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THE EFFECT OF PROCYANIDOLIC OLIGOMERS ON THE COMPOSITION OF NORMAL AND HYPERCHOLESTEROLEMIC RABBIT AORTAS*

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Abstract—Rabbits were fed with normal (group 1 and 2) and cholesterol rich diets (group 3 and 4) concomitantly to a daily peroral administration of 50 mg/kg procyanidolic oligomers (PCO) to groups 2 and 4. After 10 weeks, the cholesterol content of the blood serum and the excised aortic intima-media were significantly higher in groups 3 and 4 than in groups 1 and 2.

The DNA, hydroxyproline, uronic acid contents were similar in aortic dry weight basis in all four groups.

The intima-media samples were extracted successively with 0.15 M NaCl, 0.02 M sodium phosphate pH 7.4 (NaCl extract) and with 4 M guanidinium chloride, 0.05 M sodium acetate pH 5.8 prior (G1 extract) and following (G2 extract) hydrolysis of the collagen with collagenase.

The cholesterol contents of G1 extracts were higher in groups 2 and 4 than in groups 1 and 3.

The cholesterol content of aortic elastin increased with cholesterol feeding (group 3). With simultaneous administration of cholesterol and PCO the cholesterol content of aortic elastin in group 4 was significantly lower than in group 3.

The uronic acid contents increased in G1 extracts and in the collagenase digest with PCO treatment of both normal and hypercholesterolemic rabbits. The ratio of dermatan-sulphate to chondroitin-sulphate decreased with hypercholesterolemia (group 3) and with PCO (group 2 and 4). The parallelism between increased cholesterol and uronic acid contents and modified glycosaminoglycan composition in G1 extract, indicate that the interaction of cholesterol with macromolecules of the aorta can be modulated by PCO. This drug modifies the extractibility of aortic cholesterol and glycosaminoglycans and reduces the association of cholesterol to elastin.

Accumulation of cholesterol and lipids in the aorta and the modified metabolism of aortic smooth muscle cells during hypercholesterolemia were recognized as atherogenic factors.

Complexes of glycosaminoglycans and LDL† as well as of proteoglycans and LDL were present at physiological salt concentration in the extracts and in the collagenolytic and elastolytic hydrolysates of atherosclerotic aortas [1–3]. These results and the *in vitro* interactions of glycosaminoglycans [4], proteoglycans [5, 6], glycoproteins [7] and elastin [8] with lipoproteins indicated that different extracellular matrix components are involved in the deposition of lipids.

Procyanidolic oligomers from grape seeds were shown to modify the biochemical properties of blood vessel walls [9, 10]. However, it is not yet known if the evolution of hypercholesterolemia could be affected by this drug. To obtain further insight in the role of the extracellular matrix in the association of lipids with the aortic intima-media, we investigated the extractibility of cholesterol and glycosaminoglycans from the aorta of rabbits fed with normal and cholesterol rich diets as well as the effect of peroral administration of PCO in normal and hypercholesterolemic rabbits.

MATERIALS AND METHODS

Chemicals. Guanidinium hydrochloride, benzamidine hydrochloride and ε-aminocaproic acid were purchased from Merck, Darmstadt. Ethylenediaminetetraacetic acid (EDTA) disodium salt, from Prolabo, Paris, France. Samples of GAGs standards were generous gifts from Dr. J. A. Cifonelli and Dr. Matthews (University of Chicago, U.S.A.). Flavonoids from grape seeds (procyanidolic oligomers (PCO), Endothelon®) were obtained from Labaz Laboratories, Paris, France. All other chemicals used were of analytical grade or best grade available.

Enzymes. Collagenase, CLSPA chromatographically purified from *Clostridium hystolyticum* (425 units/mg) was purchased from Worthington Biomedical Corp. (Freehold, NJ, U.S.A.). Pronase (*Streptomyces griseus*) type IV, chondroitin ABC lyase (*Proteus vulgaris*) and chondroitin AC lyase (*Arthrobacter aurescens*) were from Sigma Chemicals Corp., Saint-Louis, MO, U.S.A. Hyaluronidase (*Strep-*

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† Abbreviations: PCO, Procyanidolic oligomers, Endothelon®; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; DS, Dermatan sulfate; CS, chondroitin sulfate; HS, heparan sulfate; GuHCl, guanidinium hydrochloride.

tomyces hyaluroniticus, 100 TRU/mg) was obtained from Seikagaku Kogyo Co., Tokyo, Japan.

