

# Effect of cholesterol-rich diets with and without added vitamins E and C on the severity of atherosclerosis in rabbits<sup>1-3</sup>

Mohamedain M Mahfouz, Hiroshige Kawano, and Fred A Kummerow

**ABSTRACT** Oxysterols as oxidation products of cholesterol are considered an atherogenic factor in the development of atherosclerosis in the arteries of cholesterol-fed rabbits. We compared the atherogenic effects of diets enriched either with 0.5% oxidized cholesterol (OC; characterized by high amounts of oxysterols) or with pure cholesterol (PC). The effects of antioxidant vitamins E and C added to the PC diet were also evaluated in view of their antioxidative properties for lipoproteins and cholesterol and how this could affect the severity of atherosclerosis. Four groups of rabbits were fed the following for 11 wk: 1) a nonpurified stock diet, 2) this stock diet plus 0.5% OC, 3) the stock diet plus 0.5% PC, and 4) the stock diet plus 0.5% PC and 1000 mg vitamin E and 500 mg vitamin C/kg diet (PC + antioxidants). The OC and PC diets were equally hyperlipidemic and hypercholesterolemic. The severity of atherosclerotic lesions was highest with the OC diet and lowest with the PC + antioxidants diet. The plasma oxysterol concentration was proportional to the severity of atherosclerosis in all three groups of cholesterol-fed rabbits.  $\beta$ -Very-low-density-lipoprotein modification was minimized by vitamins E and C as indicated by its polyacrylamide gel electrophoretic pattern and its increased binding to the rabbit liver membrane in vitro. This study indicated that OC and PC were equally atherogenic but that the addition of antioxidants to the PC diet significantly reduced its severity, even when hypercholesterolemia persisted. This indicated that atherogenesis can result from an excessive accumulation of oxidation products of cholesterol in the plasma. *Am J Clin Nutr* 1997;66:1240-9.

**KEY WORDS** Hypercholesterolemia, cholesterol oxides, antioxidant, vitamin E, vitamin C, atherosclerosis, rabbits

## INTRODUCTION

Rabbits fed a diet containing 0.2 g oxidized cholesterol (OC)  $\cdot$  kg body wt<sup>-1</sup>  $\cdot$  d<sup>-1</sup> had a 15-fold elevation of plasma cholesterol and  $\beta$ -very-low-density-lipoprotein (VLDL) concentration when compared with rabbits consuming the stock diet (1). Rabbits fed the same amount of pure cholesterol (PC) had only a threefold elevation of cholesterol concentration. These differences were thought to be related to the cholesterol oxides that are either present in OC or formed during hepatic metabolism.

Cholesterol added either as low-density lipoprotein (LDL) or as an ethanolic solution inhibited cholesterol biosynthesis in

human skin fibroblasts (2), human leukocytes (3), and rat hepatoma cells (4), but several studies (5-7) found that the addition of highly purified cholesterol to mouse fetal liver cells or mouse fibroblasts in tissue culture did not inhibit sterol biosynthesis. The oxygenated derivatives of cholesterol such as 7-ketocholesterol, 7 $\alpha$ - and 7 $\beta$ -hydroxycholesterol, and 25-hydroxycholesterol are very inhibitory of sterol biosynthesis (5-7). These results led to the conclusion that cholesterol oxides might be responsible for the effect of feeding cholesterol because cholesterol oxides are often present as impurities in preparations of cholesterol.

The barrier function of endothelial cells in culture was not significantly affected when exposed to cholesterol-enriched LDL, whereas triol (cholestane-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol)-enriched LDL resulted in a significant loss of barrier function (8). Cholesterol supplementation of smooth muscle cells in tissue culture increased the gap-junctional communication between cells whereas cholesterol oxides inhibited that communication (9), indicating that cholesterol oxides, rather than cholesterol, promote atherogenesis. The present study was designed to clarify with rabbits as an experimental model whether diets enriched with OC, in vivo formation of cholesterol oxides, or oxidation of cholesterol carried by lipoproteins may be the atherogenic source.

## MATERIALS AND METHODS

### Animals and diets

Twenty-four New Zealand white male rabbits ranging in weight from 2750 to 3100 g were housed individually in stainless steel mesh-bottomed cages and accustomed to a pelleted whole-grain commercial diet (Purina Mills, Inc, Richmond, IN) for 1 wk. Male rabbits were used to avoid any interfering effect on the results due to the female hormonal cycle. Six rabbits were assigned randomly to each of the four

<sup>1</sup> From the Burnside Research Laboratory, University of Illinois, Urbana.

<sup>2</sup> Supported by funds from the Wallace Genetic Foundation, Rye, NY.

<sup>3</sup> Address reprint requests to FA Kummerow, Burnside Research Laboratory, University of Illinois, 1208 West Pennsylvania Avenue, Urbana, IL 61801. E-mail: fkummero@uiuc.edu.

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groups. These four groups of rabbits were fed either 1) an unmodified nonpurified diet (control diet); 2) the control diet plus 0.5% OC (US Pharmacopeia cholesterol exposed to air at room temperature for > 15 y), which was characterized by a high concentration of cholesterol oxides (Table 1); 3) the control diet plus 0.5% PC; or 4) the control diet plus 0.5% PC, vitamin E (as  $\alpha$ -tocopherol acetate), and vitamin C at concentrations of 1000 mg and 500 mg/kg diet, respectively (PC + antioxidants diet). Feed and water were provided ad libitum.

PC was prepared by six recrystallizations of USP cholesterol from 95% ethanol and was kept under nitrogen at  $-20^{\circ}\text{C}$ . Its purity was checked by gas chromatography, which indicated that it contained only traces of cholesterol oxides.

The cholesterol-rich diets were prepared by dissolving the cholesterol in peroxide-free ether, spraying it over the diet, and allowing the ether to evaporate in a closed hood under nitrogen at room temperature. The cholesterol-free control diet was sprayed with an equal volume of peroxide-free ether. The diet was prepared fresh every 3 d, with any remaining diet being discarded. The free cholesterol oxide content of the cholesterol-supplemented diets was monitored weekly by a gas chromatography-HPLC method (Table 1).

