The Polyunsaturated Fatty Acid and Cholesterol Concentrations of Plasma and Aorta and Their Relationship to Avian Atherosclerosis[1]

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Growing cockerels were fed diets varying in level of protein and cholesterol and in the type of fat. Their plasma and aortas were examined for cholesterol, polyunsaturated fatty acids, and atherosclerotic involvement.

1. The cholesterol levels of plasma and aorta were increased by low-protein diets and by added dietary cholesterol, singly or in combination.
2. There appeared to be an inverse relationship between cholesterol and polyunsaturated fatty acid levels in both the plasma and aorta.
3. It was again confirmed that atherosclerotic involvement is greatest in the abdominal section of the avian aorta.
4. There was no clear intergroup relationship between polyunsaturated fatty acid levels or cholesterol concentration of the blood and the aorta and atherosclerotic involvement.
5. The birds receiving the combination of coconut oil and cholesterol showed a clear trend towards greater atherosclerotic involvement than those fed corn oil.

THE TYPE of dietary fat has been prominently implicated in the regulation of blood cholesterol (1–3). Underlying this interest in the circulating cholesterol level is the possibility that atherogenesis and cholesterol deposition in the blood vessels may be controlled by regulating the blood lipids. It has also been suggested (4) that atherogenesis may be associated with a relative, essential fatty acid deficiency.

In several studies we have attempted to clarify these and other hypotheses (5–9). In working with spontaneous atherosclerosis of hens in feeding trials of one to three years' duration, it was shown that atherosclerotic plaques occurred with considerable frequency in the abdominal aorta of animals that had been fed corn oil (5,6). These plaques contained high amounts of linoleic acid, much higher than are found in unaffected aortic tissues of hens not fed corn oil (8). In these same studies, in which the degree of dietary fat saturation was the major variable, little relationship could be established among differently-fed groups between either plasma cholesterol or aortic cholesterol level and aortic atherosclerosis (5,6).

The present study was undertaken to extend the previous observations, which were concerned with spontaneous atherosclerosis, to the dietary cholesterol-induced type. Degree of fat saturation (corn *versus* coconut oil), protein level, and cholesterol addition were the dietary variables. The major objectives were to study the relationship of blood and aorta cholesterol and polyunsaturated fatty acid levels to the extent of atherosclerotic involvement.

Methods

Day-old male chicks (Columbian x New Hampshire) were fed a practical starting ration for two weeks and then were assigned to the experimental rations for a subsequent 20-week experimental period. The composition of the various diets is given in Table I. In the experimental design different levels of protein and cholesterol and two different fats representing two levels of unsaturated fatty acids were considered. The cholesterol concentrations differed for each of the two protein levels and were chosen on the basis of the results reported by Fisher *et al.* (3).

TABLE I
Composition of Rations

Basal ration

Ingredient	Amount
	%
Soybean meal (50% protein)	20.00
Alfalfa	2.50
Dicalcium phosphate	2.50
Mico concentrate[a]	1.00
Salt	.50
B vitamin mix[b]	.10
Choline chloride	.20
A, D, and E mix[b]	.10
DL-methionine[c]	.05
Glucose (cerelose)	to 100.00

Individual experimental rations

Dietary variable[d]	Lot							
	1	2	3	4	5	6	7	8
	%							
Protein[e]	10	25	10	25	10	25	10	25
Fat—Coconut	5	5	5	5	....	....	....	....
Corn oil	....	....	....	....	5	5	5	5
Cholesterol	....	....	0.3	2	....	....	0.3	2

[a] Trace mineral mixture, containing limestone as the base, product of Limestone Corporation of America, Newton, N.J.

[b] For composition see Fisher and Johnson (10).

[c] The methionine level was adjusted proportionally when the amount of soybean meal was increased to yield a 25% protein level.

[d] Replaced an equal amount of glucose in the basal rations.

[e] Soybean meal (50% protein) was the protein source used.

