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Influence of Chlorate on Proteoglycan Biosynthesis by Cultured Human Fibroblasts*

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The influence of chlorate, an inhibitor of sulfate adenylyltransferase, on biosynthesis and secretion of proteoglycans was investigated in cultured human skin fibroblasts. At up to 10 mM concentrations, chlorate caused a reduction of [^{35}S]sulfate incorporation into small chondroitin sulfate/dermatan sulfate proteoglycan by up to 96%. Incorporation of [^{3}H]leucine and [^{3}H]glucosamine was only slightly affected. No influence was seen on the polymerization degree of the polysaccharide chain as judged by gel filtration, and on the kinetics of secretion of the proteoglycan. Concomitant with reduced sulfation, however, was an increased sensitivity toward chondroitin AC lyase which suggests a diminished epimerization of D-glucuronic acid to L-iduronic acid residues. Agarose gel electrophoresis revealed that all polysaccharide chains of control chondroitin sulfate/dermatan sulfate proteoglycan exhibited a similar sulfation degree. Chlorate treatment led to the formation of polysaccharide chains of widely varying degree of sulfation, but fully sulfated chains were synthesized even in the presence of 3 mM chlorate, and sulfate-free chondroitin was not detected. Studying the effects of chlorate treatment on the synthesis of other proteoglycan types it was found that, in cell-associated galactosaminoglycans, 6-sulfation of *N*-acetylgalactosamine residues was less affected than was 4-sulfation. In case of heparan sulfate the synthesis of sulfamate groups was less impaired than sulfate ester formation. Nitrous acid degradation at pH 4.1 indicated the presence of unsubstituted amino groups. Chlorate treatment may be considered as a means for the production of proteoglycans with defined structural alterations.

The glycosaminoglycan chains of chondroitin sulfate/dermatan sulfate proteoglycans (CS/DS-PG)[1] contain variable amounts of 4- or 6-sulfated *N*-acetylgalactosamine residues. During development the ratios of 6-sulfation to 4-sulfation may vary, but at a given developmental stage there exists a characteristic tissue-dependent sulfation pattern for the individual proteoglycan species (1–5). It might, therefore, be assumed that the sulfation pattern is of physiological significance. However, only indirect proof of the functional importance of sulfation has been obtained so far. A deficiency of adenylylsulfate kinase in cartilage seems to be the basic defect in brachymorphic mice (6). Defective processing of keratan sulfate possibly due to a deficient sulfotransferase results in macular corneal dystrophy (7, 8). Undersulfation of glycosaminoglycans in patients with Lowe syndrome has been considered to result from an elevated level of nucleotide pyrophosphatase (9), which hydrolyzes 3-phosphoadenylyl sulfate, the common substrate of all sulfotransferases, but this conclusion has not been supported by other studies (10, 11). In no case, however, has it been shown convincingly how the proposed basic defect ultimately leads to the abnormal phenotype.

Several recent reports have indicated that not only the glycosaminoglycan chains of a proteoglycan but also the core protein to which the chains are attached may be of functional significance. The core protein may be responsible for specific interactions in addition to the well-known binding of cartilage proteoglycan core protein to hyaluronate and link protein. For example, transferrin receptor properties have been ascribed to the core protein of a fibroblast heparan sulfate proteoglycan (12). The core protein of a small, iduronic acid-rich CS/DS-PG from fibroblasts has been shown to be responsible for receptor-mediated endocytosis (13) and for the interaction with fibronectin (14). Specific inhibition of type I and type II collagen fibrillogenesis by a small CS/DS-PG of tendon required an intact core protein, whereas free glycosaminoglycan chains were ineffective (15).

Considering the uncertainties about the exact contributions of glycosaminoglycan chains and core protein for the function of a proteoglycan, it could be helpful to be able to manipulate the composition of the glycosaminoglycan chains for a study of the functional consequences of such alterations. Previous studies have demonstrated that undersulfated proteoglycans are formed upon incubation of chick cartilage (16), bovine aortic endothelial cells (17), and human fibroblasts (18), respectively, in sulfate-depleted medium. In the present investigation we took advantage from the observation that chlorate is a potent inhibitor of sulfate adenylyltransferase. This enzyme is required for 3-phosphoadenylylsulfate formation, and its inhibition results in a reduced protein and carbohydrate sulfation (19). As a first approach, we have focused on the influence of chlorate on biosynthesis, posttranslational modification, and secretion of an iduronic acid-rich small CS/DS-PG which is a major secretory product of cultured human skin fibroblasts. This proteoglycan is also named proteoglycan II (PG II) to distinguish it from another small proteoglycan (PG I) being present, for example, in articular cartilage and bone (20, 21). Its core protein of M_r = 36,319, the primary structure of which has been deduced from cloned cDNA (22), is linked with only one glycosaminoglycan chain and, addi-

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[1] The abbreviations used are: CS/DS-PG, chondroitin sulfate/dermatan sulfate proteoglycans, SDS, sodium dodecyl sulfate.

tionally, with two or three asparagine-bound oligosaccharides (23). Structural alterations of cell-associated glycosaminoglycans were also studied.

