Lipid Patterns and Atherogenesis in Cholesterol-Fed Chickens. (23576)

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It is generally believed that there is a connection between blood lipids and atherosclerosis. This has led to a search for an analytical indication of tendency toward the disease that could be applied early enough in life for corrective action. The serum cholesterol concentration, the ratio of cholesterol to phospholipid(1-4), the distribution of cholesterol among the β-lipoproteins(5) and the relative amounts of cholesterol in the α- and β-lipoproteins(6-8) have all been considered with this in mind. In experimental atherosclerosis in laboratory animals the degree and character of lesions can be compared directly with analytical results. Such work in rabbits(9-11) and in chickens(12-14) has shown that correlations may vary with the experimental treatment. For example, alloxan injection in rabbits resulted in reduced aortic atheromatosis accompanied by decreased blood cholesterol/phospholipid ratio; while in chickens, administration of estrogen decreased the blood cholesterol/phospholipid ratio, but did not reduce aortic atheromatosis.

This report presents results of a study of experimental atherosclerosis produced by feeding cholesterol to a relatively large number of chickens. The degree of atheromatosis in thoracic aortas and brachiocephalic arteries has been correlated with results of analyses for total cholesterol and lipid phosphorus in plasma and for cholesterol in lipoprotein fractions.

Methods. 1) Chickens used were set out at intervals of a few weeks in 20 experiments conducted during a continuous period of 18 months starting in April 1954. They were Kerr White Leghorn cockerels, received in the laboratory at 1 or 2 days of age and housed in wire batteries. For the first 8 weeks they were fed the starter ration described in Table I. From 8 to 16 weeks of age they were fed an atherogenic diet made by replacing 7 g of corn meal in the starter ration with 2 g of USP cholesterol and 5 g of destearinated cottonseed oil. At 16 weeks of age they were fasted overnight, bled and sacrificed. 2) From the alar vein of each chicken 4 ml of whole blood were drawn and mixed with 0.7 ml of citric acid-sodium citrate-dextrose (ACD) solution (15). This yielded enough plasma for analytical procedures which follow. *Plasma fractionation.* Plasma fractionations, started the day of bleeding for all samples, were carried out on 1 ml plasma aliquots by Cohn's Method 10 as described by Lever *et al.*(16). *Cholesterol* was determined by the method of Abell, Levy, Brodie, and Kendall(17), modified by increasing the strength of alcoholic KOH solution to dissolve precipitates from plasma fractionation more easily. Plasma fractions in their centrifuge tubes, or 0.5 ml of original plasma, were dissolved in 1 ml of 33% KOH (W/W) plus 4 ml of absolute alcohol. The tubes were stoppered and placed in an incubator at 37° overnight. In the morning the mixtures were transferred to 1 oz. polyethylene bottles with 5 ml of water. Ten ml of petroleum ether were added, and the bottles were tightly closed with polyethylene screw

TABLE I. Composition of Basal Diet.

53.3 g	Yellow corn meal
30.0	Soybean meal (44% protein)
10.0	Fishmeal
2.0	Alfalfa meal
2.0	Steamed bone meal
1.5	Ground limestone
.5	Sodium chloride
.02	Manganese sulfate
.4	Choline chloride dry mix (25% choline chloride)
.2	Viadex (4000 u A and 750 u D/g)
.05	Inositol
15.0 mg	p-Aminobenzoic acid
2.0	Niacin
1.5	Calcium pantothenate
.5	Pyridoxine
.5	Riboflavin
.25	Thiamine
50.0 μg	Menadione
12.5	Biotin
5.0	Vit. B_{12}

TABLE II. Grading of Lesions in Aortas and Brachiocephalic Arteries.

