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Chemical Composition of Atherosclerotic Lesions of Aortas from Pigeons with Naturally Occurring or Cholesterol-Aggravated Atherosclerosis (38031)

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The White Carneau pigeon develops aortic atherosclerosis naturally so that by three years of age, virtually all birds have grossly visible atherosclerosis (1). The morphology of these naturally occurring lesions is similar in many ways to that of atherosclerotic lesions from human beings (2).

White Carneau pigeons also develop accelerated atherosclerosis experimentally after only a few months of consuming a diet containing cholesterol (3). These cholesterol-aggravated lesions are similar in many ways to the naturally occurring lesions of the pigeon, but differ by virtue of the presence of larger numbers of lipid-filled cells (foam cells) and the lack of a prominent "fibromuscular cap" (4).

Previous studies from this laboratory have demonstrated the high correlation between extent of grossly visible aortic atherosclerosis and total cholesterol content of aorta with naturally occurring (5) or cholesterol-aggravated (3) atherosclerosis. There are no reports, however, describing the lipid composition of specific lesion types (fatty streaks and plaques) from aortas of pigeons with either naturally occurring or cholesterol-aggravated atherosclerosis. The purpose of this study was to make such a comparison in an attempt to relate the lipid composition with the known morphologic features of the naturally occurring and cholesterol-aggravated lesions of the pigeon.

Calcification is a prominent feature of atherosclerotic lesions in human beings (6), and has also been observed morphologically in atherosclerotic plaques from pigeons (7). Since composition data from arterial tissue must be based on some measure of the amount of arterial tissue analyzed (wet weight, dry weight, lipid free dry weight, surface area, etc.), we wanted to know whether the calcium present in atherosclerotic lesions from pigeons could account for a significant proportion of the tissue weight. It was also important to know whether calcium accounted for a greater percentage of the tissue weight in plaque, compared with fatty streak or normal tissue. Such data are critical when comparing the composition of different lesion types.

Materials and Methods. Aortic atherosclerosis was produced in 50 White Carneau pigeons by feeding a cholesterol-containing diet for 7–8 months. The pigeons were obtained from the Palmetto Pigeon Plant, Sumter, South Carolina, and fed a diet of Purina Pigeon Pellets (Ralston-Purina Co., St. Louis, Missouri) coated with cholesterol and lard to a final concentration of 0.5 g cholesterol and 10 g lard per 100 g ration. The birds were less than four months of age when started on the atherogenic diet. These pigeons with cholesterol-aggravated athero-

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sclerosis were divided randomly into subgroups of 10 birds each. Atherosclerotic plaque, fatty streak, and normal aortic tissue from the 10 birds of each subgroup were pooled in order to have enough tissue for analysis. Fatty streaks were identified grossly as yellow streaks with little elevation of the endothelial surface. Plaques were also yellow, but extended significantly into the lumen of the vessel.

One hundred and eight White Carneau pigeons with naturally-occurring atherosclerosis were obtained from our pigeon facility. These birds were from 6–12 yr of age and had been maintained since hatch on a cholesterol-free, mixed grain diet consisting of corn, wheat, peas, and milo. These pigeons were divided into subgroups of 5–10 pigeons each, according to their age. Aortic tissue from each subgroup of birds with naturally-occurring atherosclerosis was pooled as above, with the exception that only normal and plaque tissue were analyzed, since fatty streaks were only rarely seen.

A third group of young White Carneau pigeons, also obtained from the Palmetto Pigeon Plant, was fed the cholesterol-free grain diet described above. These birds were less than one-year-old, had no grossly visible atherosclerosis at autopsy, and served as controls for this study. Aortic tissue from subgroups of three control pigeons each were pooled for analysis.