EXPERIMENTAL PROCEDURES

Preparation of Labeled Proteoglycans—Human skin fibroblasts from apparently healthy juvenile and adult donors were maintained in culture as described previously (24). For labeling of confluent cultures Waymouth MAB 87/3 medium (25, as formulated in the catalogue of GIBCO) was used that was modified as follows. $FeSO_4$, $MnSO_4$, and $ZnSO_4$ were omitted, and $MgSO_4$ was replaced by $MgCl_2$. The concentrations of cystine and methionine were reduced to 1% of the original value. Leucine was omitted in case of labeling with L-[4,5-^{3}H]leucine (specific radioactivity 2.55 TBq/mmol; Amersham-Buchler), and the glucose concentration was reduced to 1 mM for labeling with D-[6-^{3}H]glucosamine (specific radioactivity 814 GBq/mmol; Amersham-Buchler). The medium was supplemented with 4% dialyzed fetal calf serum (Boehringer-Mannheim), penicillin, and streptomycin. Streptomycin was omitted from cultures to be labeled with [^{35}S]sulfate (specific radioactivity 0.9–1.5 TBq/mg of S; Amersham-Buchler). Cells grown in 75-cm^2 Falcon plastic flasks were incubated in a final volume of 5 ml, whereas cells in 25-cm^2 flasks received 2 ml of medium. Labeling was terminated by either harvesting or feeding the cultures with complete tissue culture medium.

Small CS/DS-PG was extracted from the culture medium after precipitation with $(NH_4)_2SO_4$ and from the cell layer as described (23), with minor modifications (26), and purified by treatment with protein A-Sepharose (Sigma) coated with IgG from a monospecific rabbit antiserum against CS/DS-PG core protein (23). Reprecipitation of material which remained unbound during the first immune precipitation step with a second portion of antibody-coated protein A-Sepharose indicated that the first precipitation step was at least 90% complete.

Isolation and Characterization of Glycosaminoglycans—After immune precipitation, CS/DS-PG was solubilized by suspending the immune complex in 400 μl of 1% SDS and heating for 6 min at 95 °C. After drying, SDS was removed by washing with methanol, and the proteoglycan was subjected to a β-elimination reaction (0.1 M NaOH, 1 M $NaBH_4$, 24 h at 37 °C). $NaBH_4$ was destroyed by acidification. For an analysis of the glycosaminoglycan composition, oligosaccharides were first removed by chromatography on 0.7 ml columns of Dowex 1-X2, 200–400 mesh. The samples were made 0.15 M with NaCl and 0.1% with Triton X-100 and applied to the resin. The columns were washed with 3 ml of 0.3 M NaCl/0.1% Triton X-100 prior to glycosaminoglycan desorption with 3 M NaCl/0.1% Triton X-100 followed by dialysis against 0.1% Triton X-100. After parallel digestions with chondroitin ABC and AC lyase (from Seikagaku Kogyo) as described (27), the ethanol-soluble material was analyzed by high-performance liquid chromatography on a Partisil-PAC (10 μm, 25 cm × 4.6 mm, Whatman) column. The system was run at a flow rate of 1.2 ml/min with a solvent consisting of 52% acetonitrile, 12% methanol, and 36% aqueous buffer containing 0.5 M Tris, 3.6 mM H_2SO_4, and 0.1 M boric acid, pH 8.0 (28). For molecular size determinations of glycosaminoglycans, a calibrated Sephacryl S-300 column (28) was used. For an analysis of the charge density, glycosaminoglycans were electrophoresed in a 1.0% agarose gel containing 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA (pH 8.3) for 2 h at 5 V/cm. At the end of the run the gel was cut into 2-mm segments that could be solubilized in the liquid scintillation mixture (Instagel; Beckman).

Glycosaminoglycans associated with the cell membrane were prepared from the supernatant of the trypsinized cell layer (24) after dialysis against 0.1% Triton X-100 and alkaline borohydride treatment (0.1 M NaOH, 1 M $NaBH_4$; 12 h at 37 °C). The sample was then brought to pH 5 with acetic acid and dialyzed against water prior to treatment with the chondroitin lyases. The fraction of membrane associated glycosaminoglycans being resistant toward chondroitin ABC lyase was subsequently subjected to nitrous acid deamination at pH 1.5 and 4.1, respectively (29). In the low pH procedure, H_2SO_4 was substituted by HCl, and the reaction was terminated by neutralization with 2 M NaOH. The reaction mixture was subjected to high voltage electrophoresis on Whatman No. 3MM in 5% acetic acid, 0.5% pyridine, pH 3.5, for 25 min at 60 V/cm.