After 11 wk of the respective diets, the rabbits were deprived of food over night, anesthetized by intramuscular injection of ketamine (44 mg/kg) + xylazine (5 mg/kg), their chests opened, and their blood withdrawn through heart puncture into plastic tubes with EDTA (1 g/L blood) as an anticoagulant and butylated hydroxytoluene (BHT) as an antioxidant (50 mg/L blood). The plasma was collected after centrifugation at  $2000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . Four milliliters of each sample of plasma was taken for chemical analysis. The rabbits were killed by pentobarbital injection (39 mg/kg). The livers were excised immediately, washed with ice-cold saline solution, and a 5-g portion of the same lobe was immediately used from each liver to prepare liver membrane. The remaining liver was stored at  $-80^{\circ}\text{C}$ .

### Lipid analysis

Total plasma cholesterol (10), high-density-lipoprotein (HDL) cholesterol (11), and triacylglycerol (12) were determined according to the instruction manual accompanying the diagnostic kits obtained from Sigma Chemical Co (St Louis). Plasma malondialdehyde (MDA) content was measured by the thiobarbituric acid method as described previously (13). The lipid in 1 mL plasma extracted according to the Folch et al

method (14) was used for measurement of total lipid phosphorus by following the Eng and Noble method (15).

The total vitamin C content in the plasma was measured according to the method described by Roe and Kuether (16) in which the ascorbic acid was oxidized to dehydroascorbic acid by copper ion. The 2,4-dinitrophenylhydrazine derivative was dissolved in concentrated sulfuric acid and measured spectrophotometrically at 515 nm.

For liver lipid analysis, 5 g of each liver was extracted by using the Folch et al method (14) and the lipid residue taken up in 25 mL chloroform in a 25-mL volumetric flask. A known aliquot of this lipid extract was used for measuring the free and total cholesterol content by using the Zak et al method (17). Total lipid phosphorus (15) and triacylglycerol were determined at 340 nm by using Sigma kits (18).

### Measurement of cholesterol oxides in plasma

Lipids from 1 mL plasma in duplicate were extracted by using 7.5 mL chloroform:methanol (1:1, by vol) with 75  $\mu\text{g}$  BHT as antioxidant and 500 ng 20-hydroxycholesterol as an internal standard. The lipid residue obtained after washing and drying the chloroform extract under argon was saponified overnight in a shaking water bath at  $35^{\circ}\text{C}$  by using 50 mL 1 mol alcoholic KOH/L under argon in a polytetrafluoroethylene-lined, capped bottle. The unsaponifiable fraction was extracted with three, 100-mL portions of hexane. The pooled hexane extracts were washed with deionized water until neutral, then evaporated under vacuum in a rotary evaporator until dry. The residue was taken up in 10 mL chloroform:methanol (1:1, by vol) and evaporated to dryness under argon. This residue was dissolved in 500  $\mu\text{L}$  acetonitrile and vortexed, after which 500  $\mu\text{L}$  deionized water was added and vortexed again. The lipid residue in acetonitrile was passed through two sets of Sep-Pak cartridge: ammonia and C-18 cartridges, in that order (Waters Associates, Millford, MA) to separate cholesterol oxides from other lipids, then further purified by HPLC and finally analyzed by gas chromatography as described previously (19).

### Preparation of LDL and LDL-gold conjugate

Human LDL (density: 1020–1063 g/L) was obtained from fresh plasma of healthy individuals (Community Blood Service of Illinois, Urbana) and prepared by the sequential ultracentrifugation method described by Ferreri (20) at  $10^{\circ}\text{C}$  using a Beckman model L5–50 ultracentrifuge and a type 60 Ti rotor (Beckman Instruments, Fullerton, CA). The colloidal gold-LDL conjugate was prepared according to the method described by Roach et al (21).

### Measurement of the LDL hepatic membrane receptor activity

The livers from the killed rabbits were washed in ice-cold 0.154 mol NaCl/L and a 5-g portion of the same lobe of each liver was used for determination of LDL receptor activity. The liver was homogenized, and after sequential centrifugation at  $500 \times g$  (for 5 min) and  $8000 \times g$  (for 15 min), the liver membrane fraction was isolated by centrifugation at  $100\,000 \times g$  for 1 h (22). These membranes were solubilized in Triton X-100 (Sigma Chemical Co) and the detergent was removed as described previously (23). The solubilized membrane proteins

TABLE 1

Cholesterol oxide amounts in the oxidized and pure cholesterol diets<sup>1</sup>

Cholesterol oxide	Oxidized cholesterol diet (n = 3)	Pure cholesterol diet (n = 3)
	ng/kg diet	
7 $\alpha$ -hydroxycholesterol	8700 $\pm$ 2100	2200 $\pm$ 100
7 $\beta$ -hydroxycholesterol	30 600 $\pm$ 9600	1700 $\pm$ 0.0
$\beta$ -epoxide	8400 $\pm$ 700	1800 $\pm$ 500
Cholestanetriol	123 900 $\pm$ 11 600	10 000 $\pm$ 700
7-ketocholesterol	213 700 $\pm$ 24 900	18 500 $\pm$ 200
26-hydroxycholesterol	96 000 $\pm$ 15 600	1700 $\pm$ 100
Total cholesterol oxides	481 000 $\pm$ 40 700	35 800 $\pm$ 200

<sup>1</sup>  $\bar{x} \pm \text{SD}$ .

were dot-blotted on BA85 nitrocellulose membrane (Schleicher and Schuell, Keene, NH) by using a Bio-Rad apparatus (Bio-Rad Laboratories, Hercules, CA) and the LDL binding activity determined by using human LDL conjugated to colloidal gold as described previously (21). The nitrocellulose strips were soaked in 10% paraffin oil in benzene to render them transparent. For scanning densitometry we used an LKB Ultrascan XL enhanced laser densitometer (Pharmacia Biotech, Piscataway, NJ) interfaced with a microcomputer. The GELSCAN XL computer program (Pharmacia LKB Biotechnology Inc, Piscataway, NJ) was used to evaluate the scan data. The results were expressed in arbitrary absorbance units (AU). Nonspecific LDL binding was measured in parallel incubation in the presence of a 25-fold excess of unlabeled LDL, which was subtracted from total gold-LDL binding to produce specific LDL binding. Total cholesterol content was also measured in the detergent extract of liver membrane.