Pigeons were killed by decapitation and the aortas were removed and cleaned of blood and periadventitial tissue. Each aorta was opened longitudinally, weighed, and the thoracic aorta to the point of origin of the celiac axis was graded for extent of grossly visible atherosclerosis (5). Normal tissue, plaque, and fatty streak (fatty streaks were analyzed only from pigeons with cholesterol-aggravated atherosclerosis) were excised, weighed, and pooled as described above. The tissues were homogenized in ice cold 0.05 *M* Tris–HCl buffer, pH 7.5, using all-glass homogenizers, and an aliquot of the homogenate was taken for analysis. This was done so that a portion of the homogenate could also be used for enzyme studies that will be reported separately. Lipids were extracted from this homogenate with chloroform and methanol (2:1). The lipid extract was taken to dryness under nitrogen at 40°C and cholesterol-4-^{14}C (New England Nuclear, Boston, Massachusetts) was added as an internal standard. Lipids were separated by thin-layer chromatography (TLC) using Skellysolve B (Skelly Oil Co., Kansas City, Missouri):ethyl ether:acetic acid (146:50:4) as the developing solvent. After chromatography, lipids were made visible with iodine and fractions corresponding to known standards of cholesteryl oleate, tripalmitin, oleic acid, cholesterol, and lecithin were eluted. The cholesterol and cholesteryl ester fractions were analyzed by the method of Block *et al.* (8); the triglycerides, by the method of Lofland (9), as modified by Timms *et al.* (10), and phospholipids, by the method of Parker and Peterson (11). Free fatty acids were determined by gas-liquid chromatography (GLC) of the methyl esters. Methyl esters were prepared by reaction overnight with 2.5% hydrochloric acid in methanol. Separations were carried out using a MicroTek GC-2000R chromatograph at 165°C in 6-ft long, ⅛ in. stainless steel columns of 15% diethylene glycol succinate polyester on 100/120 Gas Chrom P (Applied Science Labs., State College, Pennsylvania) using nitrogen as the carrier gas. Quantification was accomplished with an Infotronics model CRS-108 digital integrator using heptadecanoic acid as the internal standard. Results for all lipid determinations were corrected for loss by recovery of the cholesterol-4-^{14}C internal standard. For this, an aliquot of the cholesterol fraction was dissolved in 10 ml of a solution of 6 g of 2,5-diphenyloxazole per liter of toluene and counted to a 2-sigma error of $<2\%$ using a Beckman LS-230 liquid scintillation counter.

The lipid free residue remaining after extraction with chloroform and methanol was dried in an oven at 85°C to constant weight for determination of lipid free dry weight. We then extracted this lipid free residue for four days with four changes of 2 ml each of 0.05 *N* hydrochloric acid. Preliminary studies indicated that this procedure extracted greater than 95% of the calcium

in the residue. An aliquot of this hydrochloric acid extract was mixed with an equal volume of 0.5% $LaCl_3$ and calcium was determined using a Jarrell-Ash atomic absorption spectrophotometer (Model 528, Waltham, Massachusetts).

Results were analyzed by a one-way analysis of variance after conversion of the data to the $\log_{10}$. We then tested the various groups for significant differences, using Duncan's Multiple Range Test (12).

Results. On the average, 43.4% of the intimal surface of the aortas from cholesterol-fed birds was covered with grossly visible atherosclerotic lesions. This ranged from aortas having no grossly visible atherosclerosis to others with 100% involvement.

Birds with naturally occurring atherosclerosis averaged 9.1% of the intimal surface covered with grossly visible lesions with a range of 1.0%–38.4%. There were no statistically significant differences in extent of naturally-occurring aortic atherosclerosis in birds ranging in age from 6–12 yr. Consequently, we have combined the analytical data from all birds with naturally occurring atherosclerosis.

The lipid composition of normal, fatty

TABLE I. Lipid Composition of Aortas from White Carneau Pigeons with Cholesterol-Aggravated or Naturally Occurring Atherosclerosis.