Other Methods—SDS-polyacrylamide gel electrophoresis (30) followed by fluorography (31) was performed as described. Uronic acids were quantitated as published previously (32).

RESULTS

Influence of Chlorate on [^{35}S]Sulfate Incorporation into CS/DS-PG—Fibroblasts were preincubated with increasing concentrations of chlorate in a sulfate-free medium and then labeled for 4 h with [^{3}H]leucine and [^{35}S]sulfate in the continuous presence of chlorate. Upon quantitative immune precipitation of small CS/DS-PG it was found that, in three different experiments, [^{3}H]leucine incorporation declined by up to 12% in the presence of ≥3 mM ClO_3^- and by 26% in the presence of 10 mM ClO_3^-. However, chlorate caused a dramatic inhibition of [^{35}S]sulfate incorporation which was only 4% (3–6% in eight different experiments) of the control value in the presence of 10 mM chlorate (Fig. 1). In the presence of 3 mM chlorate, sulfation was reduced to 8–18% of the control. SDS-polyacrylamide gel electrophoresis demonstrated that chlorate treatment did not affect the substitution pattern by asparagine-bound oligosaccharides of the core protein. It had been shown previously that the core protein carries either two or three *N*-glycosidically linked oligosaccharides (23). The amount of intracellular glycosaminoglycan-free core protein appeared also unaltered. At all chlorate concentrations, however, the mean electrophoretic mobility of CS/DS-PG during SDS-polyacrylamide gel electrophoresis was slower than that of normal CS/DS-PG (Fig. 2).

The degree of polymerization of the glycosaminoglycan chains of CS/DS-PG was not measurably altered as a consequence of chlorate treatment. Fibroblasts were incubated for 15 h in the presence of both [^{3}H]glucosamine and [^{35}S]sulfate. In three different experimental series, 10 mM chlorate caused maximally a 28% inhibition of amino sugar incorporation, whereas sulfate incorporation amounted for 5% only of the control value. The glycosaminoglycan chains of normal proteoglycans, which were liberated by a β-elimination reaction, eluted on a calibrated Sephacryl S-300 column as M_r = 37,000 reference glycosaminoglycans (Fig. 3). Glycosaminoglycans produced in the presence of 10 mM chlorate were eluted with a k_{av} value of 0.174 which would be the expected value of an M_r = 30,000 species. A molecular weight of 30,000 is calculated if 95% of the sulfate ester groups are removed from a chon-

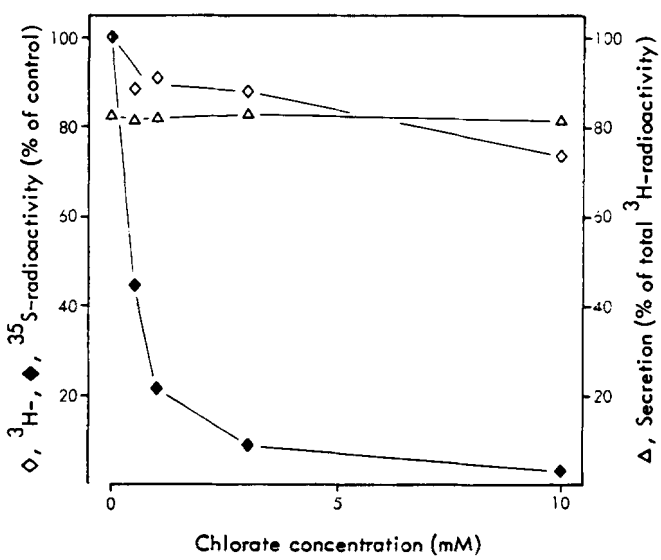

FIG. 1. **Influence of chlorate on the formation and secretion of small CS/DS-PG.** Fibroblasts were preincubated for 1 h in the presence of the sodium chlorate concentrations indicated and then labeled with [^{3}H]leucine (0.74 MBq/ml) and [^{35}S]sulfate (0.74 MBq/ml) for 4 h in the continuous presence of chlorate. Radioactivity was quantitated after immune precipitation of cell and medium extracts. Secretion of ^{35}S radioactivity varied between 88 and 94%.