#### Measurement of $\beta$ -VLDL binding to the rabbit liver membrane protein

The  $\beta$ -VLDL fractions (density  $< 1006$  g/L) were separated from the plasma of rabbits fed either the PC diet or the PC + antioxidants diet by the sequential ultracentrifugation method described by Ferreri (20). The  $\beta$ -VLDL-gold conjugates were also prepared as described previously (21). The total binding of the  $\beta$ -VLDL-gold conjugates to the solubilized liver membrane prepared from the livers of rabbits fed the PC diet and dot blotted onto nitrocellulose membrane was measured as described for LDL binding (21).

#### Gel electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (24) with a 6% separation gel and 4% stacking gel. Before electrophoresis, aliquots of the delipidated and freeze-dried  $\beta$ -VLDL were solubilized in the sample buffer, which contained 2% SDS, 10% sucrose, and 2.5%  $\beta$ -mercaptoethanol and then were heated for 3 min in a boiling water bath to destroy the disulfide bonds of proteins. The running buffer was 0.005 mol Tris/L, 0.038 mol glycine/L, pH 8.3. Electrophoresis was set at 10 V/cm and 1 mA/cm for 2.5 h at room temperature. Proteins were stained with 0.1% Coomassie brilliant blue R-250 in 30% methanol and 10% acetic acid.

#### Morphologic studies of aorta

Descending aorta tissue specimens of the same area from rabbits in each of the four groups were fixed in formalin and paraffin blocks. Sections were prepared, stained with hematoxylin and eosin (25), and the section viewed at  $50\times$  magnification in a light microscope. Other paraffin sections were stained with Verhoeff Van Gieson's stain (26) and viewed at  $140\times$  magnification.

#### Statistical analysis

All the results were subjected to analysis of variance (ANOVA) and statistically tested by Duncan's new multiple-range test (27).

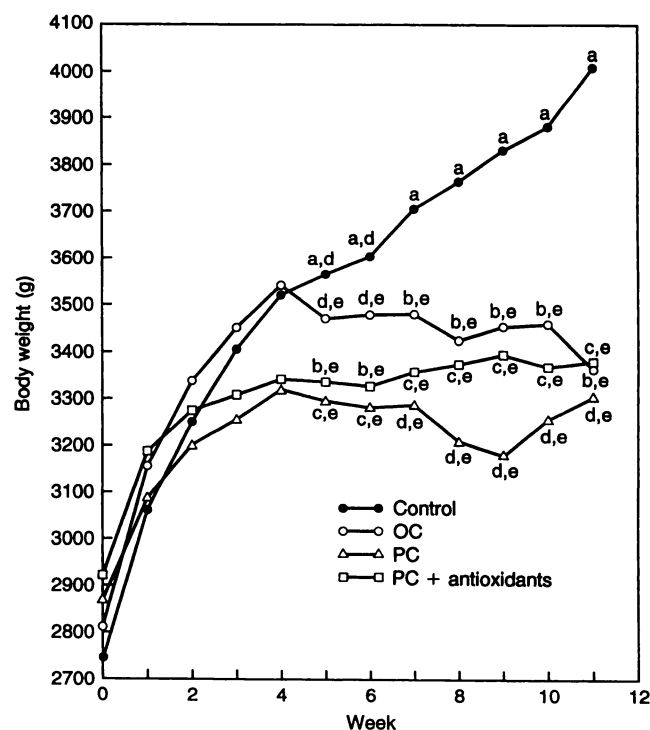
## RESULTS

The growth rate of the rabbits fed the high-cholesterol diets (PC, OC, or PC + antioxidants diet) was less than that of the control group. This decrease was significant after the fifth week of the feeding experiment (Figure 1). The decrease in the growth rate may be attributed to less average daily feed consumption by the groups fed OC ( $110.9 \pm 39.3$  g/d), PC ( $110.0 \pm 31.7$  g/d), and PC + antioxidants ( $115.3 \pm 27.1$  g/d) compared with the control group ( $164.1 \pm 23.1$  g/d).

#### Plasma chemical analysis

The OC and PC diets increased the plasma cholesterol concentration  $> 66$ - and  $70$ -fold, respectively, compared with the control group (Table 2). Supplementation of the PC diet with vitamins E and C significantly reduced the plasma cholesterol concentration by 40% compared with the rabbits fed the PC diet only. HDL concentration in the plasma did not differ between the three groups, indicating that the large increase in plasma cholesterol in the cholesterol-fed rabbits was within the low density lipoprotein fractions (LDL + VLDL) (Table 2).

The OC and PC diets increased the plasma triacylglycerol concentration by  $\approx 2.7$ - and  $2.3$ -fold, respectively, whereas the PC + antioxidant diet increased plasma triacylglycerol only 1.5-fold compared with the control group (Table 2). The plasma phospholipid concentration increased  $\approx 10$ -fold with the OC and PC diets but increased only sixfold with the PC + antioxidants diet compared with the control group. These results indicate that the OC and PC diets were equally hyperlipidemic and hypercholesterolemic, but that the addition of vi-



**FIGURE 1.** Growth of rabbits fed a control, oxidized cholesterol (OC), pure cholesterol (PC), or PC + antioxidants diet. Weights of rabbits on the same day with different superscript letters are significantly different,  $P < 0.05$ .