Diet and treatment of the rabbits. Thirty-two male New Zealand rabbits of 2.2–2.3 kg body weight were used in 4 groups of 8 animals each:

Group 1 and 2 were fed with normal UAR 112 diet 100 g/day. Group 3 and 4 were fed with UAR 112 diet supplemented with 1% cholesterol. Group 2 and 4 were treated perorally with procyanidolic oligomers (PCO) 50 mg/kg daily.

Blood samples were taken every 2 weeks after fasting for 16 hr. After 10 weeks, the rabbits were sacrificed by bleeding. Aortas were excised, cleaned from adventitia, opened longitudinally, and rinsed with sterile 0.9% NaCl.

Extraction of the aortas. In the hypercholesterolemic rabbits (groups 3 and 4), the macroscopically visible normal regions and areas with fatty streaks were not separated. The intima-media was cut into about 1 mm^2 fragments in sterile 0.9% NaCl, containing protease inhibitors: 0.1 M ε-aminocaproic acid, 0.005 M benzamidine and 0.01 M EDTA.

The fragments from the aortas were distributed into two aliquots. About 50 mg fresh weight was delipidated with chloroform–methanol 2:1 v/v according to Folch *et al.* [11] and the amount of cholesterol was determined in the extracts. The DNA, uronic acid and hydroxyproline contents of the delipidated residue were determined as described in the analytical methods.

A second aliquot of about 200 mg fresh weight was extracted according to the scheme outlined in Fig. 1. All the extracting agents were supplemented with protease inhibitors as above. The fragments were homogenized in 1 ml of the 0.15 M NaCl, 0.02 M sodium phosphate buffer pH 7.4 on an ice bath with a tissue grinder (Kontes, NJ, U.S.A.) three times for 1 min, until a suspension was formed. The suspensions were shaken at 4° overnight and centrifuged 30 min at 10,000 *g* in Beckman JA-21 centrifuge.

The supernatants were removed (NaCl extract). The sediments were extracted with 4 M guanidinium chloride, 0.05 M sodium acetate pH 5.8, at 4° overnight, and centrifuged as indicated above. Two successive extractions were carried out (G1 extracts). The pellet remaining after the extraction with 4 M GuHCl was washed with distilled water, until free of Cl^- ions, and hydrolyzed with bacterial collagenase in 1 ml 0.05 M Tris 0.01 M $CaCl_2$ buffer at pH 7.8 for 48 hr at 37°. 2 mM phenylmethanesulfonylfluoride and 10 mM *N*-ethylmaleimide were added to the samples to avoid a non-specific proteolysis [12].

The hydrolysate was centrifuged at 4000 *g* at 4°, and the supernate removed. The residue of the collagenase digest was washed with distilled water, the suspension centrifuged, the washings discarded.

The washed pellets were re-extracted with 4 M guanidinium chloride as indicated above (G2 extract). The residue was washed with distilled water to remove the guanidium chloride and was analysed for aminoacid composition.

Aliquots of extracts G1 and G2 were dialysed against running tap water and distilled water for the determination of cholesterol, hydroxyproline, uronic acid and protein contents and for the identification of glycosaminoglycans.

Glycosaminoglycans. The samples were hydrolysed successively with bacterial collagenase in the conditions described above and with pronase at pH 7.8 for 48 hr at 48°. Trichloracetic acid was added to the hydrolysates to 10% final concentration. The samples were centrifuged in a hematocrite centrifuge (Janetzki, Leipzig), and dialysed exhaustively against running tap water and distilled water. The dialysates were lyophilized.

Identification of GAGs. The samples were hydrolysed with hyaluronate lyase (EC 4.2.2.1) in 50 μl 0.1 M sodium acetate buffer at pH 5.4 and 60° for 60 min.

