Aortic Collagen Synthesis in Rabbits following Removal of Atherogenic Diet

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Male adult rabbits were divided into five experimental groups and were fed a 2% cholesterol diet using the following schedule: (A) two groups, normal control diet; (B) one group, 60 days continuous diet (60 day); (C) one group, 30 days cholesterol 30 days control diet (30-30); (D) one group, 60 days cholesterol, 30 days control diet (60-30). Aortic collagen synthesis was estimated by measuring tissue prolyl hydroxylase activity and tissue cholesterol was measured following thin layer chromatography. In the 30-30 and 60-30 animals, the removal of cholesterol from the diet caused a rapid fall in serum cholesterol values which slowed the rate of aortic cholesterol accumulation. Collagen synthetic activity was significantly elevated in the 60-30 rabbits but was at control levels of both the 60 day and 30-30 rabbits. The results demonstrate that the onset of increased aortic collagen synthesis is accompanied by an increase in the rate of cholesterol accumulation.

INTRODUCTION

The occlusive and irreversible nature of atherosclerosis is thought to be a result of the formation of fibrous aortic plaques containing increased amounts of collagen. The factors which stimulate the formation of the collagen fibers are not known. In recent experiments (Langner and Modrak, 1976a) we have shown that feeding a 2% cholesterol diet to rabbits for 90 days will result in an increase in aortic collagen synthetic rates. When rabbits were fed for 60 days or less there was no change in collagen synthetic activity even though the animals had gross aortic lesions (Langner and Modrak, 1976a, b). These results suggested that continuous cholesterol feeding in rabbits causes first a tissue cholesterol accumulation which in turn stimulates aortic collagen synthetic activity.

The effect of intermittent cholesterol feeding on aortic collagen synthesis in rabbits is unclear. The studies of Constantinides et al. (1960) have shown that intermittent cholesterol feeding for a period of 10 months does not cause reversal of the lesion but rather results in the formation of fibrous lesions. Zilversmit (1968) has suggested that the fibrous cap which forms after the removal of cholesterol from the diet may prevent the reversal of the atherosclerotic lesion. Adams et al. (1973) fed rabbits cholesterol for 12 weeks followed by a normal low cholesterol diet for 1 year. They also found that the atheroma did not regress but rather the amount of fibrous tissue in the lesion steadily increased during

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the feeding of the normal diet. These observations support the previously made suggestion that the removal of the high cholesterol diet will help to promote the formation of complex fibrous lesions (Wissler and Vesselinovitch, 1968).

In a study of the reversal of atherosclerosis Albrecht and Schuler (1965) found that the deposition of cholesterol in the arterial wall could be divided into two phases. The first phase was characterized by a slow steady increase in tissue cholesterol while the second phase was characterized by a much more rapid increase in tissue cholesterol content. This second phase appeared to be related to some unknown metabolic change since it occurred even when the rabbits were on a low cholesterol diet, provided the initial cholesterol feeding period was for a sufficiently long period of time (Albrecht and Schuler, 1965).

In this present study the relationship between aortic collagen synthesis and aortic cholesterol accumulation was studied. Rabbits were fed a high cholesterol diet followed by a period of normal, low cholesterol diet. The purpose of these studies was to: (A) determine whether the removal of cholesterol from the diet would stimulate collagen synthetic activity and (B) determine whether an alteration in collagen synthetic activity is accompanied by any change in the rate of aortic cholesterol accumulation.

METHODS

Forty male New Zealand rabbits weighing 2.0–2.5 kg were randomly divided into five equal groups. The rabbits were housed two per cage and were given water and food ad libitum. Two of the five groups served as control animals while the remaining three groups were fed a 2% cholesterol diet according to the following schedule: (A) 60 days cholesterol feeding (60); (B) 30 days cholesterol feeding, 30 days normal diet (30–30); and (C) 60 days cholesterol feeding, 30 days normal diet (60–30). The 2% cholesterol diet was prepared by adsorbing cholesterol dissolved in chloroform onto rabbit food pellets.

The rabbits were sacrificed using the following schedule. After 60 days the 30–30 and 60 day continuous feeding, and one control group were killed, and after 90 days the 6030 group and the remaining control group were killed. All animals were killed by cervical dislocation, the aortas quickly removed, and the extent of grossly visible aortic lesions estimated using the 0–4 grading system described by Lorenzen (1962). Grade 0 indicates the absence of aortic lesions while grade 4 represents the most severe lesions, usually involving both the thoracic and abdominal segments of the aorta. After grading, the aortas were carefully cleared of loosely adhering adventitial tissue, split into thoracic and abdominal segments at the celiac artery, and homogenized in 9 volumes of 0.25 M sucrose using a glass co-axial homogenizer.

