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Influence of Chondroitin Sulfate Charge Density, Sulfate Group Position, and Molecular Mass on Cu^{2+}-Mediated Oxidation of Human Low-Density Lipoproteins: Effect of Normal Human Plasma-Derived Chondroitin Sulfate[1]

Nicola Volpi*,[2] and Patrizia Tarugi†

*Department of "Biologia Animale," Biological Chemistry Section, and †Department of Biomedical Sciences, General Pathology Section, University of Modena, via Berengario 14, 41100 Modena, Italy

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The effects of chondroitin sulfate samples with decreasing charge densities, different 4-sulfate/6-sulfate ratios, and various molecular masses on Cu^{2+}-induced oxidation of human low-density lipoprotein (LDL) were evaluated by monitoring conjugated diene formation and the tryptophan fluorescence kinetics. Low-sulfated chondroitin sulfate (CS) from beef trachea had a very strong protective antioxidant effect. Quite similar behavior was observed for CS from pig trachea, and a fructose-containing polysaccharide with a chondroitin backbone from *Escherichia coli* was also strongly protective as to LDL oxidation. CS samples with decreasing charge densities proved effective in inhibiting LDL oxidation. A totally desulfated sample still exhibited a great capacity to protect LDL against oxidation. CS-4-sulfate samples (sulfate to carboxyl ratio of 0.62, about 65% 4-sulfate groups and 5% 6-sulfate groups) retained great ability to inhibit the Cu^{2+}-mediated human LDL oxidation. CS fractions with different molecular masses were examined as possible inhibitors of LDL oxidation. Samples with molecular masses lower than about 8,570 (13-15 disaccharide units) were unable to protect human LDL from Cu^{2+}-induced oxidation. Similar results were obtained on studying the degradation of tryptophan residues of the LDL protein moiety resulting from Cu^{2+} complexation through amino acid residues. A low-sulfated CS (sulfate to carboxyl ratio of 0.41, a molecular mass of about 15,600) having effective anti-oxidant properties as to metal-induced LDL oxidation was isolated from normal human plasma. The protective capacity as to Cu^{2+}-mediated LDL oxidation of CS is discussed in relation to its structure, also considering the physiological role of plasma CS in relation to factors that can alter its properties.

Key words: chondroitin, glycosaminoglycans, low-density lipoprotein.

Oxidatively modified low-density lipoproteins (LDLs) play a critical role in atherogenesis. LDL that has been oxidized by exposure to trace metals (Cu^{2+} and Fe^{2+}), lipoxygenase or endothelial cells is rapidly taken up by macrophages and smooth muscle cells. This process, independent of the intracellular cholesterol level, can lead to the formation of foam cells, with possible build up of atherosclerotic plaques (*1-3*). Moreover, oxidized LDL may cause thrombosis, acting on both endothelial cells and platelets (*4*). Agents that decrease the oxidation of LDL *in vitro*, such as ascorbate or probucol, exert a marked antiatherogenic effect in experimental animals (*5*).

The atherosclerotic arterial wall contains increased levels of copper and iron ions, which contribute to the oxidation of LDL (*1*) through the generation of free radicals. In this compartment, LDL may also interact with proteoglycans that are formed through various glycosaminoglycan (GAG) chains, mainly chondroitin sulfate (CS), dermatan sulfate, and heparan sulfate (*6, 7*). The modification in the Cu^{2+}-mediated oxidation kinetics of LDL by heparin (*8*) and CS (*9*) was recently shown, and although several studies revealed a specific LDL protein-GAGs interaction (*10, 11*), few data are available on the effect of polysaccharides with various structures and properties on ions-catalyzed LDL oxidation.

GAGs consist of alternating copolymers of uronic acids and amino sugars, and they are very heterogeneous polysaccharides in terms of relative molecular mass, charge density, and physico-chemical properties (*3, 12*). The heterogeneity of the backbone of sulfated GAGs is increased by the presence of sulfate groups in varying amounts and *O*-linked in different positions. On this basis, we investigated the capacities of various natural CS purified from animal tissues and of modified polysaccharides to alter the Cu^{2+}-mediated oxidation of human LDL. Therefore, we studied the effects of CS species desulfated to

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[2] To whom correspondence should be addressed. Tel: +39 59 243566, Fax +39 59 226769, E-mail: Volpi@unimo.it

Abbreviations: LDL, low-density lipoprotein; GAG, glycosaminoglycan; CS, chondroitin sulfate.

different extents and fractions with decreasing molecular masses in an attempt to determine the properties and characteristics that this GAG must possess to effectively inhibit human LDL oxidation *in vitro*. This last point could be of some importance in clinical applications as transition metals play a role at several levels of the lipid oxidation of LDL in addition to the initial stages (*13*), and lipid peroxidation is inhibited by iron chelation (*14*). Furthermore, we evaluated the capacity of a low-sulfated CS isolated from normal human plasma to protect against LDL oxidation. This is of importance for understanding some of the complex factors involved in protection against LDL oxidation *in vivo* under physiological and pathological conditions. As an experimental model, highly reproducible LDL oxidation mediated by Cu^{2+} was used (*15*), and several characteristic indices were determined. The effects of CS and derivatives on tryptophan residues of the protein moiety of LDL during oxidation were also investigated.