The pH of the samples was adjusted to 7.6 with 1% Na_2CO_3 and the samples were treated successively with 0.3 U chondroitin AC lyase (EC 4.2.2.5) and 0.3 U chondroitin ABC lyase at 37° for

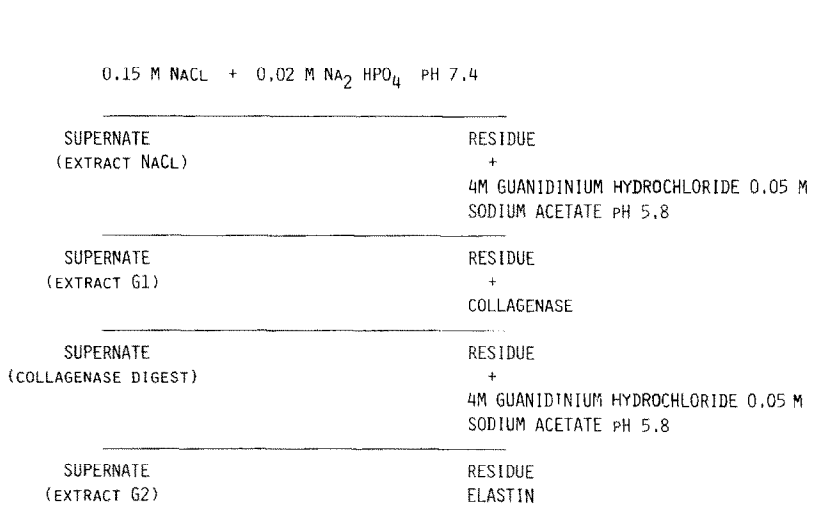

Fig. 1. Scheme of the extraction of the intima-media from rabbit aorta.

5 hr [13]. Samples of 40 μl containing about 10 μg uronic acid were exposed to 10 μl 18% $NaNO_2$ in water–acetic acid 1:1 (v/v) at room temperature for 90 min to degrade heparan sulphate [14].

The hydrolyzates were investigated by cellogel electrophoresis.

Analytical methods. Proteins were determined according to Lowry *et al.* [15], using bovine serum albumin as a standard, uronic acid according to Bitter and Muir [16] and hydroxyproline according to Bergman and Loxley [17]. The hydroxyproline content of the collagenase digest was converted to collagen using the factor of 7.46 calculated on the basis of an average hydroxyproline content of 13.4% in mammalian collagens.

Cholesterol was determined by enzymic test according to Allain *et al.* [18]. HDL in blood serum was estimated from the cholesterol content determined prior and following the precipitation of VLDL–LDL by dextran sulfate [19]. The elastin was hydrolysed with 6 N HCl in Pyrex tubes sealed *in vacuo*, the hydrolysate was evaporated to dryness, over KOH pellets under reduced pressure. The aminoacid composition of the hydrolysate was determined on an LKB aminoacid analyser (LKB, Bromma, Sweden).

The proteins were separated by polyacrylamide slab gel electrophoresis in 0.1% SDS with a linear gradient from 5% to 20% gel concentration in LKB 2001 electrophoresis unit (LKB, Bromma, Sweden). The samples were dissolved in 6 M urea pH 8.3 and reduced with dithiothreitol prior to electrophoresis [20]. The electrophoresed proteins were stained with 0.25% Coomassie Blue [21]. The electrophoretic separation of GAGs was carried out on cellulose acetate gel (Cellogel, Chemetron, Milano, Italy) in 0.1 M calcium acetate pH 5.0 [22] and in 0.1 M HCl [23]. The GAGs were stained with 0.2% alcian blue according to Bartold *et al.* [24].

The distribution of GAGs was estimated by photodensitometric scanning of the electrophoresed GAGs on cellogel at 560 nm with Sebia gel scanning system.

RESULTS

Blood serum

The cholesterol content of the blood increased continuously with the cholesterol feeding up to 10 weeks (Fig. 2). The HDL content was constant (0.34 ± 0.09 g/l) in the investigated groups throughout this period. Administration of PCO did not modify the blood cholesterol levels in rabbits fed with normal or with cholesterol rich diets. To esimate the modification of the lipoprotein pattern with hypercholesterolemia, the ratio of total cholesterol to HDL cholesterol was calculated (Table 1).