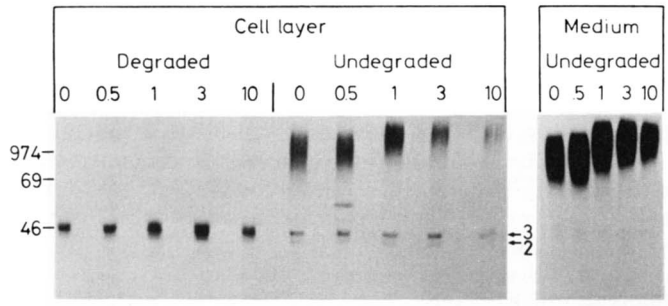

FIG. 2. **Formation and secretion of CS/DS-PG by chlorate-treated fibroblasts.** Fibroblasts were incubated as described in the legend to Fig. 1. Chlorate concentrations are indicated in millimoles per liter. The immune precipitated material from the cell layer was treated with chondroitin ABC lyase or with buffer alone prior to SDS-polyacrylamide gel electrophoresis. The acrylamide concentration of the separation gel was 12.5%. *Arrows* indicate the number of *N*-glycans.

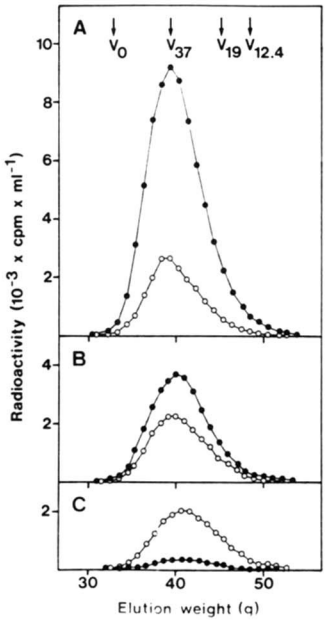

FIG. 3. **Gel chromatography on Sephacryl S-300 of [^{35}S] sulfate- (●) and [^{3}H]hexosamine- (○) labeled glycosaminoglycan chains of small CS/DS-PG.** Cultures had been incubated in the presence of [^{3}H]glucosamine (1.48 MBq/ml) and [^{35}S]sulfate (0.74 MBq/ml) for 15 h without chlorate (*A*) and with 1 mM (*B*) and 10 mM (*C*) chlorate, respectively. Glycosaminoglycan chains were liberated after immune precipitation of secreted CS/DS-PG.

droitin sulfate chain of M_r = 37,000. Similarly, the mean k_{av} value of the polysaccharide synthesized in the presence of 1 mM chlorate was 0.15, which corresponds to an apparent molecular weight of 34,000. A molecular weight of about 32,000 is calculated for the normal glycosaminoglycan having lost 80% of its sulfate groups.

Ion exchange chromatography could not be used to decide unambiguously whether the overall reduction of sulfate incorporation was due to the synthesis of completely unsulfated chains or whether there was a continuous spectrum of chains with different degree of sulfation. Upon chromatography on DEAE-Trisacryl of [^{3}H]hexosamine- and [^{35}S]sulfate-labeled chains of small CS/DS-PG, it was found that the ^{35}S/^{3}H ratio of glycosaminoglycans being desorbed with 0.4 M NaCl declined from 3.1 (control) to 0.8 (2 mM chlorate) and 0.3 (10 mM chlorate), respectively. It appears, therefore, that chains of different sulfation degree can be desorbed from the ion exchange matrix at the same ionic strength. As an alternative approach, double-labeled glycosaminoglycan chains were subjected to electrophoresis in 1% agarose. In this system, the electrophoretic mobility is not dependent on the molecular size of the glycosaminoglycans, at least in the tested M_r range of 10,000–37,000. CS/DS chains from control proteoglycans yielded a fairly symmetrical peak upon agarose gel electrophoresis (Fig. 4). The ^{35}S/^{3}H ratio of individual fractions varied in a nonsystematic manner between 2.2 and 2.8. In contrast, this ratio increased progressively from 0.5 to 2.2 in glycosaminoglycans from cells treated with 3 mM and from 0.12 to 1.2 in the material from cells treated with 10 mM chlorate, respectively. Thus, all glycosaminoglycan chains from normal CS/DS-PG appear to exhibit a similar charge density, whereas a spectrum of glycosaminoglycans with differing degrees of undersulfation is obtained from chlorate treated cells. Interestingly, even cells treated with 3 mM chlorate produce some material with normal ^{35}S/^{3}H ratio.