Grade	Appearance
	Series 1
1/4	Single small discrete plaques
1/2-1	Large single lesions
2	Multiple plaques, involving up to 1/4 of area
3	Multiple plaques, involving 1/4-1/2 of area
4	Multiple plaques, involving >1/2 of area
5	Multiple plaques, involving virtually entire surface area
	Series 2
1/2	One or 2 min discrete plaques
1	Scattered discrete plaques
2	(a) Heavy scattered "
	(b) Light uniform covering
3	Heavy uniform covering
4	Extremely heavy covering, large protruding masses, stiff wall

caps and shaken vigorously for 3 minutes. After clear phase separation suitable aliquots, usually 2 ml, of the petroleum ether were evaporated to dryness in test tubes and the amounts of cholesterol were determined with modified Liebermann-Burchard reagent(17). *Recoveries.* Analyses were carried out on single samples and recoveries were checked by comparing the sum of amounts of cholesterol in the fractions with the separately determined total cholesterol. The average recovery was 99.03%. The standard deviation for individual values was ± 7.1%. *Lipid Phosphorus* was determined by King's procedure(18) on 0.1 ml portions of plasma precipitated with trichloracetic acid as recommended by Zilversmit and Davis(19). *Grading of Lesions.* The chickens were sacrificed and the thoracic aortas and brachiocephalic arteries were removed. These were examined while fresh by 2 people working independently who graded them for degree of atheromatosis and then jointly reconciled any differences in their scores. Results from 2 series of experiments are presented here. These differ from one another in the individuals who did the scoring and in the grading systems used. Grading systems are presented in Table II. In the first series, which contained 157 birds from 11 different experiments, brachiocephalic arteries and thoracic aortas were graded separately. In the second series, which contained 98 birds from 9 experiments, brachiocephalic arteries and thoracic aortas were graded as a whole.

Results. The incidence and severity of atheromatosis observed are summarized in Table III. Plaques were found in 75% of birds examined; in 83% of the first series and 62% of the second. The results of differential examination in the first series indicated a somewhat higher incidence in brachiocephalic arteries than in aortas. Six percent of the birds had brachiocephalic lesions but no aortic lesions, but none had aortic lesions without having brachiocephalic lesions. The 2 series differed in distribution of birds with various assigned grades of involvement. In the first series 42% of aortic lesions were classified as Grade 2, whereas those in the second series were more evenly distributed between Grades 1, 2, and 3.

Figs. 1-3 present the raw individual com-

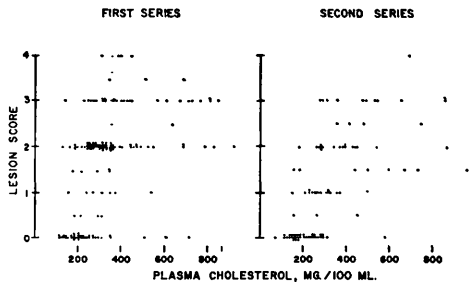

FIG. 1. Plot of plasma total cholesterol *vs* lesion score.

Table III. Distribution of Lesion scores (%)

Score	0	1/2	1	1-1/2	2	2-1/2	3	3-1/2	4
					Series 1 (157 Birds)				
Aorta	23.6	2.6	4.5	3.2	42.0	1.3	18.5	1.9	2.6
Brachiocephalics	17.2	3.2	3.8	4.5	31.2	.6	29.3	.6	9.6
					Series 2 (98 Birds)				
Aorta + Brachiocephalics	37.8	3.1	15.3	8.2	17.3	4.1	11.2	1.0	2.0

parisons of plasma cholesterol, $\frac{\alpha}{\alpha + \beta}$ cholesterol, and cholesterol/phospholipid ratio with lesion scores, the first series being illustrated only by the results from the thoracic aortas. Tables IV and V present correlation coefficients and regressions with their standard errors, calculated within groups, of lesion scores on all the various analytical results and ratios and their logarithms. Correlation coefficients of +1 or −1 indicate perfect association, either direct or inverse, of variables, whereas zero indicates no correlation. The listing under 100 r^2 in Table IV indicates the percentage of variation in lesion score that can be attributed to concomitant variation in corresponding lipid value.

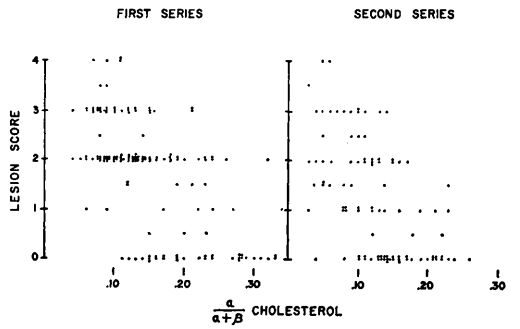

FIG. 2. Plot of plasma $\frac{\alpha}{\alpha + \beta}$ cholesterol *vs* lesion score.