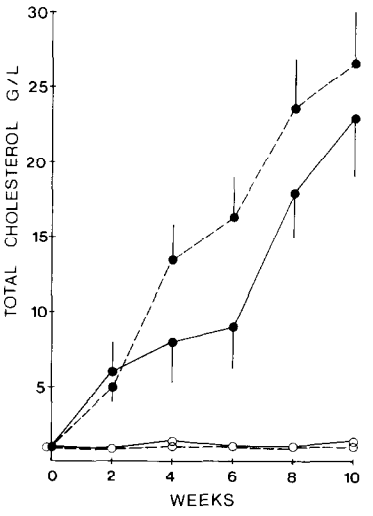

Fig. 2. Serum cholesterol concentration (mean ± S.E.) in rabbits maintained on: the standard diet (○——○), standard diet + PCO (○– –○), 1% cholesterol diet (●——●), 1% cholesterol diet + PCO (●– –●). The blood samples were taken every 2 weeks after 16 hr of starvation.

No significant difference was found in the total cholesterol/HDL cholesterol ratios between control rabbits and rabbits maintained on normal diet and treated with PCO.

The ratio of the total cholesterol to HDL cholesterol increased with hypercholesterolemia and this ratio was not modified with PCO in hypercholesterolemic rabbits.

Aorta

Composition. No macroscopically visible lesions were detected in the aortas from control rabbits. Lipid depositions in the intima were observed in both PCO-treated and non-treated hypercholesterolemic rabbits.

No significant differences were found in the hydroxyproline, uronic acid and DNA contents of aortas between the four experimental groups.

The cholesterol contents of the intima-media are

Table 1. Cholesterol content of aortic intima-media and total cholesterol HDL cholesterol ratio in the serum of rabbits after 10 weeks of different diets (mean ± S.D.)

Treatment of rabbits	Serum Total cholesterol / HDL cholesterol	Aorta Cholesterol (mg/g dry tissue)
None (controls)	2.53 ± 0.62	9.0 ± 1.7
PCO	1.78 ± 0.34	10.2 ± 6.7
Cholesterol	72.2 ± 6.2*	92.6 ± 21.8*
Cholesterol + PCO	77.9 ± 5.5*	73.6 ± 16.4*

* $P < 0.01$ compared with control and PCO groups.

given in Table 1. In rabbits kept on cholesterol rich diet for 10 weeks, the aorta cholesterol content increased about 10-fold as compared to the controls. Administration of PCO to rabbits fed with normal or with cholesterol rich diet did not modify the deposition of cholesterol in the intima-media.

Extraction of aortas

Proteins. The intima-media samples were extracted at increasing ionic strengths as shown in Fig. 1.

The distribution of the proteins in the extracts is shown in Table 2. The amount of proteins extracted at physiological salt concentrations (0.15 M NaCl extract) increased significantly in the hypercholesterolemic aortas. PCO treatment did not modify the protein content of the extracts from the control and hypercholesterolemic rabbits.

Determination of the hydroxyproline contents of the extracts indicated that about 80% of the collagen content of the extracted samples were recovered in the supernates of the collagenase digest.

The final extraction residue was analysed as elastin. It accounted for 70% of the non-collagenous proteins in the intima-media.

The ratio of collagen to elastin decreased in hypercholesterolemic aortas in the PCO treated and non-treated groups.

Polyacrylamide gel electrophoresis indicated a different protein compositon in the NaCl extract from normal and hypercholesterolemic aortas. A protein band migrating with an apparent molecular weight of 33 K was present in the NaCl extracts from hypercholesterolemic aortas. This component was absent in the NaCl extract from normo-cholesterolemic aortas. However, a protein band with electrophoretic mobility corresponding to 33 K was present in blood serum.

The electrophoretic pattern of proteins in the molecular weight range of 30–150 K in the G1 extract were similar in all the groups.