**TABLE 2**

Plasma lipids, vitamin C, and thiobarbituric acid–reactive substance (TBARS) concentrations in rabbits fed either control, oxidized cholesterol, pure cholesterol, or pure cholesterol + antioxidants diets for 11 wk<sup>1</sup>

	Control diet (n = 6)	Oxidized cholesterol diet (n = 6)	Pure cholesterol diet (n = 6)	Pure-cholesterol + antioxidants diet (n = 6)
Total cholesterol (mmol/L)	0.575 ± 0.22 <sup>a</sup>	37.65 ± 15.28 <sup>b,e,f</sup>	40.0 ± 6.26 <sup>c,e</sup>	23.8 ± 4.4 <sup>d,f</sup>
HDL cholesterol (mmol/L)	0.476 ± 0.12	0.51 ± 0.30	0.53 ± 0.13	0.57 ± 0.10
Triacylglycerol (mmol/L)	0.51 ± 0.08 <sup>a</sup>	1.41 ± 0.67 <sup>b,e,f</sup>	1.17 ± 0.36 <sup>c,e</sup>	0.76 ± 0.136 <sup>d,f</sup>
Phospholipids (mmol/L)	0.64 ± 0.08 <sup>a</sup>	6.95 ± 2.49 <sup>b,e,f</sup>	6.56 ± 1.64 <sup>c,e</sup>	3.83 ± 0.6 <sup>d,f</sup>
Vitamin C (μmol/L)	22.35 ± 5.1 <sup>a</sup>	26.6 ± 4.3 <sup>a,d</sup>	31.1 ± 6.1 <sup>b,d</sup>	59.0 ± 16.3 <sup>c</sup>
TBARS (nmol MDA/L)	617 ± 121 <sup>a</sup>	2650 ± 497 <sup>b,e</sup>	2667 ± 537 <sup>c,e</sup>	1280 ± 299 <sup>d</sup>

<sup>1</sup>  $\bar{x} \pm \text{SD}$ , MDA, malondialdehyde. Means with different superscript letters are significantly different,  $P < 0.05$ .

tamins E and C to the PC diet decreased the degree of hyperlipidemia and hypercholesterolemia.

The plasma MDA concentration was significantly increased in all the rabbits fed cholesterol. The increase was more than fourfold with the OC and PC diets and only twofold with the PC + antioxidants diet (Table 2). The addition of the antioxidants to the PC diet did, therefore, decrease the MDA concentration by 50%. The plasma vitamin C concentration was significantly increased in the rabbits fed PC + vitamins E and C and in the rabbits fed PC compared with the control group of rabbits (Table 2).

#### Liver lipids analysis

The free and total amounts of cholesterol deposited in the liver of rabbits fed high-cholesterol diets increased six- and ninefold, respectively, compared with the control group of rabbits (Table 3). Cholesterol esters represented > 55% of the total liver cholesterol in the cholesterol-fed rabbits compared with 28% in the control group of rabbits, indicating that cholesterol esterification was enhanced in the livers of the cholesterol-fed rabbits.

The phospholipid content was increased by 20–30% in the livers of the rabbits fed cholesterol, but the liver triacylglycerol concentration did not differ significantly from that of the control group of rabbits. Collectively, the results in Table 3 indicate that all cholesterol-rich diets caused an accumulation of cholesterol mainly as cholesterol ester in the liver, and feeding the PC and the PC + antioxidants diets did not reduce the amount of lipids in the liver compared with the OC diet. The accumulation of lipids in the liver tissues was, therefore, independent of cholesterol oxides or antioxidants. The liver membranes were highly enriched with cholesterol in rabbits fed cholesterol, reaching concentrations of 270.2 ± 26.9, 280.0 ±

40.9, and 310.1 ± 22.8 μmol cholesterol/g liver membrane protein in the OC, PC, and PC + antioxidants groups compared with 64.4 ± 15.2 μmol cholesterol/g liver membrane protein in the control group of rabbits.

#### Plasma cholesterol oxides

The plasma of the control rabbits contained 7α- and 7β-hydroxycholesterols, β-epoxide, cholestanetriol, 7-ketocholesterol, and 25- and 26-hydroxycholesterols (Table 4). After 11 wk of the respective diets, the cholesterol oxide concentration increased significantly in the plasma of all the cholesterol-fed rabbits. The total plasma cholesterol oxide concentrations in the rabbits fed OC, PC, and PC + antioxidants were 13 888.6 ± 1808, 8436.8 ± 1212.4, and 4452.7 ± 562.2 nmol/L plasma, respectively, compared with 889.3 ± 13.9 nmol/L in the plasma of the control rabbits. These results indicate that the rabbits fed the PC diet also accumulated cholesterol oxides in the plasma, but the addition of vitamins E and C to the PC diet significantly reduced the total plasma cholesterol oxides by 47%. Furthermore, the total plasma cholesterol oxides concentration was significantly lower in rabbits fed the PC diet compared with the rabbits fed the OC diet (Table 4).

#### Hepatic membrane LDL receptor activity

The specific binding of human LDL to the hepatic membrane protein receptors prepared from the livers of rabbits fed OC, PC, or PC + antioxidants was significantly lower compared with the specific binding of LDL to the hepatic membrane protein prepared from the control rabbits. From the same amount of membrane protein (1 μg), the density of the dots blotted from the hepatic membranes prepared from the rabbits

**TABLE 3**

Lipid contents in liver tissues of rabbits fed control, oxidized cholesterol, pure cholesterol, or pure cholesterol + antioxidants diets for 11 wk<sup>1</sup>

	Control diet (n = 6)	Oxidized cholesterol diet (n = 6)	Pure cholesterol diet (n = 6)	Pure cholesterol + antioxidants diet (n = 6)
Total cholesterol (μmol/g liver)	8.03 ± 1.55 <sup>a</sup>	80.3 ± 26.6 <sup>b,e</sup>	75.1 ± 22.8 <sup>c,e</sup>	70.2 ± 11.7 <sup>d,e</sup>
Free cholesterol (μmol/g liver)	5.77 ± 1.11 <sup>a</sup>	30.1 ± 4.66 <sup>b,e</sup>	33.9 ± 5.7 <sup>c,e</sup>	31.1 ± 3.11 <sup>d,e</sup>
Esterification of cholesterol (%)	28.4 ± 8.0 <sup>a</sup>	55.1 ± 8.6 <sup>b,e</sup>	57.4 ± 6.4 <sup>c,e</sup>	56.4 ± 7.5 <sup>d,e</sup>
Triacylglycerol (μmol/g liver)	6.11 ± 1.47	5.1 ± 0.8	5.77 ± 1.13	5.54 ± 0.8
Phospholipid (μmol/g liver)	34.4 ± 3.6 <sup>a</sup>	41.6 ± 3.1 <sup>b,e,f</sup>	44.5 ± 3.0 <sup>c,e</sup>	40 ± 1.88 <sup>d,f</sup>

<sup>1</sup>  $\bar{x} \pm \text{SD}$ . Means with different superscript letters are significantly different,  $P < 0.05$ .