MATERIALS AND METHODS

Papain [EC 3.4.22.2], trypsin [EC 3.4.21.4], collagenase [EC 3.4.24.3], DNase I [EC 3.1.21.1], RNAse A [EC 3.1.27.5], and probucol were purchased from Sigma (St. Louis, USA). Chondroitinase ABC from *Proteus vulgaris* [EC 4.2.2.4] and chondroitinase AC II Arthro from *Arthrobacter aurescens* [EC 4.2.2.5] were obtained from Sigma. Unsaturated chondroitin sulfate/dermatan sulfate disaccharides were obtained from Seikagaku Corporation, Tokyo.

Bio-Gel P10 resin (fine 200-400 mesh, 1,500-20,000 Da for globular biomolecules) was from Bio-Rad, Richmond, CA, USA. Sephadex G-75 (3,000-80,000 Da for globular biomolecules) was from Pharmacia LKB Biotechnology, Uppsala, Sweden. Ecteola-cellulose resin (condensation product of epichlorohydrin, triethanolamine, and cellulose, cross-linked fibers; capacity: 0.3-0.4 meq/g, particle size: 0.05-0.2 mm) was from Serva, Heidelberg, Germany. Cation-exchange resin Amberlite IR-120 in the Na^+ form and anion-exchange resin Amberlite IRA-400 in the OH^- form were from Supelco, Bellefonte, USA. Chelex 100 chelating resin was from Bio-Rad. Column Protein Pak 125 (300×7.8 mm; particle size: 10 μm) and Protein Pak 300 (300×7.5 mm; particle size: 10 μm) were from Waters, Milford, USA. Ten micrometer Spherisorb SAX (trimethylammoniopropyl groups $Si\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}N^+(CH_3)_3$, Cl^- form) was from Phase Separations Limited, Deeside Industrial Park, Deeside Clwyd, UK. All the other reagents were of analytical grade.

Glycosaminoglycans—CS from beef trachea (BTCS I), pig trachea (PTCS), and shark cartilage (SCCS) was donated by IBSA (Institut Biochimique SA, Lugano, Switzerland). A second preparation of bovine trachea CS (BTCS II) was purified as reported (*16*).

A fructose-containing polysaccharide with a chondroitin backbone purified from *Escherichia coli* U1-41 (05:K4:H4, Freiburg collection number 2616) (*17*) was a kind gift from Prof. Klaus Jann (Max-Planck-Institut für Immunbiologie, Freiburg, Germany). This polysaccharide consists of a backbone with the structure, -3)-β-D-glucuronyl-(1,4)-β-D-*N*-acetylgalactosaminyl (1-, to which β-fructofuranose is linked at C-3 of the glucuronic acid (*17*).

Desulfated CS species were produced according to Nagasawa *et al.* (*18*). The pyridinium salt of CS (BTCS II) was desulfated in dimethyl sulfoxide containing 10% methanol at 80°C for different times, dialyzed against distilled water, and then lyophilized. According to Nagasawa *et al.* (*18*), no appreciable depolymerization of the products was observed on high-performance size-exclusion chromatography (*19*) (see below). The progress of desulfatation was examined by degradation with lyases, and evaluation of non-sulfated and mono-sulfated disaccharides by HPLC (see below).

Regioselective 6-*O*-desulfatation of CS was performed according to Matsuo *et al.* (*20*) by treating the pyridinium salt of CS (BTCS II) in the presence of *N,O*-bis(trimethylsilyl)acetamide in dry pyridine at 80°C for different times, dialyzed against distilled water, and then lyophilized.

One gram of beef trachea CS (BTCS II) was fractionated on a 1×100 cm column packed with about 65 ml of Bio-Gel P10 resin with 0.15 M NaCl as the eluant at the flow of 1 ml/5 min. Fractions of 2 ml were collected, and then 2 volumes of ethanol was added, followed by storage at 4°C for 24 h. After centrifugation at $2,500 \times g$ for 15 min, the pellets were dried at 60°C. Fractions with a low molecular mass range, from about 19,000 to 11,000, were recovered. One gram of CS was also fractionated on a 1×100 cm column of Sephadex G-75 with elution with 0.15 M NaCl at the flow of 1 ml/10 min. Fractions of 2 ml were collected and CS species were recovered as reported above. Fractions with a low molecular mass range, from about 10,000 to 1,000, were obtained. Fractions with molecular masses greater than about 10,000 were recovered in the void volume. The peak molecular mass was determined as previously reported (*19*) by high-performance size-exclusion chromatography against a calibration curve constructed with glycosaminoglycan standards.