Cholesterol. The cholesterol content of the fractions is given in Table 3. On the basis of extracted proteins, the highest amount of cholesterol was removed with 0.15 M NaCl, in all four experimental groups. Extractions with 0.15 M NaCl proved to be insufficient to remove the total cholesterol content from the control and hypercholesterolemic intima-medias.

Guanidinium chloride solubilized further amounts of cholesterol prior to and following hydrolysis of collagen. The cholesterol found in the elastin fraction suggests that its association with the elastic fibers is mediated by bonds resisting H-bond dissociating agents.

PCO administered to rabbits fed with normal diet significantly increased the cholesterol content of fractions G1 and G2.

PCO treatment resulted however in a decrease of the cholesterol content in NaCl and G2 extracts, and in elastin of cholesterol fed animals as compared to the cholesterol fed and untreated rabbits. The increase of cholesterol in the extracts at high ionic strengths indicates a decreased extractibility of cholesterol as a consequence of PCO-treatment. The results in Table 3 show also that the association of

Table 2. Extraction of the aortic intima-media. The amount of proteins in NaCl, G1, G2 extracts and the amount of collagen and elastin are expressed as mg/g dry tissue

Treatment of rabbits	Fractions					Elastin (mg)
	0.15 M NaCl	G1	G2	Collagen	Elastin	Collagen (mg)
None (controls)	48.3 ± 6.7	92.8 ± 13.7	75.4 ± 7.2	255.4 ± 19.2	510.1 ± 24.1	2.00
PCO	56.4 ± 1.7	75.5 ± 4.6	86.4 ± 6.7	226.5 ± 18.5	487.2 ± 8.5	2.15
Cholesterol	87.6 ± 8.9*	78.0 ± 7.0	70.4 ± 8.5	256.5 ± 6.5	374.0 ± 23.4*	1.46
Cholesterol + PCO	71.4 ± 7.2†	81.7 ± 9.2	77.2 ± 2.1	245.2 ± 16.0	383.0 ± 28.3*	1.57

The collagen content was calculated from the hydroxyproline content of the collagenase digest. The scheme of extractions and the designation of the extracts are outlined in Fig. 1.

$P < 0.05$ as compared to: *control or OPC group. †cholesterol group.

Table 3. Cholesterol content (mean ± S.D.) of the fractions from intima-media of rabbit aorta

Treatment of rabbits	0.15 M NaCl	Fractions Collagenase digest		G2	Elastin
		G1			
None (controls)	75.6 ± 3.5	19.2 ± 1.5	ND	16.9 ± 5.6	6.0 ± 0.9
PCO	58.9 ± 0.5*	28.1 ± 5.1*	ND	28.8 ± 1.5*	4.9 ± 0.3
Cholesterol	770.4 ± 17.8	87.4 ± 22.4	ND	173.1 ± 39.8	42.8 ± 2.7
Cholesterol + PCO	641.0 ± 15.0†	124.5 ± 24.5	ND	100.0 ± 9.3*	21.1 ± 3.7†

The scheme of extraction and the designation of the extracts are outlined in Fig. 1. Results are expressed as mg/g protein in each extract.
$P < 0.01$ as compared to: *control group; †cholesterol group.
ND: not detectable.

Table 4. Uronic acid content of the fractions obtained by extraction of intima-media of rabbit aorta

Treatment of rabbits	0.15 M NaCl	Fractions Collagenase Digest		G2
		G1		
None (controls)	51.9 ± 8.5	8.1 ± 1.7	19.0 ± 1.1	2.6 ± 0.9
OPC	59.5 ± 5.0	18.6 ± 0.3	59.7 ± 1.8*	2.3 ± 0.1
Cholesterol	23.6 ± 4.7	11.5 ± 2.18	21.2 ± 3.0	2.2 ± 0.5
Cholesterol + OPC	33.9 ± 4.2†	20.7 ± 1.8†	48.9 ± 8.5†	1.4 ± 0.3

The scheme of extractions and the desgination of the extracts are outlined in Fig. 1. Results are expressed as mg/g protein in the individual fractions.
The uronic acid content of the supernate of the collagenase digest are expressed as mg/g hydroxyproline.
$P < 0.05$ as compared to: *control group; †cholesterol group.

cholesterol with extracellular matrix components in normal and hypercholesterolemic aortas can be modulated by peroral administration of PCO.