**TABLE 4**

Cholesterol oxide concentrations in the plasma of rabbits fed control, oxidized cholesterol, pure cholesterol, or pure cholesterol + antioxidants diets for 11 wk<sup>1</sup>

Cholesterol oxide	Control diet (n = 3)	Oxidized cholesterol diet (n = 3)	Pure cholesterol diet (n = 3)	Pure cholesterol + antioxidants diet (n = 3)
<i>nmol cholesterol oxide/L plasma</i>				
7 $\alpha$ -hydroxycholesterol	74.1 $\pm$ 4.7 <sup>a</sup>	1804.5 $\pm$ 174.9 <sup>b</sup>	1078.4 $\pm$ 210 <sup>c,e</sup>	728.6 $\pm$ 167.7 <sup>d,e</sup>
7 $\beta$ -hydroxycholesterol	230.8 $\pm$ 5.2 <sup>a</sup>	5575.8 $\pm$ 1410.4 <sup>b</sup>	3586.3 $\pm$ 1113.4 <sup>c,e</sup>	2305.5 $\pm$ 674.1 <sup>d,e</sup>
$\beta$ -epoxide	100.25 $\pm$ 12.4 <sup>a</sup>	577.9 $\pm$ 64.9 <sup>b</sup>	350.7 $\pm$ 24.1 <sup>c,e</sup>	351.2 $\pm$ 66.7 <sup>d,e</sup>
Cholestanetriol	117.4 $\pm$ 6.9 <sup>a</sup>	1367.4 $\pm$ 344.0 <sup>b</sup>	580.7 $\pm$ 131.0 <sup>c</sup>	217.1 $\pm$ 60.9 <sup>d</sup>
25-hydroxycholesterol	107.0 $\pm$ 12.2 <sup>a</sup>	587.3 $\pm$ 113.2 <sup>b,e</sup>	641.5 $\pm$ 118.9 <sup>c,e</sup>	233.1 $\pm$ 26.4 <sup>d</sup>
7-ketocholesterol	147.8 $\pm$ 12.5 <sup>a</sup>	2992.3 $\pm$ 156.0 <sup>b</sup>	1766.8 $\pm$ 348 <sup>c,e</sup>	395.0 $\pm$ 61.0 <sup>d</sup>
26-hydroxycholesterol	107.2 $\pm$ 23.6 <sup>a</sup>	935.5 $\pm$ 206.4 <sup>b</sup>	415.7 $\pm$ 84.3 <sup>c</sup>	216.2 $\pm$ 41.3 <sup>d</sup>
Total cholesterol oxides	889.3 $\pm$ 13.9 <sup>a</sup>	13 888.6 $\pm$ 1808 <sup>b</sup>	8436.8 $\pm$ 1212.4 <sup>c</sup>	4452.7 $\pm$ 562.2 <sup>d</sup>

<sup>1</sup>  $\bar{x} \pm$  SD. Means with different superscript letters are significantly different,  $P < 0.05$ .

fed OC ( $0.074 \pm 0.01$ ), PC ( $0.08 \pm 0.01$ ), and PC + antioxidants ( $0.07 \pm 0.01$ ) was lower than that of the control group ( $0.143 \pm 0.02$  AU) as measured by scanning densitometry (Figure 2). These results revealed that hepatic LDL receptor activity was significantly suppressed in all of the rabbits fed cholesterol when compared with the control rabbits. No significant differences could be detected in the LDL receptor activity among the cholesterol-fed rabbits.

#### Binding of $\beta$ -VLDL to the hepatic membrane protein

The total binding to the hepatic membrane protein of the  $\beta$ -VLDL prepared from the plasma of the rabbits fed PC + antioxidants ( $0.25 \pm 0.02$  AU) was higher than the binding of  $\beta$ -VLDL prepared from the plasma of rabbits fed the PC diet ( $0.16 \pm 0.02$  AU) as revealed in the scanning densitometry data.

#### SDS-PAGE of $\beta$ -VLDL

SDS-PAGE (Figure 3) revealed that the  $\beta$ -VLDL fractions prepared from the plasma of the rabbits fed the PC diet or the OC diet showed a dense protein band that could not penetrate even the low-concentration acrylamide gel (stacking gel, 4%). SDS-PAGE was performed under reducing conditions in the presence of mercaptoethanol to detect apoprotein covalently cross-linked by nonsulfide bonds. The nonmigrating protein

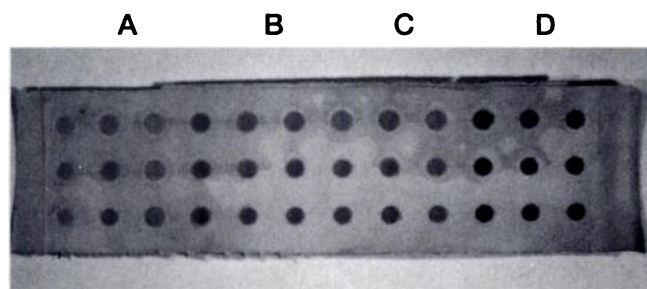
band suggested that apoproteins from the  $\beta$ -VLDL fractions from rabbits fed the PC or OC diets were extensively cross-linked by nondisulfide covalent bonds and that large protein aggregates were formed that involved covalent cross-linking between two or more polypeptide chains. This nonmigrating protein band was difficult to see in the  $\beta$ -VLDL fractions from the plasma of the rabbits fed the PC + antioxidants diet (Figure 3B).