Prolyl hydroxylase activity was measured in the 15,000g supernatant of aortic homogenate using the tritium release method of Hutton et al. (1966). This assay is based upon the stoichiometric formation of [3H2O] and [4H]hydroxyproline when a substrate consisting of a polypeptide rich in [3,4-3H]proline is incubated with aortic enzyme. Each incubation mixture contained approximately 350,000 DPM chick substrate, an aliquot of aortic enzyme, 7.5 μmoles of ascorbic acid, 0.9 μmoles of L-ketoglutarate, 0.45 μmoles of ferrous ammonium sulfate and 0.06 M Tris-HCl buffer (ph 7.5) to a final volume of 3 ml, as previously described.
The $^3$HOO formed is collected by vacuum distillation and counted in a Packard Model 3375 liquid scintillation spectrometer. All samples were corrected for counting efficiency by means of an automatic external standard.

Hydroxyproline content of aortic homogenates was estimated by the method of Kivirikko et al. (1967), and was used as an index of collagen content. Non-collagen protein concentrations were determined on the aortic homogenate and the 15,000g supernatants by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Aortic cholesterol was determined on aliquots of aortic homogenate after lipid extraction and separation of free and esterified cholesterol by thin-layer chromatography. The cholesterol was extracted from aortic homogenates by a modification of the method of Folch et al. (1957). One ml of aortic homogenate was extracted with 3 ml of chloroform-methanol (1:3) for 15 min, centrifuged and then filtered. The pellet was re-extracted two times with 4 ml of chloroform-methanol (2:1), centrifuged, and filtered. The extracts were combined and enough chloroform added to make the final proportion of chloroform to methanol 2:1. The combined extracts were then washed with 0.2 volumes of 0.04% CaCl$_2$ and dried under a stream of N$_2$ gas in a water bath at 60°C. The dried extract was stored in a freezer until ready for chromatographic separation.

The free and esterified cholesterol were separated on glass plates coated with silica gel 60 HR extra pure (E.M.) 0.5 mm thick. The plates were developed in n-heptane-diethyl ether-acetic acid (75:25:2) and visualized in iodine. The iodine was allowed to evaporate and the free and esterified cholesterol spots were scraped off into 5 ml conical centrifuge tubes. Cholesterol was determined by a modification of the method of Franey and Amador (1968). One ml of ethanol and 2 ml of a ferric chloride reagent was added to the centrifuge tube and shaken. Two ml of sulfuric acid (analytical reagent) were layered into the tubes. The tubes were shaken vigorously, left to cool, and then centrifuged. The absorbance of the supernatant was read in a Beckman DB-G spectrophotometer at 560 nm. Serum cholesterol was determined after 30, 60, and 90 days. The blood was collected from the marginal ear vein and serum cholesterol determined on the ethanolic extract of serum by the method of Franey and Amador (1968). The statistical significance of any observed differences was determined by use of the analysis of variance in multiple group comparisons and/or a student's "t" test in paired group comparisons.

Histological sections were taken from representative lesions. Three sections (arch, mid-thoracic, and abdominal) were cut before cleaning of adhering tissue and fixed in buffered formalin. The sections were embedded in paraffin, cut and stained with hematoxylin and eosin.

RESULTS

The rabbits fed the 2% cholesterol diet exhibited a very rapid rise in serum cholesterol levels. After 30 days of feeding, all the animals had serum cholesterol levels in excess of 1300 mg/100 ml (Fig. 1). When the cholesterol was removed from the diet, the serum cholesterol values fell very rapidly but still remained significantly above control values. By 60 days all rabbits fed cholesterol had
visible aortic lesions (Table I). The rabbits fed for 60 days followed by 30 days of normal diet (60-30) had the most severe lesions as shown by the greater proportion of grade 4 lesions. Histologically the lesions were characterized by having several layers of intimal foam cells, with some subintimal vacuolization. The media, however, appeared to be normal, and in comparing grade 2 lesions to grade 4 lesions, the only difference appeared to be in the thickness of the cholesterol laden intima. All of the lesions appeared to be of the foam cell variety since there was no histological indication of calcification or fibrous cap formation.

The free and esterified cholesterol levels were significantly elevated in the thoracic aortas of all the cholesterol fed rabbits (Table I). In the 30-30 group there was a significant increase in both free and esterified cholesterol when compared to control animals. The amount of cholesterol ester in the 30-30 group was lower than that seen in rabbits fed continuously for 60 days, while the amount of free cholesterol was essentially the same in both groups. The animals which were fed cholesterol for 60 days followed by 30 days of normal diet (60-30) had the highest thoracic aorta cholesterol values. The 60-30 animals had significantly elevated amounts of both cholesterol and cholesterol ester when compared to either control animals or animals fed cholesterol for 60 days.

