Aortic Cholesterol and the Plasma Lipoproteins of the Cholesterol-Fed Cockerel* (33512)

RICHARD J. JONES AND LJUBICA DOBRILOVIC
Department of Medicine, Division of Biological Sciences, University of Chicago, Chicago, Illinois 60637

Since the initial report of Gofman (1), there has been interest in the possibility that selected bands of lipoprotein macromolecules may have a particular affinity for entrance into the arterial intima. This was carried one step further when the two major low density bands were weighted differently in the selection of coefficients for an "antherogenic index" (2), where the presence of clinical disease was employed as the indicator. The relative atherogenicity of the various low density lipoproteins merits further study in view of the apparent importance given to serum triglyceride levels by Albrink (3).

While there have been several recent studies on the arterial deposition of cholesterol (4, 5), phospholipid (6, 7), triglyceride (7, 8) in the experimental animal, there has been comparatively little attention given to the physical–chemical state of blood lipids in the deposition of aortic lipid. Dayton and Hashimoto (9) showed that lipoprotein is necessary in the surrounding medium, if labeled cholesterol is to be transported through the intima, and that this is more important than the presence of living endothelial cells. In the present study correlations were examined between the lipoprotein bands and cholesterol deposited in the aortic wall. The broad lipoprotein bands were selected by density limits and quantitated by their cholesterol, phospholipid, and total fatty acid content. Arterial phospholipid and total fatty acid were also measured, but histochemical observations (10) and the work in animals of Zilversmit *et al.* (5–7) and Lofland (8) suggested that these classes of lipid may undergo rapid metabolic alteration once they enter the arterial wall.

Methods. In this experiment seventy-five 8-week-old white cockerels housed in growing batteries in an air conditioned and light (7:00 a.m.–9:00 p.m.) controlled room were fed a standard "atherogenic" ration of started mash supplemented by 1% cholesterol, dissolved in 10% cottonseed oil. From the age of 5 days they had been reared on the same Kay-Bee starter mash. Blood was drawn, after an overnight fast, from 10 cockerels initially and from all birds at 5 and 10 weeks on the diet. The blood was oxalated and each plasma sample was individually separated and subjected to sequential ultracentrifugation at densities of 1.006, 1.019, and 1.063 as modified (11) from the technique of Havel *et al.* (12). Five ml of plasma were first overlaid by 4 ml of normal saline (of density = 1.006) and spun in a 30.2 rotor in a Spinco model L ultracentrifuge at 30,000 rpm for 22 hr ($1.05 \times 10^8 g$ min) at 8–12° to obtain the very low density top fraction which was removed by a capillary pipette. Successive adjustment of the density to 1.019 and 1.063 and centrifuging for 22 hr each time allowed the similar removal of a second and third top fraction. The final bottom heavy density lipoprotein (HDL) fraction was not further subdivided. The next two fractions correspond to the usually designated low density lipoproteins (LDL), S_f0–12 and S_f12–20. The lightest fraction included all lipoproteins above S_f20 according to Lindgren *et al.* (13). Samples of the whole plasma and each of these four lipoprotein fractions were then individually refluxed—1 part in 25 parts of ethanol:ether (3:1) for 60 min—filtered and the resulting

