Metabolism of Atherosclerotic Tissue of Rabbit and Dog, with Special Reference to Esterase and Lipase

By Nelicio Maier, M.D., Ph.D., and Henry Haimovici, M.D.

In contrast to the extensive literature dealing with the chemical changes in the circulating blood in atherosclerosis and the composition of the atheromatous plaques, there is relatively little information concerning the metabolic alterations of the arterial tissue associated with the development of atherosclerosis. In previous investigations from this laboratory on the metabolism of arterial tissue, both normal and atherosclerotic, it was found that the various stages of the atherosclerotic process were accompanied by characteristic changes in the oxidative capacity of the succinic oxidase and cytochrome oxidase systems. In another series of experiments, the presence of esterase and the absence of lipase were demonstrated in the normal aortic tissue of man, rabbit, and dog.

The present investigation deals with esterase and lipase activity in atherosclerotic tissue of rabbit and dog.

Methods

Tissues studied in this investigation were aortas of rabbits and dogs. Liver and serum were used as reference tissues.

Male chinchilla rabbits, five to six months old, were placed on an atherogenic diet after a period of observation of several weeks, during which the health of the animals was ascertained. The diet consisted of Purina pellets impregnated with 0.6 g% cholesterol. The daily cholesterol intake per animal was 0.6 to 0.8 g. The control serum cholesterol level was 144.2 mg/100 ml (range: 80.8 to 198.5). Within four weeks the average concentration of serum cholesterol rose to about 700 mg/100 ml (range: 550 to 1000). The dogs were sacrificed, after nine to twenty months, by light thiopental (Pentothal) anesthesia and exsanguination.

In both rabbits and dogs, cholesterol determinations were done biweekly, by the method of Abell et al.

Studies of aortic tissue were done on its three segments, i.e., ascending and arch (hereafter referred to as "arch"), descending thoracic and abdominal. The severity of the atherosclerotic lesions was evaluated separately for each aortic segment by grading the lesions according to the percentage of surface involved. Four grades were thus established arbitrarily: 1+: 5 to 25%; 2+: 25 to 50%; 3+: 50 to 75%; 4+: 75 to 100%. The aorta and a segment of liver were removed immediately after sacrifice of the animals and placed in ice-cooled containers. The aorta was flushed with ice-cold saline solution and the adherent fat and connective tissue removed. The atherosclerotic portions used were carefully freed of surrounding tissue that appeared normal in order to study samples that were 100% atherosclerotic, by area involved. The atherosclerotic portions used were carefully freed of surrounding tissue that appeared normal in order to study samples that were 100% atherosclerotic, by area involved.

The animals on this atherogenic regimen appeared in good health and showed increase of weight, rapid in the beginning and tending to level off after several months. Animals showing a loss of weight were discarded. The rabbits were sacrificed by exsanguination after two to ten months, according to needs.

Male mongrel dogs, one to three years old, after a period of observation of several weeks, were placed on an atherogenic diet consisting of thiouracil and kibbled Purina meal containing 2.5% cholesterol. Daily intake of cholesterol was 0.6 to 0.8 g/kg body wt. Thiouracil was administered daily in meat, in amounts ranging from 70 to 100 mg/kg body wt, the dosage being adjusted according to the blood cholesterol level and the dog's appetite. During the administration of the above diet, the dogs remained in good condition. The control serum cholesterol level was 144.2 mg/100 ml (range: 80.8 to 198.5). Within four weeks the average concentration of serum cholesterol rose to about 700 mg/100 ml (range: 550 to 1000). The dogs were sacrificed, after nine to twenty months, by light thiopental (Pentothal) anesthesia and exsanguination.