Influence of Chlorate on CS/DS-PG Composition—[^{3}H] Hexosamine- and [^{35}S]sulfate-labeled glycosaminoglycan chains were obtained as described in the legend to Fig. 3. Their disaccharide constituents were analyzed by high-performance liquid chromatography after parallel digestion with chondroitin AC and ABC lyase (Fig. 5). Control material contained only trace amounts of unsulfated disaccharides. As expected, their proportion rose to up to 90% in the glycosaminoglycans from chlorate-treated cultures. With increasing undersulfation, the glycosaminoglycan chains became increasingly sensitive toward degradation by chondroitin AC lyase, suggesting a decreased epimerization of D-glucuronic to L-iduronic acid residues. Due to the low proportion of chon-

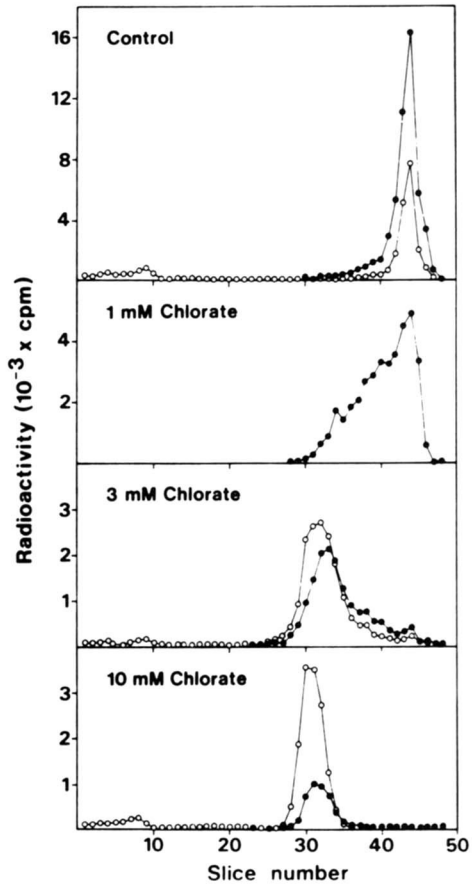

FIG. 4. **Agarose gel electrophoresis of [^{35}S]sulfate- (●) and [^{3}H]hexosamine- (○) labeled glycosaminoglycan chains of small CS/DS-PG.** Labeling and preparation of glycosaminoglycan chains were performed as described in the legend to Fig. 3.

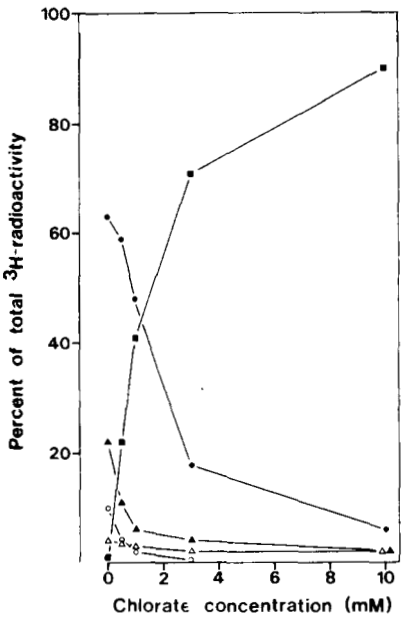

FIG. 5. **Composition of glycosaminoglycan chains of small CS/DS-PG.** Labeling conditions were as described in the legend to Fig. 3. ●, dermatan sulfate-derived monosulfated disaccharides; ○, dermatan sulfate-derived disulfated disaccharides; ▲, chondroitin 4-sulfate-derived disaccharides, △, chondroitin 6-sulfate-derived disaccharides; ■, unsulfated disaccharides.

droitin 6-sulfate-type disaccharides even in control polysaccharide chains, it could not be judged with certainty whether or not 4- and 6-sulfation are equally sensitive to chlorate treatment.

The distribution of [^{35}S]sulfate residues along the glycosaminoglycan chain of CS/DS-PG was studied by the following experiment. The [^{3}H]hexosamine- and [^{35}S]sulfate-labeled glycosaminoglycans were repeatedly digested by an extract of normal fibroblasts under conditions allowing the action of lysosomal glycosidases and sulfatases. It had been shown previously that human skin fibroblasts are devoid of endoglycosidases being able to degrade [^{35}S]sulfate-labeled chondroitin sulfate (33). Furthermore, the dermatan sulfate stored intracellularly in mucopolysaccharidosis VI fibroblasts lacks reducible end groups, although it can be degraded *in vitro* by hyaluronoglucosaminidase (34). Thus, only monosaccharides and sulfate are produced as ethanol-soluble degradation products. It can be deduced from the data presented in Table I that the polysaccharide chains from chlorate-treated cells do not contain clusters of sulfate ester groups near the nonreducing terminal end. Similar proportions of *N*-acetylgalactosamine and of sulfate were liberated during each digestion cycle. However, undersulfated glycosaminoglycans were degraded much more extensively. This indicates that the action of sulfatases may be rate-limiting during *in vitro* degradation.