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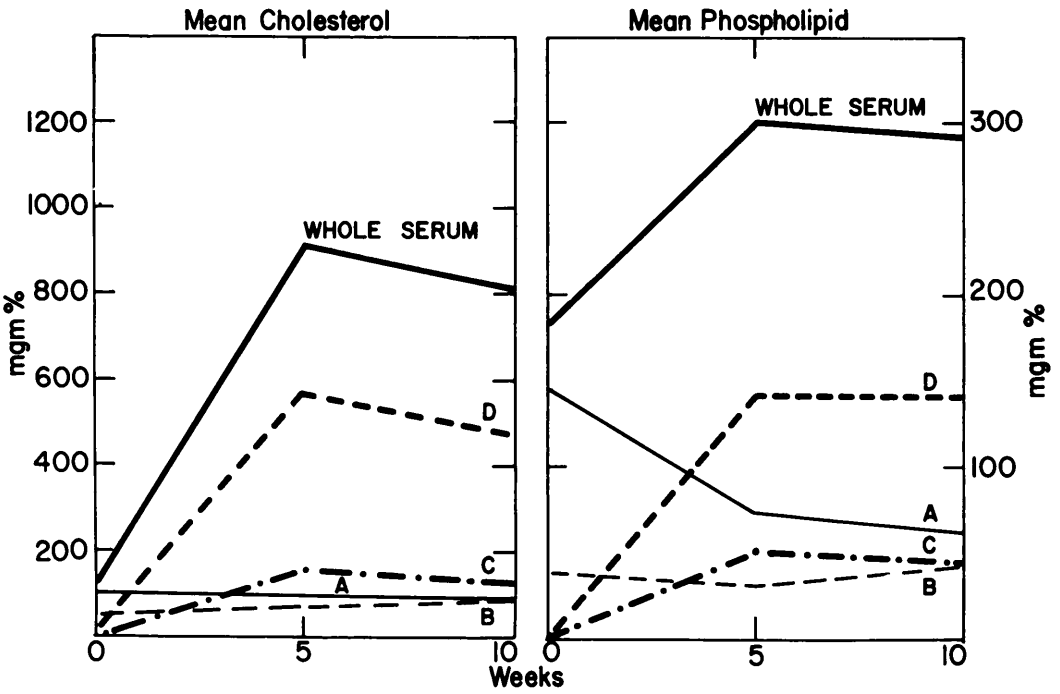

FIG. 1. Mean cholesterol and phospholipid concentrations in the plasma of chickens at 0, 5, and 10 weeks. The whole plasma values are compared with: (A) HDL; (B) S_f0–12, LDL; (C) S_f12–20; and (D) S_f>20 lipid class values. The mean is based upon plasma samples from 10 chickens at time 0 and from 75 chickens at 5 and 10 weeks of the diet.

extracts made up to volume for chemical analysis.

The birds were sacrificed at the time of the 10-week bleeding and their aortas were graded grossly (14), freed of adventitia, frozen, dried, and then later thawed, minced into fine fragments less than 1 mm in diameter and refluxed for 8 hr in alcohol:ether (3:1). The extract was filtered, tissue washings were added and the resulting extract was made up to volume for chemical analysis. In spot checks, further Soxhlet extraction of the aortic tissue residue with chloroform:methanol (2:1) for 48 hr yielded no detectable cholesterol or lipid phosphorus.

These alcohol:ether extracts of whole plasma, four lipoprotein fractions, and the aortic lipids were then analyzed in duplicate for total cholesterol (TC) by our modification (11) of the ferric chloride–sulfuric acid reaction of Zlatkis *et al.* (15), lipid phosphorus by method of Gomori (16), and total fatty acids (TFA) by the method of Albrink (17). For each plasma sample the sums of lipoprotein fraction cholesterols, phospholipids (PL),* or fatty acids each came to within ±10% of the whole plasma value *determined independently*; with rare exception they agreed to within ±5%.

Results. The mean plasma levels of cholesterol and phospholipid for the whole serum and for the four lipoprotein fractions of all 75 birds after 5 and 10 weeks on the diet are shown in Fig. 1. Note the rise in whole serum lipids during the first 5 weeks and the slight fall in the second 5 weeks. It is apparent that most of the elevation seen in whole serum cholesterol or phospholipid is due largely (72%) to an increase in the lightest (S_f>20) fraction. There is also substantial elevation in the TC of the S_f12–20 fraction, a small but significant elevation of the S_f0–12 TC and no change in the HDL TC. The changes in PL and TFA appear to be similar to those in TC with one important exception: there are significant reductions in HDL-PL

* Phospholipid was calculated as mg % lipid phosphorus × 25.

and TFA at 5 weeks, which are still maintained at 10 weeks. The TFA values parallel the PL values and hence are not presented in Fig. 1.

A correlation matrix was prepared: the various lipoprotein lipids at 5 and 10 weeks were correlated with one another and with the aortic lipid, expressed as total aortic cholesterol, phospholipid, or fatty acid, and as total cholesterol per gram of aortic tissue (wet wt.). The correlation coefficient relating gross aortic grade of atherosclerosis to aortic cholesterol (+0.64) was as high or higher than those relating it to aortic PL (+0.52) and TFA (+0.28). Except in the HDL fraction, very high correlations ($r = +0.6$ to $+0.9$) were seen between the lipid classes within each lipoprotein fraction derived from the same blood. As might be expected, whole plasma lipid values correlated best with $S_f>20$ fraction values. Lower order correlations between 5- and 10-week values, though still significantly different than zero, suggested the use of an integrated mean value of lipoprotein lipid in the correlation with aortic lipid.