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ESTERASE AND LIPASE IN ATHEROSCLEROTIC TISSUE

Results

Table 1: Esterase Activity* of Control and Atherosclerotic Rabbit Aorta

<table>
<thead>
<tr>
<th>Control</th>
<th>Entire aortic wall</th>
<th>Abdominal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atherogenic diet: 2-4 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change, % of control</td>
<td>135 ± 31.7 (7)</td>
<td>98.6 ± 5.8 (7)</td>
</tr>
<tr>
<td>P values</td>
<td>&lt; 0.05</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Degree of atherosclerosis</td>
<td>3 + to 4 +</td>
<td>2 + to 3 +</td>
</tr>
</tbody>
</table>

| Atherogenic diet: 7-10 months | | |
| Change, % of control | 79.6 ± 24.3 (7) | 50.1 ± 7.94 (7) | 46.8 ± 11.0 (6) |
| P values | > 0.50 | > 0.50 | > 0.10 |
| Degree of atherosclerosis | 4 + | 4 + | 4 + |

*Activity is expressed in micrograms of beta-naphthol liberated/mg tissue nitrogen/hour.

**Mean ± SEM.

1Degree of atherosclerosis refers to aortic segment. Portions used in experiments were free of uninvolved surrounding tissue, so as to be 100% atherosclerotic by area involved.

1Numbers in parentheses represent no. of determinations.

Each determination was made with a series of carboxylic esters, among them beta-naphthyl laurate, they differ in their reaction to activators and inhibitors. In presence of sodium taurocholate, the action of lipase is accelerated whereas esterase is inhibited. In our experiments, esterase was absent in all tissues studied, whereas lipase was measurable in all tissues except dog serum in which it was markedly increased. Thus, with the exception of dog serum, in which it was present, lipase activity was expressed in micrograms of beta-naphthol liberated per mg tissue. Enzyme activity was expressed in micrograms of beta-naphthol liberated per mg tissue and per hour by micro-Galab determination.

<table>
<thead>
<tr>
<th>Aortic intima-media</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Arch</td>
<td>80.1 ± 3.52 (9)</td>
<td></td>
</tr>
<tr>
<td>Desc. thoracic</td>
<td>59.3 ± 3.83 (9)</td>
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(grade 1+). At a later stage (seven to ten months), a reverse trend seemed to take place in the first two aortic segments. Due to the great spread of values, however, the changes appear to be at the limit of statistical significance ($P > 0.05$) when compared to the early phase.

b) Atherosclerotic Intima-Media

Since the atherosclerotic process involves primarily the intima and the immediately underlying media, it appeared important to study the atherosclerotic layer separately. In the arch, which is the site of maximum involvement in the rabbit, this layer represented about 50 to 80% of the total wet weight (approximately 40 to 65% of the total nitrogen) whereas in the descending thoracic aorta it represented about 25 to 50% of the total wet weight (approximately 15 to 30% of the total nitrogen). Table 1 (right) summarizes the results obtained with the atherosclerotic layer, separated at the cleavage plane. Esterase activity was markedly increased ($P < 0.001$) during the first two to four months. At a later stage (seven to ten months), when the atherosclerotic process was more advanced, the esterase activity showed less increase which, due to the great spread of values, appears statistically not significant ($P > 0.10$). However, when the early phase (two to four months) and the late phase (seven to ten months) of the atherosclerotic process are compared, a reverse trend is apparent and a significant decrease ($P < 0.01$) seemed to have taken place in the arch, whereas in the descending thoracic aorta it is at the limit of statistical significance ($P > 0.05$).

c) Liver and Serum

Table 2 summarizes the results obtained with the liver and serum. The activity of liver esterase was significantly increased ($P < 0.001$) during the first two to four months and reverted to normal values with further administration of the atherogenic diet. In contrast, the serum showed no change in the early period, whereas a significant decrease ($P < 0.02$) was noted later.