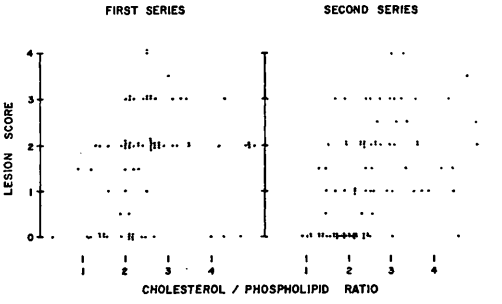

FIG. 3. Plot of plasma cholesterol/phospholipid ratio *vs* lesion score.

Discussion. In general birds with most severe atherosclerosis were also the ones with most extreme analytical values regardless of the scoring method used. The best correlations were with the logarithms of total cholesterol and β-lipoprotein cholesterol, essentially measures of the same thing in these birds. The atherosclerosis produced in chickens by

TABLE IV. Correlation Coefficients (r) of Lesion Scores on Plasma Lipid Concentrations and Ratios Expressed Arithmetically and Logarithmically.

	First series				Second series	
	Aorta score		Brachiocephalic score			
	r	(100 r^2)	r	(100 r^2)	r	(100 r^2)
Arithmetic:						
Total cholesterol, mg/100 ml	.47	(22)	.54	(29)	.55	(30)
α-Lipoprotein cholesterol, mg/100 ml	.04*	(0.1)	.09*	(0.9)	.35	(12)
β-Lipoprotein cholesterol, mg/100 ml	.50	(25)	.57	(32)	.54	(29)
$\frac{\alpha}{\alpha + \beta}$	−.57	(32)	−.58	(34)	−.57	(33)
Lipid phosphorus, mg/100 ml	.54	(29)	.57	(33)	.46	(21)
$\frac{\text{Cholesterol}}{\text{Phospholipid}}$	.29	(9)	.34	(11)	.61	(37)
Logarithmic:						
Total cholesterol, mg/100 ml	.57	(33)	.64	(41)	.65	(42)
α-Lipoprotein cholesterol, mg/100 ml	.07*	(0.5)	.13*	(1.7)	.35	(12)
β-Lipoprotein cholesterol, mg/100 ml	.60	(36)	.66	(44)	.64	(41)
$\frac{\alpha}{\alpha + \beta}$	−.49	(24)	−.51	(26)	−.29†	(8)
Lipid phosphorus, mg/100 ml	.51	(26)	.52	(27)	.54	(29)
$\frac{\text{Cholesterol}}{\text{Phospholipid}}$	.42	(17)	.41	(17)	.62	(38)

* Correlation not significant: $P > 0.1$.

† Correlation highly significant: $P = 0.005$. Correlation very highly significant in all other instances: $P < 0.001$.

TABLE V. Regression Coefficients (b), and Their Standard Errors, of Lesion Scores on Plasma Lipid Concentrations and Ratios Expressed Arithmetically and Logarithmically.

	First series		Second series
	Aorta score	Brachiocephalic score	
Arithmetic:	b ± S.E.		
Total cholesterol, mg/100 ml	.0028 ± .0005	.0034 ± .0004	.0024 ± .0004
α-Lipoprotein cholesterol, mg/100 ml	.0036 ± .0081*	.0095 ± .0084*	.0395 ± .0015†
β- " " , "	.0032 ± .0005	.0037 ± .0005	.0024 ± .0004
$\frac{\alpha}{\alpha + \beta}$	-8.6 ± 1.1	-9.2 ± 1.1	-11.1 ± 1.7
Lipid phosphorus, mg/100 ml	.43 ± .07	.46 ± .07	.21 ± .04
$\frac{\text{Cholesterol}}{\text{Phospholipid}}$	.32 ± .11†	.37 ± .11†	.89 ± .12
Logarithmic:			
Total cholesterol, mg/100 ml	3.10 ± .38	3.60 ± .37	2.95 ± .37
α-Lipoprotein cholesterol, mg/100 ml	.63 ± .73*	1.15 ± .75*	3.58 ± 1.03†
β- " " , "	2.95 ± .33	3.38 ± .32	2.72 ± .35
$\frac{\alpha}{\alpha + \beta}$	-2.57 ± .39	-2.79 ± .40	-1.01 ± .36†
Lipid phosphorus, mg/100 ml	4.26 ± .78	4.38 ± .77	4.36 ± .73
$\frac{\text{Cholesterol}}{\text{Phospholipid}}$	3.08 ± .72	3.05 ± .72	4.99 ± .68