The birds were separated into lots of 16 per treatment group. At the end of five weeks and again at 10 weeks blood was drawn from the heart (from 4 birds per lot) for plasma cholesterol and fatty acid analysis. At the end of 20 weeks blood samples were taken from all remaining birds. At each sampling period the birds were killed after bleeding, and their aortas were removed. The aorta was thoroughly cleaned from the heart to the iliac bifurcation and visually scored by one of us (H.S.W.), according to procedures previously discussed (7). This method of scoring gives greater weight to the height and area of lesions protruding into the lumen than it does to the appearance of gross lipid deposition. After scoring, the aortas were separated into thoracic and abdominal sections, and each of these was placed in a separate glass bottle with Teflon gasketed metal cap to be cold-extracted with chloroform-methanol (2:1) for 24 hrs. in a shaker. The extract was filtered, and made up to volume; aliquots were taken for cholesterol analysis by

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TABLE II

Effect of Dietary Fat, Cholesterol and Protein Level on Plasma Lipid Constituents

Dietary Variables			Plasma Values					
			Cholesterol			Fatty Acids		
Fat	Protein	Cholesterol	5 wk.	10 wk.	20 wk.	Dienoic	Tetraenoic	Other [a]
%	%	%	*mg. %*	*mg. %*	*mg. %*	*% of fat*	*% of fat*	*% of fat*
Coconut, 5	10	0	292 ± 23	251 ± 16	167 ± 18	4.3	0.9	1.9
Coconut, 5	25	0	178 ± 7	171 ± 7	172 ± 7	6.0	1.4	3.2
Coconut, 5	10	.3	1141 ± 123	1254 ± 71	700 ± 14	3.4	0.6	1.3
Coconut, 5	25	2	523 ± 93	350 ± 25	283 ± 32	6.6	1.3	3.1
Corn oil,5	10	0	267 ± 35	232 ± 6	279 ± 15	11.4	2.9	2.0
Corn oil,5	25	0	178 ± 11	153 ± 7	223 ± 23	12.0	2.6	2.6
Corn oil,5	10	.3	991 ± 138	1590 ± 188	2118 ± 107	9.1	0.9	0.9
Corn oil,5	25	2	314 ± 31	727 ± 78	344 ± 68	10.9	1.6	1.2

Pooled values for tri-, penta-, and hexaenoic acids.

the modified method of Zlatkis *et al.* (11) and for polyunsaturated fatty acid analysis, according to the procedure of Luddy *et al.* (12). The cholesterol analyses were made on individual aortic and plasma samples. Duplicate polyunsaturated fatty acid analyses were made on the pooled aortic fat extracts from each treatment group. The remaining plasma for each lot was also pooled, and 1-ml. aliquots were pipetted into individual test tubes. These samples were dried by lyophilization and stored in the cold for later analysis of polyunsaturated fatty acids. Duplicate determinations were made by using the procedure of Morris *et al.* (13).

Results

Plasma. The results of the plasma analyses are shown in Table II. Only the cholesterol values are given in complete detail for each of the three sampling periods. For greater clarity the presentation of the polyunsaturated fatty acid data has been restricted to the di- and tetraenoic acids with the tri-, penta-, and hexaenoic acids pooled together. For further clarification purposes, since there were no discernible differences between sampling periods, an average value for all three periods were computed.

Comparison of the effect of dietary fat on the polyunsaturated fatty acids of the plasma showed, as expected on the basis of the dietary fat composition, a lower dienoic and tetraenoic acid content for those birds receiving the coconut oil than for those receiving the corn oil. Within the coconut oil fed-groups those on the low-protein level generally had less of the various polyunsaturated fatty acids in the plasma than the groups receiving the higher protein diets. Adding cholesterol to the low-protein diets resulted in a further decrease of polyunsaturated fatty acid levels. In the corn oil-fed groups the feedings of low-protein diets generally resulted in lower polyunsaturated fatty acid levels as compared to the high-protein diets. The addition of cholesterol to the diet also accounted for a general decrease in fatty acid levels in comparison to the respective groups not getting cholesterol.

Irrespective of the type of dietary fat, the plasma cholesterol level was elevated on the low-protein diets as compared to the high-protein diets, and this elevation was further accentuated when cholesterol was included in the diet. At the first sampling period (5 weeks) the plasma cholesterol levels of the coconut oil-fed birds were higher than the levels for the respective corn-oil fed groups. At the final sampling period (20 weeks) the levels for the coconut oil-fed groups had decreased and those for the corn oil-fed groups had increased so that at this time the levels for the corn oil-fed groups were actually higher than the levels for the respective coconut oil-fed groups.