II DOG

a) Entire Atherosclerotic Aortic Wall

Table 3 (left) summarizes the results obtained with the entire aortic wall from dogs on the atherogenic diet for five to twenty months. This table indicates that no change in esterase activity is apparent in either the atherosclerotic or the nonatherosclerotic aortic segments. Changes occurring in the innermost atherosclerotic intima-media may have been masked, however, by the great mass of underlying normal tissue which represented about 75 to 90% of the total wet weight or 90 to 95% of the total nitrogen.

b) Atherosclerotic Intima-Media

Table 3 (right) summarizes the results obtained with the abdominal atherosclerotic intima-media layer, separated at the cleavage plane. It reveals markedly increased esterase activity ($P < 0.001$) during all phases of the atherosclerotic process, in contrast to the

<table>
<thead>
<tr>
<th>TABLE 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Esterase Activity</strong> of Rabbit Serum and Liver</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td><strong>Atherogenic diet: 2-4 months</strong></td>
</tr>
<tr>
<td>Change, % of control</td>
</tr>
<tr>
<td>$P$ values</td>
</tr>
<tr>
<td>Atherogenic diet: 6-10 months</td>
</tr>
<tr>
<td>Change, % of control</td>
</tr>
</tbody>
</table>

*Activity is expressed in micrograms of $\beta$-naphthol/mg of tissue nitrogen or ml serum/hour.

**Mean ± SEM.

†Numbers in parentheses represent no. of determinations.
absence of significant change in the innermost intima-media of the uninvolved descending thoracic aorta.

Table 4 summarizes the results obtained with the innermost intima-media of the uninvolved abdominal and descending thoracic aorta from dogs with marked atherosclerosis. The character of the atherosclerotic intima-media was markedly increased (P < 0.001), in contrast to the nonatherosclerotic intima-media which decreased significantly (P < 0.001). In the atherosclerotic intima-media, the esterase activity was markedly increased (P < 0.001) while the lipase activity remained unchanged (P > 0.10).

Table 5 summarizes the results obtained with the innermost intima-media of the abdominal and nonatherosclerotic portions of the abdominal aorta at the stage of patchy atherosclerosis (grade 1 to 3+). The character of the nonatherosclerotic portions were those of normal tissue, i.e., smooth, glistening intima and a normal histologic picture of the arterial wall. This table indicates no significant change (P > 0.10) in the esterase activity of the nonatherosclerotic intima-media in contrast to the atherosclerotic one in which esterase activity was markedly increased (P < 0.001). In the esterase activity of the aorta it was noted at that stage.

Table 6 reveals significantly increased esterase activity (P < 0.001) in liver during three to thirteen months on the atherogenic diet and reversal to normal values thereafter. In contrast, serum showed decreased esterase activity (P < 0.001) throughout the administration of the atherogenic diet while lipase activity was not affected for several months (P > 0.10), decreased at a later stage.

### Discussion

Investigation of esterase by histochemical techniques in normal human and mammalian arterial tissue, has yielded conflicting results. With few exceptions, esterase was either present inconstantly or was not detected. In the atherosclerotic tissue, the above methods yielded positive results. Using biochemical methods, we could also detect a significant increase in esterase activity (P < 0.001).

### Table 3

**Esterase Activity** of Control and Atherosclerotic Dog Aorta

<table>
<thead>
<tr>
<th></th>
<th>Entire aortic wall</th>
<th>Aortic intima-media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arch</td>
<td>Desc. thoracic</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>86.1 ± 3.22** (13)</td>
<td>62.5 ± 3.25 (13)</td>
</tr>
<tr>
<td><strong>Atherogenic diet: 5-20 months</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change, % of control</td>
<td>-6.1</td>
<td>+10.4</td>
</tr>
<tr>
<td>P values</td>
<td>&gt;0.10</td>
<td>&gt;0.50</td>
</tr>
<tr>
<td>Degree of atherosclerosis†</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

For symbols see footnote table 1.
TABLE 4
Esterase Activity* of Aortic Intima-Media from Dogs with Marked Atherosclerosis in Peripheral Vessels, and None in Aorta

<table>
<thead>
<tr>
<th></th>
<th>Desc. thoracic</th>
<th>Abdominal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>66.6 ± 2.08** (17)†</td>
<td>66.6 ± 1.74 (21)</td>
</tr>
<tr>
<td>Atherogenic diet: 6-11 months</td>
<td>60.4 ± 3.26 (6)</td>
<td>58.2 ± 4.30 (6)</td>
</tr>
<tr>
<td>Change, % of control</td>
<td>- 9.40</td>
<td>- 12.9</td>
</tr>
<tr>
<td>P values</td>
<td>&gt; 0.10</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

For symbols see footnote table 1.