All polysaccharide preparations were further submitted to purification on Chelex 100 chelating resin to eliminate possible trace metals, in particular Cu^{2+}.

Normal Human Plasma-Derived Chondroitin Sulfate—CS was purified from normal human plasma as previously reported (*21*). Two liters of human plasma from healthy volunteers was submitted to exhaustive proteolytic digestion, β-elimination in alkaline medium, purification on anion-exchange resin, and precipitation with organic solvents. Heparin and heparan sulfate were degraded by nitrous acid treatment, and CS was further purified on anion-exchange resin, with elimination of proteins by precipitation with 10% trichloroacetic acid. This preparation of human plasma polysaccharides (2.8 mg) comprised about 100% chondroitin sulfate. The purity of the sample and properties were checked as reported above (*21*).

General Analytical Methods for Glycosaminoglycans—The peak molecular mass and polydispersity of GAG samples were determined by high-performance size-exclusion chromatography according to Volpi and Bolognani (*19*). The amount of the constitutive disaccharides, and the sulfate-to-carboxyl ratio of galactosaminoglycan preparations and desulfated CS were determined by degradation with chondroitinase ABC and chondroitinase ACII, and separation of unsaturated disaccharides by SAX-HPLC (*16*).

Preparation of Low-Density Lipoprotein—Venous blood samples were obtained from healthy donors at the S. Agostino Hospital (Modena) after overnight fasting.

Plasma LDL was isolated from freshly drawn EDTA (1 mg/ml)-containing blood samples by continuous density gradient ultracentrifugation in a Beckman SW 41 rotor at 38,000 rpm for 20 h at 10°C (*22*). After centrifugation, aliquots of 500 μl (fraction 1) and 400 μl (fractions 2-24) were collected, and their protein concentrations were measured according to Lowry *et al.* (*23*). The fractions corresponding to the LDL peak were pooled (about 10 ml) and dialyzed at 4°C against three changes (about 15 liters) of 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl for 24 h (PBS). The LDL was purged with nitrogen, stored at 4°C, and used within 5-6 days (*15*).

Low-Density Lipoprotein Oxidation—Samples of LDL corresponding to 50 μg protein/ml were oxidized with 0.9 μM $CuSO_4$. This concentration is of the same order of magnitude as those described for atherosclerotic plaques (*14*). The oxidation of LDL (150 μg protein) was performed in PBS at 30°C, in the absence and presence of 600 or 1,200 μg of GAGs (see legends to figures). These concentrations were chosen to evaluate and compare, on a weight basis, the effects of various polysaccharide samples (a proportional effect of GAGs was observed in the range from 100 to 1,200 μg/150 μg of LDL protein, see Fig. 1). Oxidation was monitored continuously by measuring the increase in absorbance at 234 nm due to the formation of conjugated CC double bonds (*15*). Several characteristic indices were determined according to Puhl *et al.* (*15*), such as the length of the lag phase (t_{lag}), the maximum rate of oxidation during the propagation phase, and the maximum concentration of conjugated dienes before the onset of decomposition.

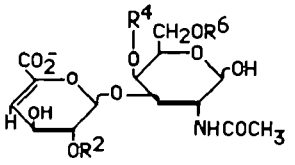
Measurement of LDL-Tryptophan Fluorescence Kinetics—Tryptophan fluorescence was examined with a Kontron SFM 25 spectrofluorometer connected to a Kontron plotter 800 (excitation at 282 nm and emission at 331 nm). Samples of LDL corresponding to 50 μg protein/ml PBS were oxidized with 0.9 μM $CuSO_4$, and then the decrease in tryptophan fluorescence, corresponding to the composition of this amino acid (*9*), was measured in the absence or presence of CS and derivatives (600 μg/150 μg of LDL protein) at 30°C.

RESULTS

Structures and Properties of Natural CS Samples—The various CS preparations showed evident differences in structure and properties (Table I). Shark cartilage CS had a greater percentage of 6-sulfated disaccharide (4-sulfate/6-sulfate ratio of 0.51) and disulfated disaccharides with greater charge density (1.21). Pig trachea CS had large amounts of 4-sulfated disaccharide (4-sulfate/6-sulfate ratio of 2.41) and a sulfate to carboxyl ratio less than 1, whilst beef trachea CS had a lower percentage of 4-sulfated disaccharide (4-sulfate/6-sulfate ratio of 1.37) (Table I). Beef trachea CS was found to strongly inhibit the Cu^{2+}-mediated LDL oxidation (see below). To confirm these data, another sample of this CS was prepared since differences in the structure and properties of GAGs have been reported to depend on the preparative processes used (*24*). Small differences were indeed noted between the two preparations (Table I).