Thoracic Aorta. The results of the analysis on the aortic segments are presented in Tables III and IV. As for the plasma values, the polyunsaturated fatty acids have been averaged for all three periods. The di- and tetraenoic acids are presented separately while the tri-, penta-, and hexaenoic acids have been combined into one pooled value. The visual scores were also averaged for the three sampling periods since there was excellent agreement between periods.

In the thoracic section of the aorta the coconut oil-fed groups had lower di- and tetraenoic acid levels than the corn oil-fed groups while there were no appreciable differences in the tri-, penta-, and hexaenoic acid fractions. With but one exception and irrespective of other dietary variations, the low protein-fed groups showed lower levels of the polyunsaturated fatty acids. While either low-protein or cholesterol-supplemented diets produced variable effects on the dienoic acid level, the combination of dietary cholesterol and low protein invariably resulted in lower dienoic acid concentrations.

TABLE III

Effect of Dietary Fat, Cholesterol, and Protein Level on Atherogenesis and Lipid Components of the Thoracic Aorta

Dietary Variables			Thoracic Aorta Values						
			Cholesterol—Dry defatted tissue				Fatty Acids		
Fat	Protein	Cholesterol	5 wk.	10 wk.	20 wk.	Score	Dienoic	Tetraenoic	Other [a]
%	%	%	*mg./g.*	*mg./g.*	*mg./g.*		*% of fat*	*% of fat*	*% of fat*
Coconut, 5	10	0	15.4 ± 1.1	16.8 ± 1.0	10.2 ± 0.3	1.1	4.8	2.1	2.8
Coconut, 5	25	0	22.5 ± 12.4	17.6 ± 4.8	8.5 ± 1.7	1.0	4.2	4.8	3.5
Coconut, 5	10	.3	23.3 ± 8.4	44.3 ± 8.5	28.4 ± 4.7	1.2	3.5	2.3	2.8
Coconut, 5	25	2	13.8 ± 3.2	16.6 ± 1.5	9.3 ± 2.2	1.1	8.6	4.1	1.2
Corn oil, 5	10	0	14.8 ± 2.1	13.8 ± 0.3	10.2 ± 0.8	1.1	8.6	5.4	2.8
Corn oil, 5	25	0	14.1 ± 0.5	13.6 ± 1.0	9.8 ± 0.7	1.1	9.0	6.4	3.1
Corn oil, 5	10	.3	18.8 ± 2.4	48.0 ± 7.7	41.1 ± 10.2	1.1	8.0	2.9	2.4
Corn oil, 5	25	2	17.5 ± 0.9	20.4 ± 0.9	16.3 ± 2.6	1.1	13.2	5.4	3.1

[a] Pooled values for tri-, penta-, and hexaenoic acids.

TABLE IV

Effect of Dietary Fat, Cholesterol, and Protein Level on Atherogenesis and Lipid Components of the Abdominal Aorta

Dietary Variables			Abdominal Aorta Values						
Fat	Protein	Cholesterol	Cholesterol—Dry defatted tissue			Score	Fatty Acids		
			5 wk.	10 wk.	20 wk.		Dienoic	Tetraenoic	Other[a]
%	%	%	mg./g.	mg./g.	mg./g.		% of fat	% of fat	% of fat
Coconut, 5	10	0	20.9 ± 2.1	23.7 ± 2.8	15.6 ± 2.2	1.4	4.7	2.0	2.3
Coconut, 5	25	0	32.3 ± 8.7	16.0 ± 1.2	12.7 ± 2.0	1.6	6.5	2.7	2.7
Coconut, 5	10	.3	26.2 ± 2.4	37.3 ± 4.1	46.6 ± 7.3	1.7	3.9	1.2	2.3
Coconut, 5	25	2	17.8 ± 1.4	23.9 ± 4.0	18.4 ± 2.0	1.8	8.9	1.8	3.1
Corn oil, 5	10	0	18.7 ± 1.4	19.6 ± 0.9	13.3 ± 1.4	1.4	13.6	1.8	2.6
Corn oil, 5	25	0	23.6 ± 6.0	19.3 ± 0.5	12.9 ± 1.7	1.7	18.5	2.5	2.6
Corn oil, 5	10	.3	30.7 ± 5.0	30.4 ± 2.6	72.2 ± 24.1	1.5	10.0	1.3	1.9
Corn oil, 5	25	2	22.9 ± 2.4	32.2 ± 6.0	26.2 ± 8.1	1.6	19.3	2.2	2.3