Influence of Chlorate on CS/DS-PG Secretion—Chlorate-pretreated fibroblasts were pulse-labeled for 10 min with [^{3}H]leucine and then subjected to different chase periods. It can be seen in Fig. 6 that chlorate treatment did not exert any influence on CS/DS-PG secretion.

Sulfation of Cell-associated Chondroitin Sulfate/Dermatan Sulfate and of Heparan Sulfate—In fibroblasts from adult and juvenile donors a large chondroitin sulfate/dermatan sulfate proteoglycan and heparan sulfate proteoglycans are preferentially located in the pericellular matrix (27). The glycosaminoglycan chains of the large proteoglycans are shorter than those from small CS/DS-PG and contain more chondroitin 6-sulfate-type disaccharides. Only a minor proportion of per-

TABLE I

Degradation of glycosaminoglycan chains of CS/DS-PG by fibroblast homogenates

Glycosaminoglycans were prepared from CS/DS-PG secreted by cells that had been incubated with [^{35}S]sulfate and [^{3}H]glucosamine as indicated in Fig. 8. The following radioactivities were recovered: control, 12,300 counts/min ^{3}H, 36,400 counts/min ^{35}S; 1 mM chlorate, 9,600 counts/min ^{3}H, 8,500 counts/min ^{35}S; 10 mM chlorate, 8,800 counts/min ^{3}H, 1,600 counts/min ^{35}S. The glycosaminoglycans were incubated with a dialyzed extract of normal fibroblasts (0.45 mg of cell protein) in 20 mM sodium acetate buffer, pH 4.5, containing 10 mM NaCl and 0.1% Triton X-100 for 24 h at 37 °C, final volume 100 μl. Ethanol-soluble degradation products were obtained after addition of 4 volumes of ethanol. Fibroblast homogenate was added to the ethanol-insoluble material, and incubation continued for two more cycles of digestion.

Digestion cycle	Ethanol-soluble radioactivity (% of total)					
	Control		1 mM $NaClO_3$		10 mM $NaClO_3$	
	^{3}H	^{35}S	^{3}H	^{35}S	^{3}H	^{35}S
1	4.8	4.1	6.9	7.0	17.0	14.1
2	4.5	4.4	7.2	7.0	20.1	21.0
3	5.0	4.8	7.6	8.1	16.2	18.0

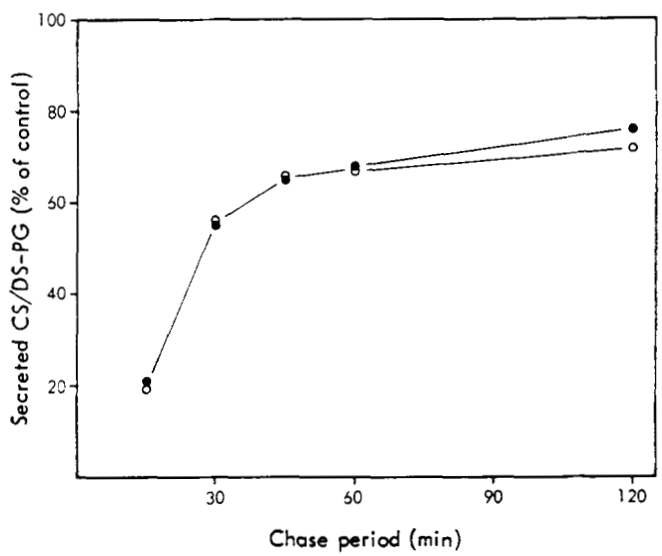

FIG. 6. **Secretion of CS/DS-PG.** Fibroblasts were preincubated for 1 h in the absence or presence of 3 mM sodium chlorate and then labeled with [^{3}H]leucine (approximately 3 MBq/ml) for 10 min. The same radioactive medium was used for sequential labeling of up to five flasks. Radioactivity was quantitated after immune precipitation of cell and medium extracts. ●, chlorate-treated cells; ○, control cells.

TABLE II

Composition of surface-associated glycosaminoglycans

Cultures were incubated for 15 h in the presence of [^{35}S]sulfate (0.55 MBq/ml) and the chlorate concentrations indicated. Abbreviations: GAG, glycosaminoglycans; HS, heparan sulfate; C-4-S, chondroitin 4-sulfate; C-6-S, chondroitin 6-sulfate; DS, dermatan sulfate.