Effects of Natural CS and Derivatives on Cu^{2+}-Mediated LDL Oxidation—The kinetics of 0.9 μM Cu^{2+}-induced oxidation of human LDL were followed by continuously recording the diene *vs.* time profile (Fig. 1). Six repeated tests on different days for the same LDL preparation gave a coefficient of variation of lower than about 10% for t_{lag}, the maximum concentration of conjugated dienes and the maximum rate of oxidation (*15*). The low coefficient of variation value for the LDL oxidation curves is mainly due to the total elimination of the EDTA from the LDL preparation on 24 h dialysis against 15 liters of PBS, to the

TABLE I. **Physico-chemical properties, and unsaturated nonsulfated and sulfated disaccharide percentages derived from the polysaccharide chains of chondroitin sulfates (CSs) from different sources (PT: pig trachea; SC: shark cartilage; BT: beef trachea) by chondroitin lyases cleavage.**



	R^2	R^4	R^6	PTCS	SCCS	BTCS I	BTCS II
ΔDi-0S	H	H	H	6.1	2.2	7.3	4.4
ΔDi-6S	H	H	SO_3^-	25.6	49.0	40.6	37.9
ΔDi-4S	H	SO_3^-	H	66.3	27.7	51.4	56.0
ΔDi-2,6diS	SO_3^-	H	SO_3^-	1.9	16.1	0.7	0.8
ΔDi-4,6diS	H	SO_3^-	SO_3^-	0.0	3.0	0.0	0.8
ΔDi-2,4diS	SO_3^-	SO_3^-	H	0.0	0.0	0.0	0.0
ΔDi-2,4,6triS	SO_3^-	SO_3^-	SO_3^-	0.0	1.9	0.0	0.0
M_r (×1,000)				18.10	>50.00	23.76	26.95
SO_3^-/COO^- (*)				0.96	1.21	0.93	0.98

The amount of each identified disaccharide was determined using purified standards and is reported as a weight percentage. Under the experimental conditions, cleavage of galactosaminoglycan samples with chondroitinases produces 100% disaccharides. ΔDi-0S: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-D-galactose], ΔDi-4S: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-D-galactose 4-sulfate], ΔDi-6S: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-D-galactose 6-sulfate], ΔDi-2,6diS: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid 2-sulfate)-D-galactose 6-sulfate], ΔDi-2,4diS: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid 2-sulfate)-D-galactose 4-sulfate], ΔDi-4,6diS: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-D-galactose 4,6-disulfate], and ΔDi-2,4,6triS: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid 2-sulfate)-D-galactose 4,6-disulfate. M_r: molecular mass.

use of nitrogen to eliminate oxygen (*15*), and to the short period for which LDLs were used (5-6 days).

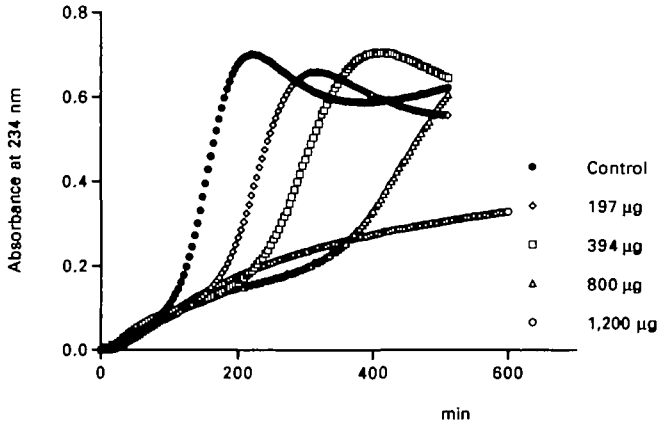


Fig. 1. **Effect of the beef trachea chondroitin sulfate (BTCS I) concentration (from about 200 to 1,200 μg) on the kinetics of Cu^{2+}-induced human LDL (150 μg of protein) oxidation.** The initial absorbance (about 0.150) was subtracted from all data.

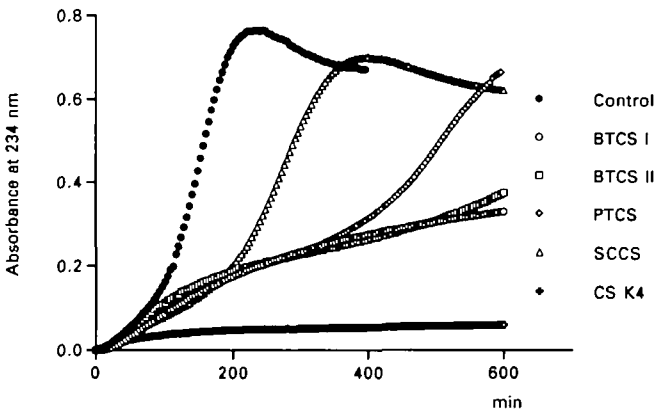


Fig. 2. **Effects of chondroitin sulfates from beef trachea (BTCS I and BTCS II), pig trachea (PTCS), and shark cartilage (SCCS) on Cu^{2+}-induced oxidation of human LDL (150 μg of protein).** The strong protective properties as to LDL oxidation of a fructose-containing polysaccharide with a chondroitin backbone (CS K4) purified from *Escherichia coli* (*17*) are also illustrated. All glycosaminoglycans were examined at the concentration of 1,200 μg. The initial absorbance (about 0.150) was subtracted from all data.