Tissue	Phospholipid	Cholesterol	Free fatty acids[a]	Triglycerides	Cholesteryl ester
	(Results as mg/g wet weight)				
Control					
1. Normal	6.02 ± 0.543[b]	0.974 ± 0.07	0.316 ± 0.058	2.72 ± 0.40	0.186 ± 0.06
N[c]	5	5	5	5	4
Cholesterol-aggravated					
2. Normal	5.68 ± 1.1	1.08 ± 0.10	0.290 ± 0.071	1.94 ± 0.36	0.305 ± 0.02
N	4	4	4	4	3
3. Fatty streak	12.3 ± 2.2	6.45 ± 1.8	1.174 ± 0.371	4.20 ± 1.37	6.30 ± 1.5
N	4	4	4	4	3
4. Plaque	18.7 ± 2.3	19.7 ± 2.2	1.510 ± 0.437	6.74 ± 0.59	31.8 ± 5.6
N	4	4	4	4	4
Naturally occurring					
5. Normal	4.22 ± 0.27	1.64 ± 0.135	0.292 ± 0.015	2.12 ± 0.28	0.493 ± 0.02
N	17	17	5	17	16
6. Plaque	10.2 ± 0.93	26.8 ± 5.0	1.000 ± 0.373	4.28 ± 0.51	18.4 ± 2.8
N	16	17	5	17	17
Statistical significance[d]					
1 vs 2	NS	NS	NS	NS	S
1 vs 5	S	NS	NS	NS	S
2 vs 3	S	S	S	S	S
2 vs 4	S	S	S	S	S
2 vs 5	NS	NS	NS	NS	NS
3 vs 4	S	S	NS	NS	S
5 vs 6	S	S	S	S	S
4 vs 6	S	NS	NS	S	S

[a] Represents an average of 94% of free fatty acids in arterial tissues.

[b] Mean ± standard error of the mean.

[c] *N* = number of determinations on homogenates of pools of pigeon aortas. Each pool was composed of tissue from 3–10 aortas.

[d] NS = not significant at $P < 0.05$; S = significant at $P < 0.05$.

TABLE II. Lipid Composition of Aortas from White Carneau Pigeons with Cholesterol-Aggravated or Naturally Occurring Atherosclerosis.

Tissue	Phospholipid	Cholesterol	Free fatty acids[a]	Triglycerides	Cholesteryl ester
	(Results as mg/g lipid free dry weight)				
Control					
1. Normal	31.2 ± 2.8[b]	5.06 ± 0.45	1.678 ± 0.214	13.97 ± 1.91	0.997 ± 0.36
N[c]	5	5	5	5	4
Cholesterol-aggravated					
2. Normal	28.4 ± 5.5	5.44 ± 0.61	1.798 ± 0.381	10.45 ± 1.93	1.70 ± 0.07
N	4	4	4	4	3
3. Fatty streak	54.6 ± 6.1	28.2 ± 5.8	5.558 ± 2.06	19.34 ± 5.76	31.1 ± 8.3
N	4	4	4	4	3
4. Plaque	93.0 ± 3.9	102.0 ± 16.0	7.883 ± 2.41	32.91 ± 3.64	166.2 ± 35.0
N	4	4	4	4	4
Naturally occurring					
5. Normal	19.6 ± 1.8	6.72 ± 0.49	1.556 ± 0.175	10.18 ± 1.49	2.10 ± 0.14
N	15	15	5	15	14
6. Plaque	46.8 ± 5.3	94.4 ± 17.0	5.631 ± 2.571	21.00 ± 3.53	81.0 ± 14.0
N	13	13	5	13	13
Statistical significance[d]					
1 vs 2	NS	NS	NS	NS	S
1 vs 5	S	NS	NS	S	S
2 vs 3	S	S	S	NS	S
2 vs 4	S	S	S	S	S
2 vs 5	S	NS	NS	S	NS
3 vs 4	S	S	NS	NS	S
5 vs 6	S	S	S	S	S
4 vs 6	S	NS	NS	S	S

[a] Represents average of 94% of the total free fatty acids in arterial tissues.

[b] Mean ± standard error of the mean.

[c] *N* = number of determinations on homogenates of pools of pigeon aorta. Each pool was composed of tissue from 3–10 aortas.

[d] NS = no significance at $P < 0.05$; S = significant at $P < 0.05$.

streak, and plaque tissue from aortas of pigeons with either cholesterol-aggravated or naturally occurring atherosclerosis is shown in Tables I and II. The results in Table I are presented as mg per gram wet weight, while those in Table II are expressed as mg per gram lipid free dry weight. Generally speaking, the conclusions from these two tables are the same. Where there are differences in statistical significance, these values represented results that were borderline and happened to fall on opposite sides of the statistical limit of significance chosen here as $P < 0.05$.