* Regression not significant: $P > .1$.
† Regression significant: $P = .001$-$.006$. Regression very highly significant in all other instances: $P < .001$.

cholesterol feeding seems to be closely associated with resulting high plasma cholesterol concentration, most of which is in the β-lipoprotein fraction. The logarithmic expression counteracts the tendency for the data to have greater scatter at higher values.

The α-lipoprotein cholesterol was significantly related to lesion score only in the second series. There was no tendency for it to be greater in birds with little atheromatosis.

In agreement with Barr(8) it was found that the $\frac{\alpha}{\alpha + \beta}$ cholesterol ratio was the arithmetic expression most closely associated on the whole with lesion score. It offered no advantage, however, over the logarithmic expression of total cholesterol, and thus in experiments with cholesterol-fed birds the fractionation is probably not worthwhile. The cholesterol/phospholipid ratio ranked low as an indicator of lesion score in the first series, but high in the second. It is attractive to speculate that this difference may be related to differences in scoring systems, one of which considers only area of the lesions while the other tries to take into account thickness of deposit as well. However, examination of aortas from additional birds, ranked first in order of area covered and then in order of lesion thickness, did not support this conclusion. In a similar study with chickens fed ¼% cholesterol in the diet for 35 weeks Stamler and Katz(20) found that birds with aortic lesions had higher blood cholesterol than those without lesions, but found no relation with cholesterol/lipid phosphorus ratio. It would seem that degree of association of lesion score with cholesterol/phospholipid ratio varies from one experiment to another for reasons not apparent at present.

Most of the regression coefficients listed in Table V have high mathematical significance, and thus it is most improbable that the observed relationships between lipid values and lesion score are due to chance. The large number of observations contributes to this significance, and the relationships apply to the population as a whole. General conclusions can be drawn from the data. An important one is that treatments that lower the plasma cholesterol of cholesterol-fed cockerels will probably retard plaque formation also.

For individual birds, however, the plasma

lipid pattern forms an uncertain basis for estimating lesion score, just as it does for the corresponding estimation in individual human beings. If a cholesterol-fed cockerel has a plasma cholesterol concentration of 100 mg%, the chances are 1 in 2 that he will have aortic lesions. If, however, his plasma cholesterol concentration is in the neighborhood of 300 mg%, there is still 1 chance in 10 that he has no lesions at all.

The observed variance of the data can be used to calculate the reliability of the cholesterol-fed cockerel as an experimental animal. Two groups, each containing 10 birds on the regimen described here, may be expected to differ through chance from one another in average lesion score by one-half grade 3 times in 10, and by one full grade 1 time in 20. They may be expected to differ in average plasma cholesterol concentration by 10% 2 times in 3, and by 20% 1 time in 3. It is only as the difference approaches 50% that the probability of a chance occurrence decreases to 1 in 100. Thus, conclusions based upon moderate changes in lesion scores or blood cholesterol concentrations must be supported by repeated experiments.

Summary. 1) The degree of atheromatosis in the thoracic aortas and brachiocephalic arteries has been compared with plasma lipid pattern in 255 cholesterol-fed cockerels. Correlation was good with all determined variables except α-lipoprotein cholesterol concentration. It was best with logarithms of total cholesterol and β-liproprotein cholesterol concentrations, and with arithmetic $\frac{\alpha}{\alpha + \beta}$ cholesterol ratio. 2) General conclusions drawn from determined regressions are valid for population as a whole. Thus, a treatment which lowers the plasma cholesterol of cholesterol-fed cockerels may be expected to retard plaque formation also. For individual birds, however, estimation of lesion score from plasma lipid pattern is an unsatisfactory procedure. The observed variance of the data can also be used to calculate the reliability of the cholesterol-fed cockerel as an experimental animal.

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