#### Morphologic changes in aorta

The stained sections of the aorta specimen from the rabbits fed the control diet revealed a normal intima (Figure 4A and Figure 5A). The stained section of the aorta from the rabbits fed OC revealed intimal thickening (atherosclerosis) (Figure 4B, Figure 5B). The stained section of the aorta from the rabbits fed PC showed less intimal thickening than in rabbits fed OC (Figure 4C, Figure 5C). The stained section of the aorta from the rabbits fed PC + antioxidants showed little intimal thickening and little atherosclerosis (Figure 4D, Figure 5D).

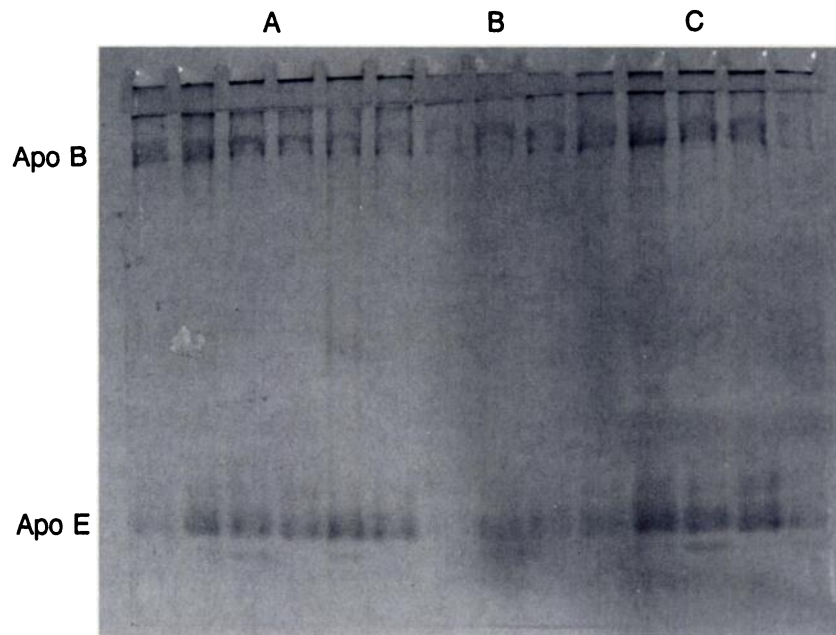
#### DISCUSSION

Our results agree with those of Schwenk et al (28) and Higley et al (29) but not those of Kosykh et al (1), who found that feeding rabbits an OC diet for 6 wk caused a greater elevation of plasma cholesterol than did a PC diet. However, when Kosykh et al extended the feeding period to 12 wk (which was similar to our feeding period), they found that rabbits fed the PC diet reached the same degree of hypercholesterolemia as rabbits fed an OC diet. The plasma cholesterol concentration has been shown to be regulated by the rate of hepatic secretion (30, 31) and clearance of lipoproteins from plasma (32). The rabbits fed PC or PC + antioxidants consumed the same amount of dietary cholesterol. If one assumes that vitamins E and C do not interfere with cholesterol absorption, an equal amount of dietary cholesterol would therefore be delivered to the livers of rabbits fed PC or PC + antioxidants. Because the cholesterol supplied to the hepatocytes is a major determinant of apolipoprotein (apo) B synthesis and secretion of  $\beta$ -VLDL (33), one would expect that the rate of  $\beta$ -VLDL secretion by the livers of rabbits fed PC or PC + antioxidants diet would be similar.



**FIGURE 2.** Binding of gold-LDL conjugates to liver membrane protein. Solubilized liver membrane protein prepared from rabbits fed pure cholesterol + antioxidants (A), pure cholesterol (B), oxidized cholesterol (C), or a control (D) diet were dot-blotted onto nitrocellulose paper (1  $\mu$ g protein). The dot blots were incubated with 20 mg LDL protein/L as colloidal gold-LDL conjugates of human LDL followed by photochemical silver staining.





**FIGURE 3.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of  $\beta$ -VLDL fractions isolated from rabbits fed a pure cholesterol (PC, A), pure cholesterol + antioxidants (B), or oxidized cholesterol (C) diet. The gel was loaded with 10  $\mu$ L of the delipidated and freeze-dried  $\beta$ -VLDL fractions (total volume: 200  $\mu$ L) derived from equal volumes of plasma (1.5 mL). Apo, apolipoprotein.

OC exerts an inhibitory effect on cholesterol catabolism to bile acids through inhibition of hepatic microsomal  $7\alpha$ -hydroxylase (34). Chronic vitamin C deficiency decreased the catabolism of cholesterol to bile acids in guinea pigs (35). This is unlikely to have been the case in our study. If cholesterol catabolism had increased in the livers of the rabbits fed PC + antioxidants, we would have observed a decrease in their hepatic as well as their cell membrane cholesterol concentrations resulting in an elevation of LDL receptor activity (36). This was not the case because the hepatic LDL receptor activity was down-regulated in all the cholesterol-fed rabbits to the same degree as compared with the control group. The rabbits also failed to develop high rates of bile acid production in response to dietary cholesterol (37).

We observed that the *in vitro* binding to the liver membrane protein of the  $\beta$ -VLDL fraction isolated from the plasma of rabbits fed PC + antioxidants was higher than the binding of  $\beta$ -VLDL from rabbits fed PC. This may not necessarily represent the hepatic uptake of  $\beta$ -VLDL *in vivo* that occurs through parenchymal and nonparenchymal cells such as endothelial and Kupffer cells (38). This implies, however, a metabolic difference between these  $\beta$ -VLDL fractions.