TABLE II. **Characteristic indices (means±standard deviation for at least three independent experiments) of the LDL-oxidation kinetics with the conjugated diene method in the absence and presence of various natural chondroitin sulfates (1,200 μg).** BTCS, beef trachea chondroitin sulfate. PTCS, pig trachea chondroitin sulfate. SCCS, shark cartilage chondroitin sulfate.

Sample	t_{lag}	b	c
Control	119	0.315	0.764
BTCS I	>600	n.d.	n.d.
BTCS II	>600	n.d.	n.d.
PTCS	410	0.1015	n.d.
SCCS	205	0.1730	0.699

t_{lag}: length of the lag phase in minutes. b: Maximum rate of LDL oxidation determined from the slope of the tangent to the propagation phase. c: Maximum concentration of conjugated dienes before the onset of decomposition. n.d.: Not determined.

As already observed (*9*), CS from beef trachea had a very strong protective effect against Cu^{2+}-induced human LDL oxidation (1,200 μg of CS/150 μg of LDL protein) (Fig. 2), since the maximum concentration of conjugated dienes was not reached even after 10 h. Thus, it was not possible to calculate t_{lag} or the maximum rate of oxidation (Table II). These results were confirmed when the second preparation of beef trachea CS was examined. Similar behavior (Fig. 2) was observed for CS from pig trachea, which markedly increased t_{lag} (410 min *vs.* 100 min in controls) and the maximum rate of oxidation (0.1015 *vs.* 0.315 in controls) (Table II). Shark cartilage CS modified the kinetics of LDL oxidation to a lower extent than the other CS preparations,

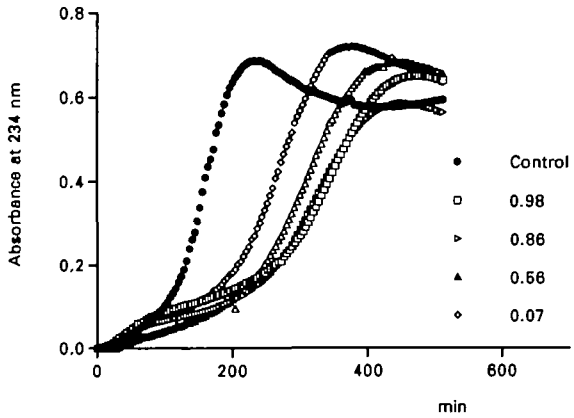


Fig. 3. **Effects of chondroitin sulfate samples (BTCS II) desulfated to various extents (sulfate to carboxyl ratios of 0.98 to 0.07) on the kinetics of Cu^{2+}-induced LDL (150 μg of protein) oxidation.** All glycosaminoglycan samples were examined at the concentration of 600 μg. The initial absorbance (about 0.150) was subtracted from all data.

TABLE III. **Characteristic indices (means±standard deviation for at least three independent experiments) of the LDL-oxidation kinetics with the conjugated diene method in the absence and presence of chondroitin sulfate samples (CSb, beef trachea chondroitin sulfate, 600 μg) with various sulfate to disaccharide ratios (charge density), and of chondroitin sulfate fractions of decreasing molecular mass.**

Sample	t_{lag}	b	%
Control	119	0.315	0
CSb 0.98	280	0.100	100
CSb 0.86	272	0.125	95.0
CSb 0.75	261	0.121	88.2
CSb 0.56	227	0.151	67.1
CSb 0.38	216	0.179	60.2
CSb 0.07	212	0.193	57.8
CSb 26,950 (0.022 μmol, 600.0 μg)	280	0.100	100
CSb 14,430 (0.022 μmol, 317.5 μg)	270	0.103	93.8
CSb 11,380 (0.022 μmol, 250.4 μg)	280	0.108	100
CSb 8,570 (0.070 μmol, 600.0 μg)	122	0.290	1.9
CSb 5,430 (0.110 μmol, 600.0 μg)	120	0.329	0.6
CSb 3,530 (0.170 μmol, 600.0 μg)	131	0.305	7.4
CSb 1,140 (0.526 μmol, 600.0 μg)	110	0.310	0.0

t_{lag}: length of the lag phase in minutes. b: Maximum rate of LDL oxidation determined from the slope of the tangent to the propagation phase. %: Percentage values representative of the capacities of different chondroitin sulfate samples to modify the kinetics of Cu^{2+}-induced LDL oxidation considering 100% inhibition is produced by natural chondroitin sulfate (having a charge density of 0.98 and a molecular mass of 26.950, see also Table I), and 0% inhibition of the control.

even though it induced an increase in t_{lag} and a marked decrease in the maximum rate of oxidation (Fig. 2 and Table II). A fructose-containing polysaccharide with a chondroitin backbone purified from *Escherichia coli* (*17*) had a very strong protective effect against LDL oxidation (Fig. 2).