Table I is extracted from the larger correlation matrix and indicates the correlation of the milligrams of cholesterol per gram of aorta with the concentrations for the various lipid classes of the whole plasma and the several lipoprotein fractions either at 5 weeks, at 10 weeks or as their integrated mean. It may be seen that the highest correlation coefficient is seen with the $S_f>20$ fraction PL or TC. The correlation coefficients for each of the chemical determinations of this fraction approximate those for the whole plasma. The lipids of the other fractions do not have as high a correlation coefficient with aortic cholesterol even though there is in general a rather high correlation between the S_f12–20 and $S_f>20$ fractions at 10 weeks ($r = +0.6$). The correlation coefficient between aortic cholesterol and $S_f>20$ fraction TC is highest for the integrated value.

Because so many intercorrelations existed among all of these parameters, a generalized stepwise regression program was employed. The various integrated mean lipoprotein cholesterol values were treated as independent variables, related to aortic cholesterol, Y mg /g, as the dependent variable in the equation,[1] $Y = c_0 + c_1[A] + c_2[B] + c_3[C] + c_4[D]$.[2]

On the basis of largest initial reduction in the mean squared deviation, the D fraction was taken as the first entering independent variable. Judged on the basis of the F-ratios, subsequent stepwise inclusion of the additional variables did not significantly improve the fit to the data of the equation, $Y = 0.405 + 0.00182[D]$.

Discussion. In this experimental situation, then, the aortic deposition of cholesterol, and probably all lipids, may thus be in part related to this turbid, triglyceride-rich $S_f>20$ fraction, which includes all particles that float at plasma density (1.006). This is perhaps not surprising in view of the fact that this fraction makes the greatest contribution to the hyperlipidemia. However, the S_f12–20 fraction cholesterol still accounted for 28% of the mean elevation in whole serum cholesterol without making any significant improvement in the regression equation. It is also pertinent that certain birds with moderately severe gross lesions and heavy aortic cholesterol deposition had fasting S_f0–12 values which remained within the normal range. Nevertheless, since a significant correlation did exist between both the S_f0–12 and S_f12–20 fraction cholesterols and aortic cholesterol, the possibility that small increases in their concentration might enhance the aortic deposition of cholesterol could not be excluded, especially if they were greatly favored in their selection by the intima.

From this experiment, it may be concluded

[1] In this equation $[A]$, $[B]$, $[C]$ and $[D]$ refer to integrated mean cholesterol concentrations (mg%) in plasma lipoprotein fractions HDL, S_f0–12, S_f12–20 and $S_f>20$, respectively; c_0, c_1, c_2, c_3 and c_4 are constants.

[2] The regressions were computed using a version of Generalized Stepwise Regression Program BIMD 34 [See BIMD Computer Programs Manual (1961), Division of Biostatistics, Department of Medicine and Public Health, School of Medicine, University of California, Los Angeles] modified by Peter M. Neeley, Biological Sciences Computation Center, University of Chicago.

TABLE I. Correlation Coefficients between Aortic Cholesterol (mg/g) and Chemical Constituents of the Plasma and Plasma Lipoprotein Fractions at 5 Weeks, 10 Weeks, or Integrated[a] for 10 Weeks.

Fraction	Cholesterol			Phospholipid			Fatty acids		
	5 weeks	10 weeks	Int.[a]	5 weeks	10 weeks	Int.[a]	5 weeks	10 weeks	Int.[a]
Whole plasma	.54	.54	.57	.55	.52	.59	.37	.34	.41
HDL ($d > 1.063$)	.09[b]	.06[b]	.08[b]	.22[b]	.04[b]	.18[b]	.14[b]	.31	.25
LDL (d 1.019–1.063)	.27	.39	.34	.32	.34	.37	.17[b]	.26	.24
S_f12–20 (d 1.006–1.019)	.20[b]	.53	.30	.23	.50	.34	.20[b]	.38	.28
$S_f > 20$ ($d < 1.006$)	.55	.47	.57	.59	.45	.60	.35	.34	.41

[a] Integrated as indicated in the formula: integrated mean $= \frac{1}{2}(O + 2V + X)$ mg $\times$ 10/100 ml; where $O =$ mean value at beginning of experiment; $V =$ individual value at 5 weeks; $X =$ individual value at 10 weeks.