The aortic wet weight to body weight ratios were increased in both the 60 and 60-30 groups of rabbits. The increase in the 60 day animals was statistically significant whereas the increase in the 60-30 animals was not statistically significant at the 0.05 level (Table II). Since the color formed in the protein assay procedure (Lowry et al. 1951) is heavily dependent upon the presence of amino acids which are essentially absent in collagen, this procedure has been used by us as a crude estimate of the aortic non-collagen protein content. The amount of non-collagen protein expressed as protein per gram of thoracic aorta...
Cholesterol Content of the Thoracic Aorta From Rabbits Fed a 2% Cholesterol Diet For Varying Periods of Time

<table>
<thead>
<tr>
<th>Groupa</th>
<th>µg Free cholesterol</th>
<th>µg Cholesterol ester</th>
<th>No. rabbits with lesions graded</th>
<th>mg protein</th>
<th>mg protein</th>
<th>0-1-2-3-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.0 ± 0.4 (8)b</td>
<td>—c</td>
<td>8-0-0-0-0</td>
<td>0-1-2-3-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>19.8 ± 4.5 (6)d</td>
<td>23.4 ± 9.1 (6)</td>
<td>0-1-4-1-1</td>
<td>0-4-3-1-0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-30</td>
<td>17.4 ± 0.8 (8)d</td>
<td>11.5 ± 1.1 (6)</td>
<td>0-1-4-1-1</td>
<td>0-4-3-1-0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.8 ± 0.6 (8)</td>
<td>—c</td>
<td>8-0-0-0-0</td>
<td>0-1-2-3-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60-30</td>
<td>48.9 ± 8.9 (8)d</td>
<td>83.3 ± 22.6 (8)</td>
<td>0-1-2-3-4</td>
<td>0-1-2-3-4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

30-30 = 30 days of 2% cholesterol diet followed by 30 days of normal diet. 60 = 60 days of 2% cholesterol diet. 60-30 = 60 days of 2% cholesterol diet followed by 30 days of normal diet.

* Mean ± SE (N).

c Cholesterol content less than 0.3 µg/mg protein which was the lower limit of our assay system.

d P < 0.05 compared to control.

Wet weight (Table II) did not change in any of the groups during the experiment. Similarly, the collagen content expressed as the amount of hydroxyproline per gram of tissue or per thoracic aorta segment showed no significant change when compared to control values (Table II).

The prolyl hydroxylase activity of the thoracic aorta was unchanged in either the 30-30 or the 60 day animals (Table III). The 60-30 animals however, showed a significant 3-fold increase in their prolyl hydroxylase activity. These animals also exhibited a significant increase in their rate of total cholesterol accumulation when compared to 30-30 day animals. This increase in total cholesterol accumulation was due primarily to a large increase in the rate of cholesterol ester accumulation. The rate of free cholesterol accumulation did not increase as much as the cholesterol ester rate but was still significantly greater than the free cholesterol accumulation rate of the 30-30 day animals (Table III).

Hydroxyproline and Protein Content of the Thoracic Aorta From Rabbits Fed a 2% Cholesterol Diet For Varying Periods of Time

<table>
<thead>
<tr>
<th>Groupa</th>
<th>Thoracic aorta wt. (gms)</th>
<th>mg protein</th>
<th>mg hyprob</th>
<th>mg hypro</th>
<th>Thoracic aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body wt. (kg)</td>
<td>gm tissue</td>
<td>gm tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.29 ± 0.01 (8)c</td>
<td>127.0 ± 3.6 (8)</td>
<td>5.46 ± 0.30 (8)</td>
<td>4.98 ± 0.18 (8)</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0.33 ± 0.01 (7)d</td>
<td>121.9 ± 7.8 (7)</td>
<td>4.63 ± 0.35 (7)</td>
<td>4.42 ± 0.31 (7)</td>
<td></td>
</tr>
<tr>
<td>30-30</td>
<td>0.27 ± 0.01 (8)</td>
<td>129.6 ± 7.0 (8)</td>
<td>5.35 ± 0.21 (8)</td>
<td>4.61 ± 0.14 (8)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.26 ± 0.02 (8)</td>
<td>123.3 ± 7.6 (8)</td>
<td>5.22 ± 0.33 (8)</td>
<td>4.35 ± 0.40 (8)</td>
<td></td>
</tr>
<tr>
<td>60-30</td>
<td>0.35 ± 0.04 (8)</td>
<td>130.6 ± 4.9 (8)</td>
<td>4.71 ± 0.16 (7)</td>
<td>5.08 ± 0.23 (8)</td>
<td></td>
</tr>
</tbody>
</table>