Figure 3 illustrates the effects of desulfated CS samples (600 μg of CS/150 μg of LDL protein) having sulfate to disaccharide ratios ranging from 0.98 to 0.07. As is more evident from Table III, removal of sulfate groups does not abolish the antioxidant effect of this polysaccharide but only reduces its capacity. It is noteworthy that CS species desulfated by about 19% (sulfate to disaccharide ratio of 0.75) retain a great part of their antioxidant properties, and that nearly totally desulfated CS still possesses a great capacity to protect against LDL oxidation (57.8% of native CS, Table III).

The effect of molecular mass on the capacity of CS (600 μg of CS/150 μg of LDL protein) to inhibit LDL oxidation *in vitro* is shown in Fig. 4. A CS fraction with a molecular mass of 8,570 and formed of about 13-15 disaccharide units prepared by gel-filtration chromatography is unable to protect human LDL from Cu^{2+}-induced oxidation. Table III better illustrates the effect of CS molecular mass on the kinetics of LDL oxidation. In fact, as evidenced by the numeric values of the kinetic curves of LDL oxidation, t_{lag} and the slope of the tangent to the propagation phase, CS fractions with molecular masses of 8,570, 5,430 (8-10 disaccharide units), 3,530 (4-6 disaccharide units), and 1,140 (about 2 disaccharide units) are ineffective. On the contrary, CS fractions with molecular masses of 14,430 (23-25 disaccharide units) and 11,380 (18-20 disaccharide units) retain the full capacity to inhibit the Cu^{2+}-mediated human LDL oxidation.

Regioselective 6-*O*-desulfatation of CS performed according to Matsuo *et al.* (*20*) permitted us to produce CS derivatives composed of 4-*O*-sulfate groups esterified on the galactosamine unit. Also in this case, two derivatives (sulfate to carboxyl ratio of 0.65, 4/6 sulfate ratio of 10.4, and 5.4% 6-sulfate groups; and sulfate to carboxyl ratio of 0.62, 4/6 sulfate ratio of 12.8, and 4.5% 6-sulfate groups) retained the capacity to inhibit the Cu^{2+}-mediated human LDL oxidation (1,200 μg of CS/150 μg LDL protein) (not shown).

Cu^{2+}-Induced LDL Oxidation in the Presence of Normal Human Plasma-Derived Chondroitin Sulfate—Low-sulfated CS was isolated and purified from normal human plasma. As previously reported (*21*), this glycosaminoglycan has a molecular mass of about 15,600 and a sulfate to carboxyl ratio of 0.41; it is formed from about 59% Δ-Di0s, 3% Δ-Di6s, and 38% Δ-Di4s. We examined 343.2 μg (0.022 μmol) and 686.4 μg (0.044 μmol) of human plasma CS as to its possible capacity to inhibit LDL oxidation *in vitro*. The results, illustrated in Fig. 5, confirm

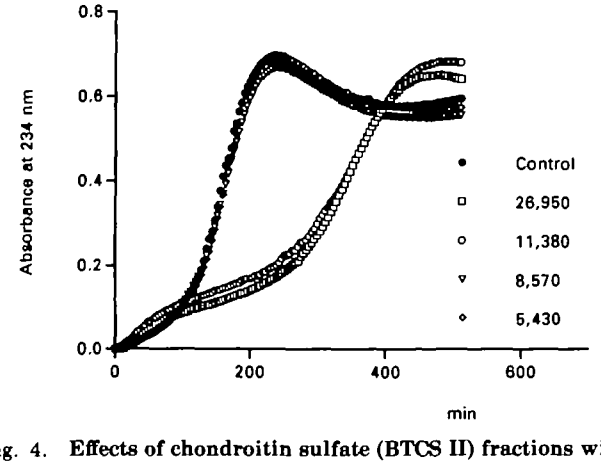


Fig. 4. **Effects of chondroitin sulfate (BTCS II) fractions with decreasing molecular masses (Da) on the kinetics of Cu^{2+}-induced LDL (150 μg of protein) oxidation.** See Table III for the polysaccharide amounts examined. The initial absorbance (about 0.150) was subtracted from all data.

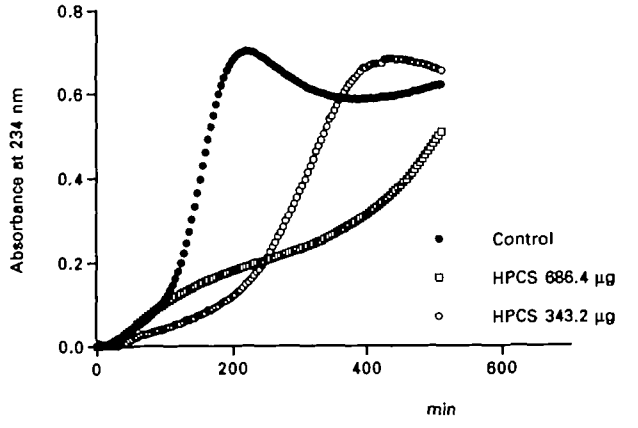


Fig. 5. **LDL (150 μg of protein) oxidation kinetics in the presence of human plasma-derived chondroitin sulfate (HPCS) at different concentrations (343.2 μg, 0.022 μmol, and 686.4 μg, 0.044 μmol).** The initial absorbance (about 0.150) was subtracted from all data.