[a] Pooled values for tri-, penta-, and hexaenoic acids.

The cholesterol content of the thoracic section of the aortas decreased from the first to the third sampling period except for the groups receiving the low-protein diets which contained cholesterol. While the coconut oil-fed groups had somewhat higher cholesterol levels at the first sampling period, these were generally slightly lower than those of the corn-oil fed groups at the last sampling period. Regardless of the type of fat, the low-protein groups and those groups with added cholesterol had higher cholesterol levels than the groups receiving high-protein diets or diets with no added cholesterol, respectively, As with plasma the di- and tetraenoic acids of thoracic fat were inversely related to the cholesterol concentration of thoracic fat.

There were no differences in the thoracic visual scores among any of the treatment groups. As in previous studies, no appreciable proliferative atherosclerotic lesions were discernible in the thoracic section of the aorta (5,7,9).

Abdominal Aorta. In comparing the effect of dietary coconut and corn oil on the polyunsaturated fatty acids of the abdominal section of the aorta, it was observed that the dienoic acid levels in the abdominal aortic fat of the coconut oil-fed birds were lower but that the other unsaturated fatty acids were unaffected by the type of dietary fat. The low-protein diets produced lower di- and tetraenoic acid levels than the higher-protein diets regardless of the type of fat. In the corn oil-fed groups the addition of cholesterol resulted in lower tetraenoic and slightly lower values for the pooled tri-, penta-, and hexaenoic acids. As was true for both plasma and thoracic fat, the di- and tetraenoic acids were invariably lowest on the combination of low protein-added cholesterol.

It seems in order at this point to compare briefly the relative amounts of the fatty acids in plasma and thoracic and abdominal aortic fat. The greatest discernible difference appears in the tetraenoic acid fraction of the fat from the thoracic section of the aorta, which is more than twice as high as the comparable values for plasma and abdominal aorta. On the other hand, the relative dienoic acid concentration of plasma and abdominal aorta fat is higher than that of thoracic aorta fat. While it seemed more appropriate to present and compare the relative composition of the tissue and plasma fat, calculation of the absolute amounts of dienoic acid per gram of tissue or ml. of plasma gave essentially the same pattern.[2]

Consistent with previous studies, the cholesterol content of the abdominal section of the aorta was greater than that of the thoracic section (5,6,7,9). It showed a decrease between the first and last sampling periods when no cholesterol was added to the diet but increased when cholesterol was present in the diet. Dietary fat did not appear to influence the cholesterol content of the abdominal aorta in any particular direction. The most significant dietary effect was exerted by the low-protein diets and by the combination of low protein-added cholesterol which were responsible for higher tissue-cholesterol levels than were observed on any of the other diets. In agreement with the observation on plasma and thoracic aortic fat the di- and tetraenoic acids of the abdominal aortic fat are generally lowest on dietary combinations resulting in the highest cholesterol levels.

Visual score for the abdominal region of the aorta was consistently greater than the score for the thoracic segment for all groups. Neither dietary cholesterol nor level of protein appeared to affect the score of the groups fed corn oil despite highly significant differences in the cholesterol content of abdominal aortic fat. On the other hand, birds receiving the combination of coconut oil and cholesterol showed a clear trend towards greater atherosclerotic involvement.

Discussion

The present studies on dietary-cholesterol-induced atherosclerosis agree with and extend previous observations on spontaneous atherosclerosis of chickens (5,6,8,9). In confirmation of several recent reports (17,18,19,3) dietary protein appeared to exert the greatest single effect of the dietary variables tested upon the various measurements taken.