[b] Correlation coefficient is not significantly greater than zero at the 5% level of confidence. Degrees of freedom = 73.

that deposition of aortic cholesterol in the cholesterol-fed chick is not correlated with variations in HDL lipid. Rather it is correlated with the low density lipoproteins of the plasma, particularly the lipoproteins less dense than $S_f > 20$. While a significant correlation is found between aortic cholesterol and S_f0–12 and S_f12–20 TC, these parameters do not improve the regression of $S_f > 20$ TC upon aortic cholesterol. Consequently, this does not favor the possibility that S_f0–12 and S_f12–20 fractions might be still contributing significantly to aortic cholesterol. Although somewhat higher correlation coefficients could be obtained using the integrated mean value instead of the 5- or 10-week value of a lipoprotein lipid, it cannot be claimed that this provided any real increase in precision.

The work of Courtice and co-workers has shown that capillary intima is most permeable to lipoproteins of S_f0–12 (18), but also allows the passage under some circumstances of lipoproteins of density less than 1.019 (19). No limiting size or density has been found in his studies for excluding lipoproteins from transintimal passage, although the proportion of those blood-borne lipoprotein particles entering the intima seems to decrease as particle size increases beyond a certain point. This should be further tested in atherogenesis by studying further subdivisions of the low density lipoprotein spectrum above $S_f > 20$.

Summary. In an experiment with 75 cholesterol-fed cockerels, in which deposition of cholesterol in the aortic wall was correlated with the various lipoprotein lipids, it was found that no significant correlation existed with the heavy density lipoprotein fraction. Significant correlations were present with the S_f0–12, S_f12–20 and $S_f > 20$ cholesterol after 10 weeks. In fact, the variance in aortic cholesterol could be largely explained by $S_f > 20$ lipoprotein cholesterol. None of the measured plasma lipid parameters correleated with aortic phospholipid or total fatty acids as well as with the aortic cholesterol.

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Labeling of Marrow Cells of Vitamin E-Deficient Monkeys by ^{3}H-Precursors of Nucleic Acids and Protein* (33513)

CALVIN HANNA, ORVILLE G. NEUFELD,[1] AND COY D. FITCH[2]

Departments of Pharmacology and Medicine, University of Arkansas School of Medicine, Little Rock, Arkansas 72201

When young rhesus monkeys are fed diets deficient in vitamin E, they develop a characteristic syndrome of anemia and muscular weakness after 1–3 years (1, 2). During the anemia the lifespan of the circulating erythrocytes is reduced by about two thirds of normal (3), and the bone marrow is hypercellular (4). There is a granulocytosis but only a slight reticulocytosis (1, 2, 4). Treatment with *alpha*-tocopherol induces reticulocytosis and rapid remission of the anemia and muscular weakness (1–4).

Although erythrocyte survival is reduced, abnormal erythropoiesis is accorded the primary role in producing the anemia of vitamin E deficiency (1, 4, 5). Not only is the bone marrow hypercellular as judged from morphological studies (4) and from measurements of DNA and RNA content (6), there are also abnormalities of the nucleated erythroid cells (4). Porter *et al.* (4), found many of the erythroid cells to be multinucleated, and they describe the nuclei of all nucleated erythroid cells of anemic, vitamin E-deficient monkeys as more deeply staining and homogenous than normal. To further define this abnormality of erythropoises, autoradiographic techniques were used in the present study of thymidine, deoxyuridine, uridine, and leucine incorporation into bone marrow cells.

Materials and Methods. Young rhesus monkeys (*Macaca multatta*) were fed a soybean protein-based, vitamin E-deficient diet [see Table I of Ref. (7)] supplemented with 2% of calcium carbonate and 0.1% of ferrous sulfate heptahydrate at the expense of a similar weight of corn starch. This extra supplement of iron is more than enough to prevent the occurence of iron-deficiency anemia, which otherwise may occur in monkeys fed this diet (7). Control monkeys received on their food 80 mg of *dl-alpha*-tocopheryl acetate dissolved in ethanol three times weekly. Control and vitamin E-deficient monkeys were always studied stimultaneously.

To monitor the course of the anemia, blood was obtained at intervals from an ear vein for determination of hemoglobin concentra-

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[1] Present address: Department of Pharmacology, University of California Medical Center, San Francisco, California 94122.

[2] Present address: Division of Biochemistry, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C. 20012.