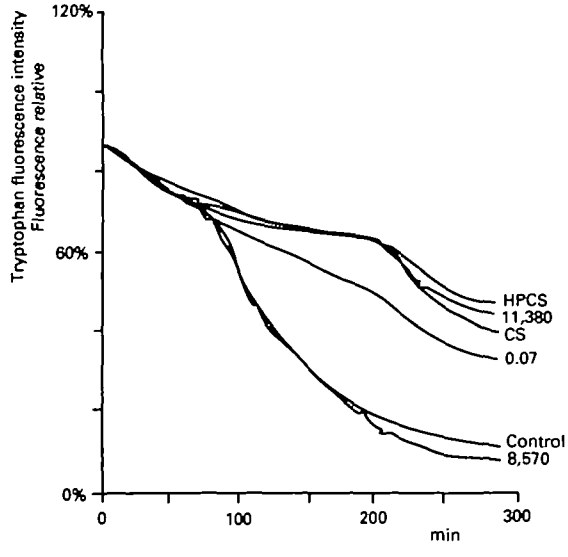


Fig. 6. **Kinetics of LDL-tryptophan fluorescence during copper-induced oxidation.** LDL (150 μg of protein) was oxidized in the absence (control) and presence of chondroitin sulfate (CS), a desulfated species (charge density of 0.07), two fractions with different molecular masses (11,380 and 8,570), and normal-human plasma derived chondroitin sulfate (HPCS). All glycosaminoglycan samples were examined at the concentration of 600 μg.

that human plasma low-sulfated CS has effective antioxidant properties. The capacity of low-sulfated human plasma CS to protect LDL against Cu^{2+}-induced oxidation confirms the relative importance of the polysaccharide charge density in LDL oxidation kinetics.

Effects of CS Samples on LDL-Tryptophan Fluorescence Kinetics—Figure 6 shows the effects of natural CS, a totally desulfated CS, two samples with different molecular masses (11,380 and 8,570), and normal human plasma-derived CS on copper-induced tryptophan quenching. The kinetics of tryptophan fluorescence decrease during copper-mediated LDL oxidation, and the natural CS, desulfated CS, fraction with a molecular mass of 11,380, and plasma derived GAG are able to reduce tryptophan quenching. On the contrary, the CS fraction with a molecular mass of 8,570 is ineffective.

DISCUSSION

Although several reports have appeared on the effects of some polysaccharides on Cu^{2+}-mediated human LDL oxidation (*8, 9*), no study has been reported on the capacity of various GAGs to influence LDL oxidation kinetics depending on their structures. This is of some importance due to the great variability of polyanion structures which exhibit macroheterogeneity, mainly due to the presence of different monosaccharide units (grouped in galactosaminoglycans and glucosaminoglycans), and, within the same polysaccharide species, microheterogeneity due to variously sulfated disaccharides, polydispersity of the chains, and specific oligosaccharide sequences (*3, 12, 24*). In this study we compared the effects of CS species desulfated to different extents and fractions with decreasing molecular masses in an attempt to clarify the structure-function relationship. CS samples extracted and purified from tissues were used, and this could be of a possible biologic relevance since natural polyanions with structures and properties similar to those examined could have protective effects against *in vivo* LDL oxidation. We chose to examine GAGs on a weight basis and not on a molar basis due to the significant dispersity of their polysaccharide chains. Nevertheless, a comparison on a molar basis of some polyanions was also attempted to better understand the effect on *in vitro* LDL oxidation in relation to GAG properties.

Albertini *et al.* (*9*) reported evidence that CS has a strong LDL protective effect that depends on the polysaccharide nature and structure. CS from pig trachea and CS from beef trachea each exhibit a strong protective effect against human LDL oxidation (under the experimental conditions adopted, CS has a greater protective effect than 10 μM probucol, not shown). The more highly sulfated CS from shark cartilage was found to affect LDL oxidation to a lower extent when evaluated on a weight basis. Due to the strong capacity of beef trachea CS to inhibit LDL oxidation, chemical modification of its structure was undertaken. Molecular mass was found to be a more critical factor than charge density or the positions of sulfate groups along the polysaccharide chains determining the protective effect of human LDL. In fact, completely desulfated CS samples retained a great antioxidant capacity as to the native glycosaminoglycans, further confirming that polysaccharide chains deprived of sulfate groups retain protective effects against Cu^{2+}-induced LDL oxidation. Albertini *et al.* (*9*) reported that chondroitin-4-sulfate is more effective than related chondroitin-6-sulfate, and that the sulfate group of position 4 of *N*-acetylgalactosamine was crucial for the protective activity. We found that samples of chondroitin-4-sulfate (with no 6-sulfate groups) retained a strong protective effect against LDL oxidation, but, on the other hand, it was evident that a totally desulfated CS also showed anti-oxidant properties. Besides, a natural non-sulfated polysaccharide composed of a backbone with the structure of chondroitin containing fructose purified from *Escherichia coli* (*17*) had a very strong protective capacity. Further studies are in progress to evaluate the contribution of fructose belonging to this polysaccharide on the protective effect on LDL. We also found that CS fractions with molecular masses lower than about 8,600 (13-15 disaccharide units) were completely ineffective in protecting LDL against oxidation, even when large numbers of moles were used (see Table III).