The most important observation recorded in the present investigation was the lack of relationship between plasma or aortic cholesterol concentration and the degree of atherosclerotic involvement. This is particularly true for the corn oil-fed groups, which had higher plasma and aortic cholesterol concentrations but generally lower scores than the coconut oil-fed birds. Since this observation is entirely consistent with previous findings from this laboratory (6), it raises the question whether, on corn oil diets, sterols other than cholesterol are being measured. The high cholesterol values might conceivably also result from interference from the relatively high dienoic acid concentration of plasma or aortic fat, as recently suggested by Kahn and Yacowitz (14).

Although among the cholesterol-fed groups those receiving corn *versus* coconut oil generally had a lower incidence of atherosclerosis. Sinclair's suggestion (4) of a relative, essential fatty acid deficiency as the cause of atherogenesis is not fully supported by the

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[2] This was of particular interest since some of the hypercholesteremic plasmas were also lipemic so that the total quantity of fat differed between samples.

present data. Thus the birds fed coconut oil and no cholesterol had essentially the same visual score as any of the four groups fed corn oil despite the fact that the latter groups had at least twice the dienoic acid concentration of the aorta as found in the former groups. This does not rule out the possibility that the type of fatty acid does play a role in plaque formation, as suggested by the higher scores but low essential fatty acid intake of the birds fed coconut oil and cholesterol.

The striking similarity of relationships between cholesterol level and polyunsaturated fatty acid content of plasma and each of the aortic segments supports the suggestion of Hirsch and Nailor (15) that aortic lipids reflect passive deposition from plasma. Close inspection of the comparative fatty acid analyses indicate however considerable differences between plasma and aortic fat, the interpretation of which must await further investigation.

The data in Table II show that dietary coconut oil, at 5 weeks, resulted in higher plasma cholesterol values than did dietary corn oil (16). After 5 weeks the cholesterol levels decreased on the coconut oil diets and increased on the corn oil diets. At 20 weeks corn oil-fed groups thus had the higher plasma cholesterol levels. While there is no ready explanation for the steady increase with time in plasma cholesterol for the corn oil groups, the decrease observed on the low protein-coconut oil diets is very likely caused by the increasingly greater ability of the low-protein level to satisfy the protein needs of the older animal. Whereas 10% protein satisfies no more than half the protein needs of a young chick, this amount is fully adequate to meet the requirements of an essentially mature bird at 20 weeks of age. Whatever the explanation for these findings however, they do point up the danger of evaluating short experiments in terms of dietary variables on atherogenesis, a process which presumably continues over the entire life-span of the animal.

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Glyceride Structure of Vegetable Oils by Countercurrent Distribution. V. Comparison of Natural, Interesterified, and Synthetic Cocoa Butter[1]

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The glyceride structure of cocoa butter is of considerable practical importance and of particular theoretical interest. Countercurrent distribution, gas chromatography, and isotopic dilution methods are employed in its study. The observed fractionation of glycerides is accounted for by assuming that palmitic and stearic acids are randomly esterified on the 1 and 3 positions of glycerol and that oleic is on the 2 position, as demonstrated by other workers. Complete randomization of the specific structure of cocoa butter through the application of interesterification catalysts greatly alters its physical properties, including its countercurrent distribution pattern. A glyceride synthesized according to the "1,3 random palmitostearo-2-olein" concept has properties similar to natural cocoa butter.

A CONFECTIONERY coating fat with properties equal or superior to cocoa butter could provide a new, large market for domestic fats and vegetable oils. Two necessary steps are the learning of the glyceride structure of natural cocoa butter and the synthesizing of a fat of equivalent structure on a laboratory scale.

Fundamental interest in the glyceride structure of cocoa butter stems from a nonrandom distribution of palmitic, stearic, oleic, and linoleic fatty acids among its glyceride molecules (1,2). Recent and current work, which exploits the high-resolving power of countercurrent distribution for fractionating glycerides, has shown that constitutionally different glycerides in linseed (3), soybean (4,5), corn (6), and safflower (7) oils agree with a random pattern. These results are contrary to those obtained by Hilditch

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[2] This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.