Chlorate	Cell surface GAG					Secreted GAG
	Total cpm	Composition (% of total)				Total cpm
		HS	C-4-S	C-6-S	DS	
mM						
0	71,100	70.9	13.9	4.1	11.1	209,000
0.5	55,800	75.1	9.6	4.2	9.3	162,000
1.0	38,100	83,2	6.3	3.8	6.8	117,000
3.0	26,900	93.8	2.3	1.8	2.1	38,600
10.0	11,500	96.7	1.6	1.6	0.6	5,400

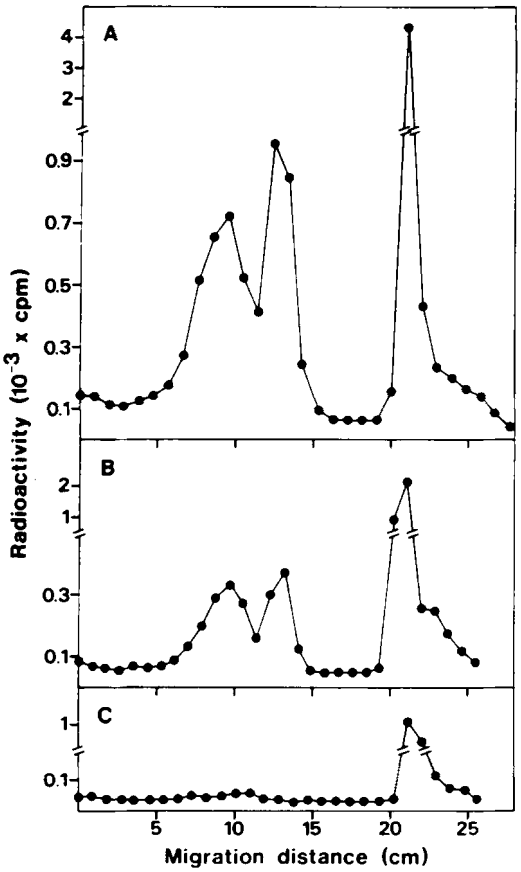

FIG. 7. **High voltage electrophoresis of nitrous acid deamination products at low pH.** Cultures had been incubated in the presence of [^{35}S]sulfate (0.55 MBq/ml) for 15 h without chlorate (*A*) and with 1 mM (*B*) and 10 mM (*C*) chlorate, respectively. Cell-associated heparan sulfate was subjected to nitrous acid degradation at low pH followed by high voltage electrophoresis. The mobilities of reference substances were 21 cm for [^{35}S]sulfate, 13–14 cm for 2-sulfoiduronosyl-6-sulfo-[1-^{3}H]anhydromannitol, and 9–10 cm for unsaturated monosulfated disaccharides obtained from CS/DS-PG by chondroitin ABC lyase treatment.

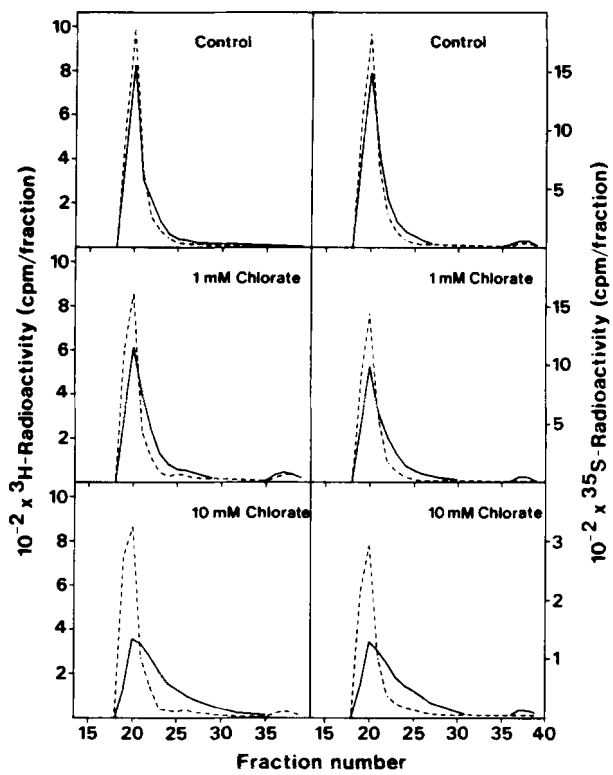

FIG. 8. **Chromatography of nitrous acid deamination products at pH 4.1 on a TSK G 2000 SW column.** Cultures had been incubated in the presence of [^{35}S]sulfate (0.74 MBq/ml) and [^{3}H] glucosamine (1.48 MBq/ml) for 15 h in the presence of the chlorate concentrations indicated. Cell-associated heparan sulfate was subjected at pH 4.1 to nitrous acid (*solid lines*) or acetic acid (*broken lines*) treatment followed by chromatography on a 7.5 × 500 mm TSK G 2000 SW column equilibrated with 0.1% SDS, 50 mM sodium phosphate, pH 6.0. Fractions of 0.5 ml were collected at a flow rate of 0.25 ml/min. ^{3}H radioactivity profiles are presented on the *left*, ^{35}S radioactivity profiles on the *right*. Note the different scales for ^{35}S radioactivity.