The lipid free dried residue accounted for from 19.1 to 23.9% of the wet weight of the tissue and was not significantly different among any of the tissues studied.

The concentration of all lipid classes was greater in fatty streaks compared with normal tissue from aortas of pigeons with cholesterol-aggravated atherosclerosis. There was a further increase in the content of all lipid classes in plaques from cholesterol-fed birds although this increase was not statistically significant for free fatty acids or triglycerides. All lipid classes were significantly increased in plaques from birds with

naturally occurring atherosclerosis compared with normal tissue from the same aortas.

Although plaque tissue from both lesion types contained increased concentrations of all lipid classes, this increase was not of equal magnitude in the two types of plaques. Based on both wet weight and lipid free dry weight, there was relatively less phospholipid, triglyceride, and cholesteryl ester in plaques from pigeons with the naturally occurring disease compared to those with cholesterol-aggravated atherosclerosis. There was no significant difference, however, in the free cholesterol or free fatty acid concentration of these two types of plaques.

Phospholipids comprised the single largest class of lipids in normal and fatty streak tissue, while cholesteryl esters made up the largest lipid class in atherosclerotic plaques. While phospholipids, triglycerides, and free fatty acids increased in concentration 2–4 fold in plaque tissue, the free cholesterol content increased 25-fold and cholesteryl esters, up to 170-fold. The cholesteryl ester concentration in plaques from birds with cholesterol-aggravated atherosclerosis was about twice as large as the cholesteryl ester content of plaques from birds with the naturally occurring disease. As mentioned above, there was no such difference for free cholesterol.

Normal tissue from aortas of pigeons with cholesterol-aggravated or naturally occurring atherosclerosis contained significantly more cholesteryl ester than the nonatherosclerotic control aortas. Normal tissue from aortas with naturally occurring atherosclerosis differed from normal tissue from both control and cholesterol-fed birds by containing significantly less phospholipid.

Table III shows the calcium content of aortic tissues from pigeons with cholesterol-

TABLE III. Calcium Content of Aortas from White Carneau Pigeons with Cholesterol-Aggravated or Naturally Occurring Atherosclerosis.

Tissue	Wet Weight (Results as mg/g)	Lipid free dry weight (Results as mg/g)
Control		
1. Normal	0.133 ± 0.01[a]	0.714 ± 0.05
Cholesterol-aggravated		
2. Normal	0.133 ± 0.08	0.680 ± 0.04
3. Fatty streak	0.244 ± 0.03	1.03 ± 0.09
4. Plaque	0.307 ± 0.06	1.55 ± 0.28
Naturally occurring		
5. Normal	0.144 ± 0.01	0.743 ± 0.02
6. Plaque	1.56 ± 0.72	7.68 ± 3.6
Statistical significance[b]		
1 vs 2	NS	NS
1 vs 5	NS	NS
2 vs 3	NS	NS
2 vs 4	S	S
2 vs 5	NS	NS
3 vs 4	NS	NS
5 vs 6	S	S
4 vs 6	S	S

[a] Mean ± standard error of the mean. Results are the mean of 5 determinations on homogenates of pools of pigeon aortas. Each pool was composed of tissue from 10 aortas of birds with naturally occurring or cholesterol-aggravated atherosclerosis and 3 aortas from control birds.

[b] NS = not significant at $P < 0.05$; S = significant $P < 0.05$.

aggravated or naturally occurring atherosclerosis. No significant differences were seen among any of the normal tissues. In birds with cholesterol-aggravated atherosclerosis, there was a progressive increase in the calcium content of fatty streak and plaque tissue. The calcium content of plaques from birds with naturally occurring atherosclerosis was approximately ten times greater than normal tissue from the same birds and was significantly greater than plaques from birds with the cholesterol-aggravated disease. A similar correlation between calcium and lipid content has been observed morphologically in White Carneau pigeons with naturally occurring atherosclerosis (7). In naturally-occurring lesions, calcium accounted for, at the most, only about 0.2% of the plaque wet weight and less than 1.0% of the lipid free dry weight of the plaque.

