined between 2 and 10 months.

The selective proliferation of intimal smooth muscle cells overlying the Ch, ChP, and Ch-DPL implants was an unexpected finding. Nonsignificant but measurable thickening occurred over the TP and PA implants; it was seldom seen with DPL, platinum, albumin, or subintimal stab. It is well-known that endothelial disruption predisposes to intimal fibromuscular thickening.\textsuperscript{34, 35} A certain amount of local intimal damage almost certainly resulted from the implantation process and/or the distorting presence of the implants. This was suggested by the localized absence of endothelium over all types of implants in about one-third of the cases, seen only at the time of earliest necropsy (3 days). However, a sustained and significant intimal thickening occurred only over the Ch, ChP, and Ch-DPL implants. Since the autoradiographic determinations show that all lipids are transported to the intima, the proliferative response appears to be biochemically specific for Ch and ChP or for some product of their metabolism. Thus we are drawn to conclude that intimal concentrations of cholesterol in the order of 400 mg/dl and its palmitic ester in the order of 20 mg/dl are associated with accelerated replication and growth of intimal smooth muscle cells in this preparation. Possibly, cholesteryl ester is the sole proliferative stimulant and the observed response to cholesterol is a result of its partial esterification by the arterial cells.\textsuperscript{36-38}

The nature of the relationship between cholesterol and intimal proliferation has not been defined in these studies.
The fate and fibrogenic potential of subintimal implants of crystalline lipid in the canine aorta. Quantitative histological and autoradiographic studies.

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