The protective capacities as to Cu^{2+}-mediated human LDL oxidation of CS samples depending on structure were confirmed by studying the degradation of a proportion of the tryptophan residues of the LDL protein moiety. As previously reported (*9*), CS reduces tryptophan degradation resulting from Cu^{2+} complexation through amino acid residues (*25*). By monitoring LDL-tryptophan fluorescence we obtained further evidence that the protective effect against LDL oxidation by CS is highly specific, depending on the polysaccharide backbone having the structure, -3)-β-D-glucuronyl-(1,4)-β-D-*N*-acetylgalactosaminyl(1-. The highly-specific mechanism of protection against Cu^{2+}-mediated LDL oxidation by CS also seems to be confirmed by several considerations. Copper-binding sites, involved in the initiation of LDL oxidation, are localized on the Apo-B-100 protein (*25, 26*), and Camejo *et al.* (*6*) reported increased access of Cu^{2+} ions in hydrophobic regions. Therefore, we assume that the protective effect of GAGs against Cu^{2+}-mediated LDL oxidation is due to their capacity to interact, mainly through their hydrophobic groups, with hydrophobic regions of the LDL protein. This model explains the strong capacity of CS to inhibit LDL oxidation, mainly due to the presence of *N*-acetyl groups rather than sulfate (and iduronic acid) residues. In fact, CS has large hydrophobic patches in its secondary structure due to the presence of *N*-acetyl residues with strong hydrophobic bonding potential able to interact with hydrophobic regions of proteins (*27, 28*). On the other hand, the mechanism of protection against LDL oxidation does not seem to be related to the capacity of CS to chelate Cu^{2+} ions. In fact, the interaction between GAGs and ions is of an electrostatic nature and depends on negatively charged groups of polyanions (*29*). According to this model, highly-sulfated CS from shark cartilage should have a greater effect than other CS as to inhibition of LDL oxidation. Low-sulfated CS with a charge density of about 0.41 and a molecular mass of 15,600 extracted and purified from normal human plasma (*21, 30*) exhibits a strong inhibitory capacity during Cu^{2+}-mediated LDL oxidation. On the other hand, this protective effect against ion-mediated LDL oxidation could be exercised *in vivo* by low-sulfated CS normally present in the blood compartment as a product of the degranulation of several haemopoietic cells (*e.g.*, lymphocytes, granulocytes, platelets, and monocytes) that mainly contain simple homopolymeric chondroitin-4-sul-

fate chains (*31*).

The relative content of chondroitin-4-sulfate/chondroitin-6-sulfate isomers in the human aorta varies with age, with a decrease in the 4-sulfate/6-sulfate ratio (*32*). The loss of the chondroitin-4-sulfate isomer and the concomitant increase in chondroitin-6-sulfate is even more pronounced in atherosclerotic lesions (*32*). We suggest that modifications of the numbers and positions of sulfate groups of the CS chains of human aorta PGs, and then of the 4-sulfate to 6-sulfate ratio and charge density of CS are not factors responsible for the greater susceptibility of LDL to oxidation. In light of the results presented here, we assume that a decrease in the CS content or a reduced CS molecular mass (approx. lower than 8,600, 13-15 disaccharide units) might contribute to the inability of CS to protect LDL from oxidation, and to the deposition of oxidatively modified human LDL in the extracellular matrix of the arterial intima, predisposing to atherosclerosis (*33*). Upon stimulation with bacterial antigens, polymorphonuclear leukocytes produce reactive oxygen species, such as the superoxide radical anion and hydrogen peroxide, which further interact in the presence of transition metal ions to produce hydroxyl radicals (*34*). Many of these reactive oxygen species are unable to discriminate between host and pathogen macromolecules, resulting in losses of structural integrity and function of tissues (*34*). CS present in connective tissues is depolymerized by oxygen-derived species (*35*), and CSs from different sources are degraded by a free-radical process mediated by ions (*36, 37*) with marginal desulfation. These considerations strengthen the view that free radicals may contribute to the oxidation of LDL, acting as direct agents producing oxidation, and also degrading CS, thus altering the ability of this polysaccharide to protect LDL from further oxidation.

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