Discussion. The results from this study confirm that biochemical as well as morphological differences exist between naturally occurring and cholesterol-aggravated atherosclerotic lesions of pigeons. Although the total lipid content of the cholesterol-aggravated plaques was only slightly greater than that of the naturally-occurring plaques (78 compared with 61 mg/g wet weight, respectively), there was a major difference in the proportion of the individual lipids in these two types of plaques. There was an increase in the concentration of all lipid classes in plaques, but this increase was greatest for cholesterol and cholesteryl esters. The free cholesterol concentration was similar for both types of atherosclerotic plaques and was some 16–18 times higher in concentration than in normal tissue. On the other hand, there was greater than a 100-fold increase in the cholesteryl ester content in the cholesterol-aggravated plaques compared with a 37-fold increase in the naturally occurring plaques.

This greater amount of cholesteryl ester in the plaques from cholesterol-fed birds is consistent with the predominance of "foam cell" containing lesions seen in these aortas. Similar findings have been reported for lesions containing "fat-filled" cells in human beings (13, 14). In these lesions in human beings, the major cholesteryl ester is cholesteryl oleate, which is also the major cholesteryl ester of atherosclerotic plaques from cholesterol-fed pigeons (15).

Although we have no absolute measure of the origin of this high content of cholesteryl oleate, there is suggestive evidence that a significant proportion may come from esterification (15), probably by the "foam cell" itself (16). Significant amounts of cholesteryl esters, however, must also come from influx from the blood (17) although which of these processes, increased influx or increased synthesis, occurs first is not clear. This is emphasized by the observation that in the pigeon aorta, biochemical differences between susceptible and resistant areas of the same aorta can be detected in normal-appearing aortas (18). Cholesterol esterification is, however, one of the earliest biochemical events to be stimulated in aortas from pigeons fed cholesterol for short periods of time (3).

Also apparent from this study is the fact that normal-appearing arterial tissue from pigeons with either naturally occurring or cholesterol-aggravated atherosclerosis is not normal chemically. This is seen principally in the significantly greater content of cholesteryl esters in normal aorta from pigeons with cholesterol-aggravated or naturally occurring atherosclerosis. Insull and Bartsch (19) have also reported that normal-appearing intima from human aorta can accumulate cholesterol before grossly visible lesions appear. These workers observed that grossly visible fatty streaks appeared only after the total cholesterol content of the intima had exceeded about 10 mg/g wet weight. In the present study, fatty streaks had an average total cholesterol content of 12.7 mg/g wet weight, which is surprisingly similar to the above described threshold for fatty streaks in human aorta.

Whenever lipid composition data on arterial tissue are presented, one is faced with the problem of how best to express the results. Differences in the cellularity of the tissue or in other components such as connective tissue, glycosaminoglycans, water, minerals, etc., can all produce apparent changes in lipid composition when data are

presented as per mg wet weight or as lipid free dry weight. Although differences in lipid composition may have resulted in this study due to changes in some of these factors, these differences were probably relatively small. Differences between normal and diseased tissue in such factors as wet weight, calcium content, or as determined in a previous study, DNA content (20), would not be expected to alter the wet weight or lipid free dry weight by more than a few percent. Likewise, the most consistent difference observed in this study was a change in the proportion of cholesteryl esters to other lipid components and such a change in the proportion of cholesteryl esters to other lipid components would not be altered by the basis on which the data are expressed.

Summary. The content of free and esterified cholesterol, phospholipid, free fatty acid, triglyceride, and calcium of normal, fatty streak, and atherosclerotic plaque was determined in aortas from pigeons with cholesterol-aggravated or naturally occurring atherosclerosis. The concentration of all lipid classes was greater in fatty streak and plaque than in normal tissue. Normal tissue from pigeons with atherosclerosis, although having no grossly visible lesions, had a higher concentration of cholesteryl esters than aortic tissue from pigeons without atherosclerosis. Atherosclerotic plaques from pigeons with cholesterol-aggravated atherosclerosis contained about twice the concentration of cholesteryl esters as plaques from birds with naturally occurring atherosclerosis, even though there was no difference in the content of free cholesterol. Calcium content was greatest in plaques, particularly in the naturally-occurring disease, but never accounted for more than 1% of the lipid free dry weight of the aorta.

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