Glycosaminoglycans. The uronic acid contents of the extracts are given in Table 4. About 40%, 21% and 20% of the aortic uronic acids were recovered in the NaCl, G1 extracts and collagenase digest respectively. Traces of uronic acids were detected in the fraction containing elastin.

On the basis of extracted proteins, the amount of uronic acid decreased in the NaCl extract with hypercholesterolemia. Administration of PCO to

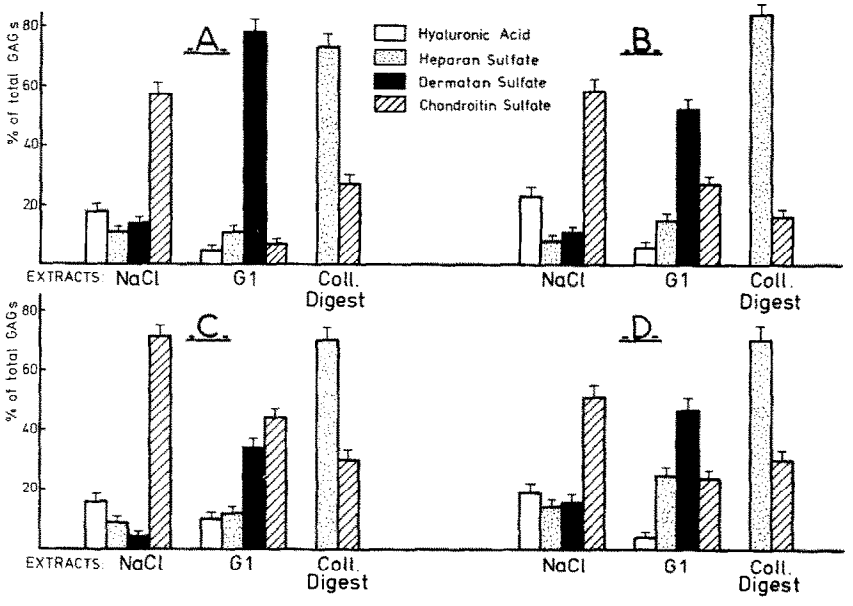

Fig. 3. The distribution of the glycosaminoglycans in the rabbit aorta extracts. The scheme of the extraction and the designation of the extracts are outlined in Fig. 1. The results are expressed as % of total GAGs in each extract. A—control (standard diet); B—standard diet + PCO; C—1% cholesterol diet; D—1% cholesterol diet + PCO. The bars represent the S.E.M. of three photodensitometric determinations.

control and cholesterol-fed rabbits increased the uronic acid contents in G1 extract and in the collagenase digest.

Hypercholesterolemia of PCO treatment induced different modifications in the distribution of uronic acids in the extracts. Unlike hypercholesterolemia, PCO treatment increased the amount of uronic acid associated with collagen.

The distribution of the GAGs in the extracts was investigated by electrophoresis and specific enzymic assay. The NaCl, G1 extracts and the supernate of the collagenase digest exhibited different GAG compositions as shown schematically on Fig. 3 A–D.

In the NaCl extract, and the collagenase digest, chondroitin sulphate and heparan sulphate were the major GAG components in the four experimental groups.

Hypercholesterolemia and PCO treatment modified the composition of G1 extract. The ratio of dermatan sulphate to chondroitin sulfates was about 10:1 in the control groups (Fig. 3A). This ratio changed to 2:1 in groups treated with PCO (Fig. 3, B–D) and it decreased to 1:1 with hypercholesterolemia (Fig. 3C). The data in Table 4 and in Fig. 3 A–D indicate that qualitative changes were observed at the level of GAGs in the G1 extract with hypercholesterolemia. Administration of PCO to both normo- and hypercholesterolemic rabbits produced qualitative and quantitative changes in the GAG-patterns.

Considering the uronic acid contents of the collagenase digest (Table 4) and the data in Fig. 3 A–D, administration of PCO produced an increase of the amount of the heparan sulphate and chondroitin sulphate associated with collagen both in control and in cholesterol-fed rabbits.