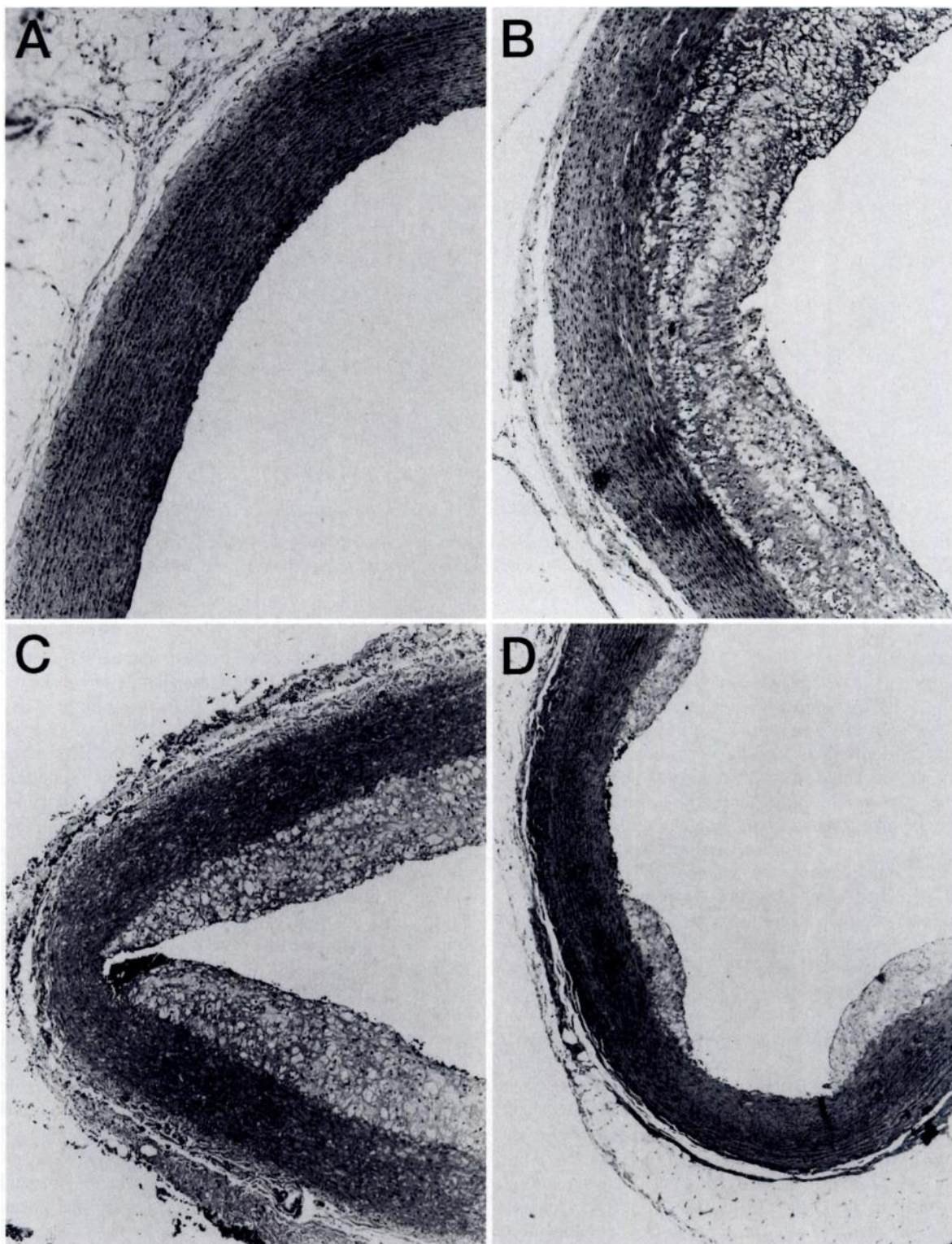
Reactive aldehydes such as MDA have been known to cross-react with proteins (39, 40) to form both intra- and intermolecular cross-linkages of  $\beta$ -VLDL apoproteins by nondisulfide bonds resulting in the dense protein band we noted in the SDS-PAGE of  $\beta$ -VLDL. A nonmigrating protein band was observed previously in lipoproteins oxidatively modified by copper ions (41), nitrogen dioxide in air (42), and cigarette smoke (43).

Similar to MDA-altered LDL (44) and HDL apolipoprotein modified by aldehyde (40), the MDA alteration of  $\beta$ -VLDL in cholesterol-fed rabbits may be responsible for their recognition by scavenger receptors on the macrophages and may explain

cholesterol ester accumulation in human monocyte macrophages. The  $\beta$ -VLDL from hypercholesterolemic rabbits is taken up by macrophages without modification (45). Modification of the arginine residues of  $\beta$ -VLDL also blocked their uptake by apo B, E–LDL receptors of the liver parenchymal cells (46). Antioxidant supplementation of a cholesterol-rich diet may protect  $\beta$ -VLDL from alteration and consequently decrease its atherogenic effect and increase its binding affinity to the hepatic receptors, which may enhance its clearance from the circulation. An increase of lipid peroxidation products (47) in the plasma may play an important role in the accumulation of modified lipoproteins and their involvement in the development of atherosclerotic degeneration of cells of the vessel walls.

Cholesterol feeding increases lipid peroxidation (48, 49) and indications of *in vivo* modification of  $\beta$ -VLDL were noted previously (50, 51). LDL and  $\beta$ -VLDL from hypercholesterolemic rabbits were also found to be more susceptible to *in vitro* oxidation and had lower  $\alpha$ -tocopherol concentrations than LDL from normolipidemic rabbits. This lends support to the possible *in vivo* modification of  $\beta$ -VLDL in hypercholesterolemic rabbits (52).