*a See Table I.

b Hypro (hydroxyproline) per gram wet weight.

c Mean ± SE (N).

d P < 0.05 compared to control.
The fibrous atherosclerotic lesion is characterized as having a fibrous cap covering a core of lipids and cellular debris. To produce this lesion experimentally in rabbits several investigators have fed an intermittent high cholesterol diet (Constantinides et al., 1960; Adams et al., 1973). The results of these studies have suggested that the period of low cholesterol feeding stimulates aortic collagen synthetic activity. In the present experiments, the relationship between intermittent cholesterol feeding and collagen synthesis was investigated by measuring prolyl hydroxylase activity. Prolyl hydrosylase (E.C. 1.14.11.2) is the enzyme which hydroxylates appropriate peptide prolines to collagen hydroxyproline.

Several studies (Takeuchi et al., 1967; Halme et al., 1970; and Langner and Fuller, 1973) in different tissues have shown an increase in prolyl hydroxylase activity before there was a significant increase in tissue hydroxyproline levels. These changes in enzyme activity have been interpreted as being an early indicator of increased collagen synthetic activity (Halme et al., 1970; Langner and Fuller, 1973). The data in Table III shows that the removal of cholesterol from the diet after 30 days of cholesterol feeding had no effect on stimulating collagen synthetic activity. In rabbits fed cholesterol for 60 days followed by 30 days of normal diet (i.e., 60–30) there was a significant increase in collagen synthetic activity (Table III). This increase was of a similar magnitude as that previously observed (Langner and Modrak, 1976a) when rabbits were fed cholesterol continuously for 90 days. These data, therefore, indicate that after 30 or 60 days of cholesterol feeding the removal of cholesterol from the diet has by itself no immediate effect upon stimulating aortic collagen synthetic activity. Rather, it appears that the duration of cholesterol feeding is more important in determining whether or not aortic collagen synthesis will be elevated.

The lesions which we observed in all experimental groups were histologically of the foam cell type. The cholesterol and cholesterol ester content were significantly increased in all of the aortas from experimental animals (Table I). Colorimetric determination of collagen content based upon hydroxyproline content

### TABLE III

<table>
<thead>
<tr>
<th>Groups</th>
<th>Prolyl hydroxylase activity (dpm/µg prot.30 min)</th>
<th>Total cholesterol accumulation (µg/mg protein-day)</th>
<th>Cholesterol ester accumulation (µg/mg protein-day)</th>
<th>Free cholesterol accumulation (µg/mg protein-day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4,571 ± 833 (7)</td>
<td>0.721 ± 0.02 (6)</td>
<td>0.390 ± 0.15 (6)</td>
<td>0.331 ± 0.07 (6)</td>
</tr>
<tr>
<td>60-30</td>
<td>4,266 ± 642 (7)</td>
<td>0.476 ± 0.03 (6)</td>
<td>0.192 ± 0.02 (6)</td>
<td>0.290 ± 0.137 (8)</td>
</tr>
<tr>
<td>Control</td>
<td>4,002 ± 762 (7)</td>
<td>1.47 ± 0.03 (8)</td>
<td>0.04 ± 0.025 (8)</td>
<td>0.34 ± 0.09 (8)</td>
</tr>
<tr>
<td>60-30</td>
<td>12,695 ± 1,246 (8)</td>
<td>0.37 ± 0.03 (8)</td>
<td>0.39 ± 0.07 (8)</td>
<td>0.37 ± 0.09 (8)</td>
</tr>
</tbody>
</table>

* See Table I for explanation.

* Prolyl hydroxylase activity is estimated by the formation of \(^3\)H\(\cdot\) following incubation at 30° of a [3,4-\(^3\)H\(\cdot\)] proline rich substrate with 15,000g thoracic aorta enzyme preparation for 30 min.

* Daily accumulation of thoracic aorta, total cholesterol, cholesterol ester, or free cholesterol was estimated by dividing the final content by the number of days in the experimental period.

* Mean ± SE (N).

* P < 0.05 compared to control.

/ P < 0.05 compared to 30–30 group.

### DISCUSSION

The fibrous atherosclerotic lesion is characterized as having a fibrous cap covering a core of lipids and cellular debris. To produce this lesion experimentally in rabbits several investigators have fed an intermittent high cholesterol diet (Constantinides et al., 1960; Adams et al., 1973). The results of these studies have suggested that the period of low cholesterol feeding stimulates aortic collagen synthetic activity. In the present experiments, the relationship between intermittent cholesterol feeding and collagen synthesis was investigated by measuring prolyl hydroxylase activity. Prolyl hydroxylase (E.C. 1.14.11.2) is the enzyme which hydroxylates appropriate peptide prolines to collagen hydroxyproline.