DISCUSSION

The extractibility of extracellular matrix components depends on several factors such as interactions with each other or their anatomical localization. It is likely, that association of blood serum components to the aortic wall could modify the solubility of the macromolecular constituents of the aorta *in vitro*.

Aortic intima-media exhibits a characteristic glycosaminoglycan composition [25, 26]. Thus, the release of these macromolecules by the different extracting agents could be an indication of the modifications of the biochemical properties of the aorta produced by the experimental conditions. It also can be used as a criterion to evaluate a potential effect a drug on hypercholesterolemic blood vessel wall.

Starting from these premises, the deposition of cholesterol in aorta was investigated in terms of the distribution of cholesterol in aorta extracts obtained at increasing ionic strengths.

The rise of the blood cholesterol levels resulted as expected in an increase of the cholesterol content of the aorta wall. The analytical finding on the cholesterol contents of the extracts confirmed that extractions at physiological salt concentration remove only part of the cholesterol of rabbit aortas as was shown previously in human [27] and rat [28] aortas. The electrophoretic patterns of proteins in the NaCl extract and in the blood serum suggests that the NaCl extract from hypercholesterolemic aortas contains serum proteins. Accordingly, the uronic acid contents were lower in the NaCl extracts from hypercholesterolemic aortas, than from the controls.

The cholesterol resisting extractions with 0.15 M NaCl exhibited a characteristic distribution pattern in the fractions obtained from both normal and hypercholesterolemic aortas. Cholesterol was present in the guanidinium chloride extracts and in elastin, but it was not detectable in the supernatant of the collagenase digest.

Unlike the GAG composition of NaCl extract, the GAG pattern of G1 extract was modified with hypercholesterolemia. One explanation of the modified chondroitin sulphate/dermatan sulphate ratios in the G1 extract obtained from the hypercholesterolemic aortas could be a modification of the composition of aortic proteoglycans or an altered extractibility of the proteoglycans due to their interactions with lipoproteins. The recovery of relatively high amounts of cholesterol in the G2 extract and in elastin from cholesterol-fed rabbit aortas indicates that the accumulation of cholesterol cannot be attributed exclusively to the interactions of proteoglycans with liproproteins. We reported previously that guanidinium chloride extracts obtained prior to and following hydrolysis of collagen contain glycoproteins insoluble at low ionic strengths [29]. It was shown that aortic structural glycoproteins interact with LDL *in vitro* [7]. Considering the distribution pattern of cholesterol in the extracts and previous results [27, 28], it seems likely that besides glycosaminoglycans and elastin, structural glycoproteins also interact with cholesterol or with lipoproteins transporting cholesterol in the aorta.

The findings indicate also that cholesterol associates more strongly with elastin than with collagen or heparan sulphate. The present results further confirm the existence of matrix-bound cholesterol in the intima-media of rabbit aorta.

Cholesterol interacting with elastin induces conformational changes in the elastin polypeptide sequences [30]. It was postulated that one of the biological consequence of the elastin–lipid interactions could be an activation of elastolysis [31].

An increase of the GAG content in G1 extracts and in collagenase digests and modified DS/CS ratio in G1 extract were observed as a consequence of peroral administration of PCO to normal and hypercholesterolemic rabbits. As the uronic acid contents of aorta on a dry weight basis did not change, the modification induced by the treatment seems to be a decreased extractability of proteochondroitin-dermatan-sulphate from aortas of PCO treated rabbits. As heparan sulphate is the major GAG component of the collagenase digest (Table 4, Fig. 3 A–D), the increased uronic acid content of this fraction is an indication of a stronger association of HS to collagen in PCO treated aortas.

Although the penetration of cholesterol into the aorta during hypercholesterolemia was not reduced by PCO treatment, significantly lower amounts of cholesterol were bound to aortic elastin in the PCO

treated, hypercholesterolemic rabbits, than in the untreated hypercholesterolemic rabbit aortas.

These findings suggest that the selective deposition of cholesterol in the arterial tissue is modified by perorally administered PCO and more specifically that PCO-treatment decreases the cholesterol–elastin interaction.

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