Lower concentrations of OC found in the PC and PC + antioxidants diets may partially account for the plasma elevation of OC during bioaccumulation during cholesterol feeding because OC can be absorbed and transported in lipoproteins (53). The *in vitro* formation of OC by human blood polymorphonuclear leukocytes and by rat lung macrophages suggests a possible intravascular lipid peroxidation of cholesterol *in vivo* (54). Endogenous sources of OC also include the action of tissue cholesterol enzyme hydroxylases, epoxidases, and dehydrogenases along with intravascular sources. Recent studies have shown that probucol, an antioxidant, reduced the plasma concentration of OC in hypercholesterolemic (55) and WHHL



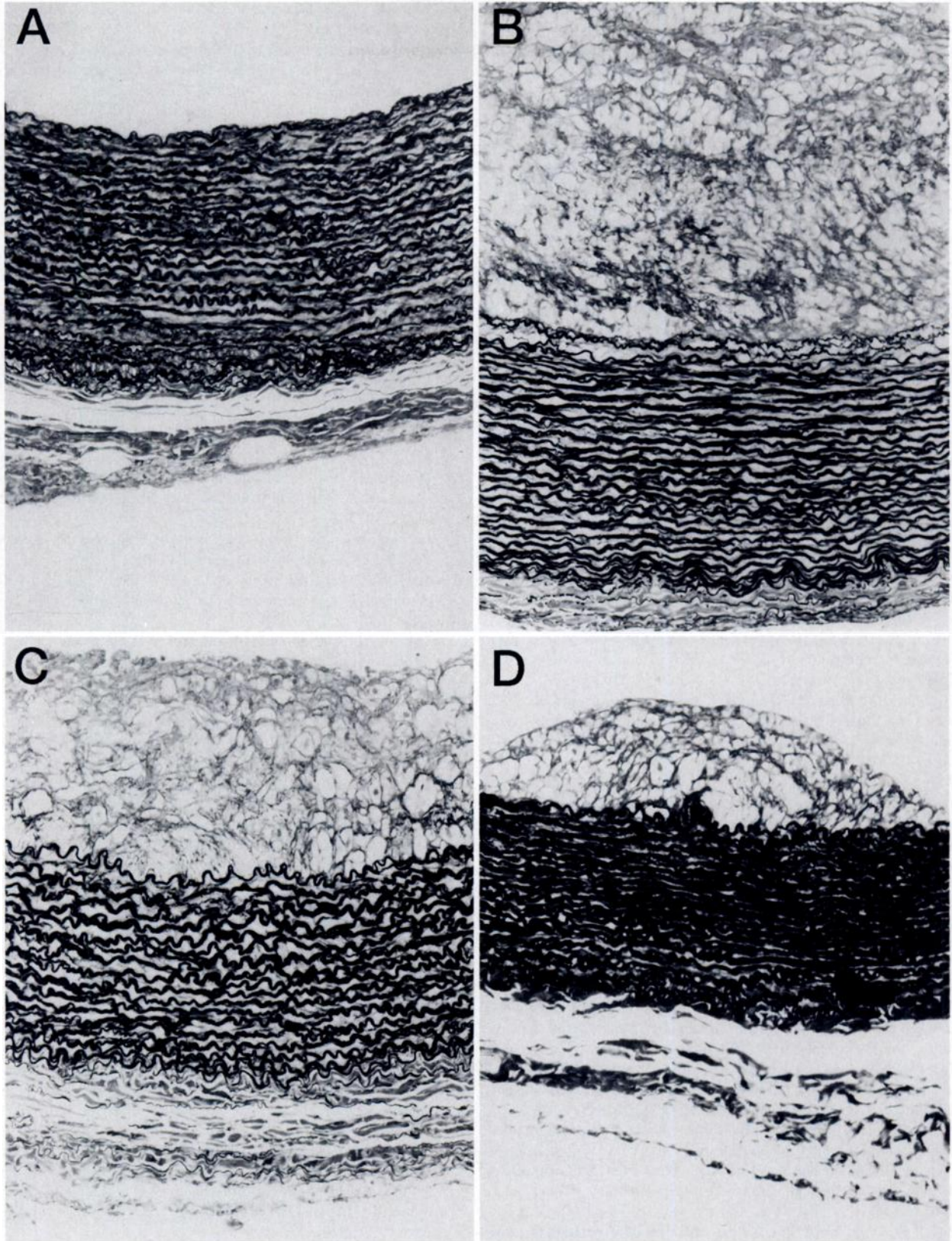
**FIGURE 4.** Hematoxylin and eosin staining of descending aorta from rabbits fed a control (A), oxidized cholesterol (B), pure cholesterol (C), or pure cholesterol + antioxidants (D) diet (original magnification  $\times 140$ ).

(56) rabbits by  $> 50\%$ , thus providing evidence for the in vivo formation of OC and the involvement of free radical mechanisms.

Vitamin E is lipophilic in nature and is primarily transported in lipoproteins. It can provide protection of lipoproteins against

in vivo peroxidation by decreasing free radical oxidative damage to lipids. As suggested previously (55), under supraphysiologic cholesterol concentrations the antioxidant protection by plasma vitamin E may be insufficient to scavenge radical processes within a large lipid substrate pool and cholesterol





**FIGURE 5.** Elastica-Van Gieson-stained cross sections of descending aorta from rabbits fed control (A), oxidized cholesterol (B), pure cholesterol (C), or pure cholesterol + antioxidants (D) diet (original magnification  $\times 50$ ).



may become oxidized (57). Supplementation with antioxidant vitamins E and C may lead to a significant rise in their plasma concentrations, thus preventing or minimizing cholesterol oxidation. The lower plasma concentration of OC may also reduce its cytotoxic and atherogenic effects (58), as we found in the rabbits fed a PC + antioxidants diet.

Vitamin C has a synergistic activity with vitamin E and preserves the  $\alpha$ -tocopherol concentration during oxidative stress by converting the  $\alpha$ -tocopheryl radical back to the reduced state so that it may function again as an antioxidant (59), thereby permitting the continued inhibition of lipid peroxidation. Under oxidative stress, a combination of moderate concentrations of vitamins E and C may be more beneficial than a high concentration of vitamin E alone, which has been shown to increase oxidation and atherogenesis rather than enhance protection (60, 61).

Our results show that supplementation of a cholesterol-rich diet with vitamins E and C still produced hypercholesterolemia in rabbits but the degree of lipid peroxidation and lipoprotein modification was significantly reduced as was the severity of atherosclerosis.

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