Several studies (Takeuchi et al., 1967; Halme et al., 1970; and Langner and Fuller, 1973) in different tissues have shown an increase in prolyl hydroxylase activity before there was a significant increase in tissue hydroxyproline levels. These changes in enzyme activity have been interpreted as being an early indicator of increased collagen synthetic activity (Halme et al., 1970; Langner and Fuller, 1973). The data in Table III shows that the removal of cholesterol from the diet after 30 days of cholesterol feeding had no effect on stimulating collagen synthetic activity. In rabbits fed cholesterol for 60 days followed by 30 days of normal diet (i.e., 60–30) there was a significant increase in collagen synthetic activity (Table III). This increase was of a similar magnitude as that previously observed (Langner and Modrak, 1976a) when rabbits were fed cholesterol continuously for 90 days. These data, therefore, indicate that after 30 or 60 days of cholesterol feeding the removal of cholesterol from the diet has by itself no immediate effect upon stimulating aortic collagen synthetic activity. Rather, it appears that the duration of cholesterol feeding is more important in determining whether or not aortic collagen synthesis will be elevated.

The lesions which we observed in all experimental groups were histologically of the foam cell type. The cholesterol and cholesterol ester content were significantly increased in all of the aortas from experimental animals (Table I). Colorimetric determination of collagen content based upon hydroxyproline content
(Table II) showed no change from control values, also suggesting that the observed lesions were not fibrous in nature. These observations are consistent with our previous findings that significant amounts of cholesterol must accumulate before fibrous tissue formation is stimulated (Mondrak and Langner, 1976a). The 60–30 rabbits which had an increased collagen synthetic rate as indicated by elevated prolyl hydroxylase levels (Table III) also had no increase in hydroxyproline content (Table II). This apparent contradiction can be explained by assuming that the 60–30 rabbits are in the beginning stage of fibrous lesion formation. Since there is a significant amount of collagen normally present in the aorta it could take several weeks of elevated collagen synthesis before an increased collagen content could be detected by histological or colorimetric techniques.

Even though the presence of increased collagen in the fibrous lesion has been long recognized, the significance of this deposition is unknown. Some investigators (Wissler and Vesselinovitch, 1968) have suggested that the laying down of collagen fibers simply represents an attempt of the artery to repair itself, while other investigators (Smith, 1974) have questioned whether the elaboration of collagen fibers might somehow alter the lipid accumulating abilities of the aortic tissue.

Previous studies by Albrecht and Schuler (1964) demonstrated that the accumulation of cholesterol followed a biphasic pattern which they attributed to some unknown metabolic alteration. Zilversmit (1968) also observed a similar biphasic accumulation of aortic lipid which he attributed to a loss of an undefined permeability barrier. In these present studies, the daily net accumulation of cholesterol was calculated by dividing the final tissue cholesterol content in Table I by the appropriate number of experimental days. In doing this we also observed a biphasic cholesterol accumulation rate going from an initial total cholesterol accumulation rate of approximately 0.6 µg/mg protein/day in the 30–30 or 60 day groups to 1.5 µg/mg protein/day in the 60–30 rabbits (Table III). The actual increase in cholesterol accumulation rate during the last 30 days of the experiment was probably much greater than the 1.5 µg/day would indicate. If we assume from Table I that after 60 days there was a total thoracic aorta cholesterol content of 42 µg/mg protein and after 90 days the content increased to approximately 130 µg/mg protein, then the cholesterol accumulation rate during the last 30 days was approximately 3 µg/mg protein/day, which is approximately 5 times greater than the initial accumulation rate observed during the first 60 days.

The exact reason for this increase in cholesterol accumulation is not known. In our experiments we have observed this increase in cholesterol accumulation rate only in tissues which have an elevated collagen synthetic rate. This observation is consistent with our hypothesis that increased collagen deposition plays an active role in the retention of aortic cholesterol. There are however, many other possible explanations of our data. At the present time it is impossible to determine how or if the parameters of cholesterol accumulation and collagen synthesis relate to each other. Many other factors such as endothelial permeability, the rate of non-collagen protein synthesis, the rate of collagen breakdown, and the rate of tissue cholesterol synthesis must be investigated in order to fully understand the relationship of connective tissue metabolism to atherogenesis.
ACKNOWLEDGMENTS

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REFERENCES