TABLE 5
Esterase Activity* of Atherosclerotic and Adjacent Nonatherosclerotic Intima-Media of Dog Abdominal Aorta

<table>
<thead>
<tr>
<th></th>
<th>66.8 ± 1.74** (21)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atherogenic diet: 6-15 months</td>
<td>Nonatherosclerotic</td>
</tr>
<tr>
<td>Change, % of control</td>
<td>+ 16.6</td>
</tr>
<tr>
<td>P values</td>
<td>&gt; 0.10</td>
</tr>
</tbody>
</table>

For symbols see footnote table 1.

TABLE 6
Esterase and Lipase Activity* of Serum and Esterase Activity of Liver from Dogs on Atherogenic Diet

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Lipase</th>
<th>Hematocrit</th>
<th>Liver Esterase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>63.2 ± 2.35** (24)†</td>
<td>2763 ± 250 (14)</td>
<td>46.7 ± 0.95 (23)</td>
<td>1675 ± 94.7 (21)</td>
</tr>
<tr>
<td>Atherogenic diet: 2-4 months</td>
<td>34.8 ± 3.28 (9)</td>
<td>2345 ± 342 (8)</td>
<td>44.5 ± 0.96 (9)</td>
<td>2255 ± 76.8 (13)</td>
</tr>
<tr>
<td>Change, % of control</td>
<td>- 44.9</td>
<td>- 15.1</td>
<td>- 4.7</td>
<td>+ 34.6</td>
</tr>
<tr>
<td>P values</td>
<td>&lt; 0.001</td>
<td>&gt; 0.10</td>
<td>&gt; 0.10</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Atherogenic diet: 6-20 months</td>
<td>37.6 ± 2.25 (14)</td>
<td>1591 ± 219 (13)</td>
<td>41.0 ± 1.18 (14)</td>
<td>1653 ± 223 (10)</td>
</tr>
<tr>
<td>Change, % of control</td>
<td>- 31.5</td>
<td>- 42.4</td>
<td>- 12.3</td>
<td>- 1.4</td>
</tr>
<tr>
<td>P values</td>
<td>&lt; 0.001</td>
<td>&lt; 0.01</td>
<td>&lt; 0.001</td>
<td>&gt; 0.50</td>
</tr>
</tbody>
</table>

For symbols see footnote table 2.

ways detect esterase in normal arterial tissue of man, rabbit, and dog. It may be thus assumed that the conflicting results obtained with histochemical techniques were due to the fact that the esterase activity of normal arterial tissue was near or below the threshold of sensitivity of the histochemical methods, whereas in the atherosclerotic tissue the activity was enhanced and the enzyme became detectable.

In our experience and that of others, the extent of aortic lesions in the rabbit, despite some individual variations, may be predictable since it appears to depend upon the duration and level of cholesterolemia. It was thus possible to separate the development of atherosclerosis into two phases, according to the duration of the atherogenic diet, namely: early phase, two to four months, and late, seven to ten months.

In contrast to the rabbit, the dog presents greater individual variation and the extent of
the lesions does not seem to parallel the level and duration of cholesterolemia.\textsuperscript{22} This individual variation seems to be related to the breed. The development of the atherosclerotic lesions in the dog follows, however, a definite pattern of distribution, namely, involvement of the peripheral vessels first, and subsequently the abdominal aorta. The descending thoracic aorta, usually refractory to atherosclerosis, may in some instances develop, at a later stage, minimal lesions as compared to the abdominal aorta. For these reasons, early and late atherosclerosis in the dog could not be grouped according to the duration of the atherogenic diet. The severity of involvement of the abdominal aorta was chosen therefore as criterion for the separation into two phases: grade 1+ to 3+ (early), and grade 4+ (late).

Our results indicate that in both the rabbit and the dog, the relatively early phase of the atherosclerotic process was associated with increased esterase activity in the atherosclerotic intima-media (tables 1 and 3). At the later stage, a reverse trend was noted in the rabbit, whereas in the dog the esterase activity remained markedly increased. Our results agree with a previous biochemical investigation indicating increased esterase activity in the entire wall of the atherosclerotic thoracic aorta of rabbits fed cholesterol for two months.\textsuperscript{23}

While increased esterase activity in the early phase may be the result of "adaptation" due to accumulation of lipids, the reverse trend noted later in the rabbit may be attributed to the known development of fibrosis and degenerative changes accompanying the "death" of the tissue. The nature of the markedly increased esterase activity during the late phase of atherosclerosis in the dog, is difficult to interpret. Similar findings were obtained in histochemical observations (unpublished data) on a series of hydrolytic enzymes in dog aorta, which revealed that the severe or late form of atherosclerosis was associated with markedly increased activity for some enzymes, and decreased activity for others. The enzymatic increase did not seem to be due to invading macrophages.

In a previous investigation\textsuperscript{3} on the oxidative capacity of the succinic oxidase and cytochrome oxidase systems in the atherosclerotic aortic intima-media of dogs and rabbits we noted a similar increased activity in the early phase of the atherosclerotic process. In the late phase, however, the oxidative capacity was decreased in both species. Parallelling the findings with aortic tissue at the early phase, the liver of both species showed first increased esterase activity and a reversal to normal values later. In contrast, the rabbit serum esterase remained unaffected for several months and showed decreased activity later, whereas the dog serum showed decreased activity throughout the administration of the atherogenic diet.

In the rabbit, the serum enzymatic decrease paralleled hematocrit changes of similar magnitude (table 2), suggesting a relation to hemodilution. In the dog, decreased esterase activity in the first two to four months was not associated with hematocrit changes. It is noteworthy that during this phase the lipase activity was not affected, although it decreased later. At this later stage, an average hematocrit decrease of about 10% was noted, whereas esterase and lipase activities were decreased by about 30% and 40% respectively (table 6).

The enzymatic changes described above were noted in tissues already atherosclerotic. The question arose whether any enzymatic changes preceded the development of the lesions. In a previous investigation\textsuperscript{3} it was found that the oxidative capacity of the cytochrome oxidase system was decreasing prior to the development of atherosclerosis, whereas that of the succinic oxidase system was not affected. In the present investigation, the results obtained with uninvolved innermost intima-media of aorta at stages which may be considered pre-atherosclerotic according to the known sequence of appearance of the lesions in the dog, seem to indicate that the esterase activity was not affected at these stages (tables 3-5).
The results obtained in the present investigation expand our previous findings with other arterial enzymes and indicate further that metabolic alterations of the arterial wall are associated with the development of atherosclerosis. The role these alterations may play in the evolution of the atheroma must await further investigation.

Summary

Esterase and lipase were studied in the aorta, liver, and serum of dogs and rabbits on an atherogenic diet. With the exception of dog serum, lipase was not present in any of the tissues studied.

At a relatively early stage the atherosclerotic intima-media layer, separated at the cleavage plane, showed increased esterase activity in both animals. At later stages, a reverse trend was noted in the rabbit, whereas in the dog, the esterase activity remained markedly increased. No pre-atherosclerotic enzymatic changes were noted.

The liver of both species showed increased esterase activity first and reversal to normal values later. In contrast, rabbit serum displayed no change for several months and decreased activity subsequently whereas in the dog, decreased esterase activity was noted throughout the administration of the atherogenic diet. Lipase activity in dog serum, which was unaffected for several months, decreased later.

Our results expand previous findings with other arterial enzymes and indicate further that metabolic alterations of the arterial wall are associated with the development of atherosclerosis.

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Metabolism of Atherosclerotic Tissue of Rabbit and Dog, with Special Reference to Esterase and Lipase
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