icellular proteoglycans consists of small CS/DS-PG (27). We have, therefore, analyzed the glycosaminoglycan composition of the material that can be released from the cell monolayer by mild trypsin treatment. From the data of Table II one can conclude that 4-sulfation is more affected by chlorate treatment than 6-sulfation. At a given chlorate concentration, sulfation of heparan sulfate is much less reduced than that of chondroitin sulfate/dermatan sulfate. Nitrous acid degradation at low pH which leads to the hydrolysis of sulfamate groups indicated that heparan sulfate from chlorate-treated cells was less affected with respect to its *N*-sulfate than to its ester sulfate content (Fig. 7). However, some amino sugar residues must contain an unsubstituted amino group. Nitrous acid degradation at pH 4.1 resulted in a partial depolymerization of undersulfated heparan sulfate (Fig. 8). At this pH, *N*-sulfated amino sugar residues are only minimally attacked (29), and only trace amounts of inorganic sulfate were therefore formed. The similarity of the profiles of ^{3}H and ^{35}S radioactivities indicated that the unsubstituted amino groups did not cluster at over- or undersulfated regions along the sulfate chains.

DISCUSSION

In contrast to previous reports (17, 18), incubation of fibroblasts in sulfate-depleted medium alone did not result in the production of undersulfated proteoglycan. This failure has also been observed in case of Swiss mouse 3T3 cells (35). The divergent results could be explained theoretically by differences in the capacity of endogenous synthesis of sulfate from methionine and cysteine. In contrast to the use of sulfate-depleted medium alone, the addition of chlorate to this medium led to a rapid and predictable decline of the incorporation of [^{35}S]sulfate into proteoglycans. The availability of a monospecific antiserum against the CS/DS-PG core protein made the quantitative isolation of this proteoglycan possible without relying on its polyanionic charge. From the results the following conclusions can be drawn.

First, the degree of polymerization of the glycosaminoglycan chains is not dependent on the extent of their sulfation. It had been suggested previously that chondroitin sulfate chain elongation stops after the formation of *N*-acetylgalactosamine 4,6-bis-sulfate residues at the nonreducing terminal position (36), and a 6-sulfotransferase with strong activity toward terminal *N*-acetylgalactosamine 4-sulfate residues of dermatan sulfate had been purified from human serum (37). In the present investigation, the nonreducing terminus of the glycosaminoglycan chains has not been characterized but it was shown that the sulfate ester groups do not cluster at the nonreducing portion as a result of chlorate treatment. From the studies on the biosynthesis of CS/DS-PG in the presence of monensin, it was suggested that chain polymerization could occur in an earlier compartment of the Golgi complex than 4-

sulfation (38), which also implies the independence of glycosyltransferase and subsequent sulfotransferase actions.

Second, completely unsulfated glycosaminoglycan chains could not be detected even at the highest chlorate concentrations used, but chlorate caused the biosynthesis of polysaccharides varying widely in their degree of sulfation. However, some fully sulfated chains were synthesized even in the presence of 3 mM chlorate where the majority of the material contained between 0.15 and 0.7 sulfate ester groups per disaccharide unit. This could be explained by the assumption that, upon the formation of a critical number of sulfate ester groups, further sulfation is stimulated. In conjunction with this assumption is the finding that keratan sulfate sulfotransferases reacted best with keratan sulfate segments exhibiting already a relative high degree of sulfation (39). The observation of glycosaminoglycan chains of varying degrees of sulfation is in contrast to the previous observation of undersulfation of a cartilage proteoglycan (16), where fully sulfated and unsulfated polysaccharide chains were linked to the same core protein. Because of the substitution of CS/DS-PG core protein with a single chain only, the above explanation cannot be valid in the latter case.

Third, the transit time of CS/DS-PG through the various biosynthetic compartments was unaltered in the presence of chlorate. Sulfation, therefore, cannot be considered as a transport signal. A fourth observation, an inverse relation between undersulfation and epimerization of GlcA residues, was not unexpected. It had been shown previously that epimerization of D-glucuronic acid to L-iduronic acid residues is tightly coupled with sulfation of the adjacent *N*-acetylgalactosamine residues (40).

In addition to CS/DS-PG which could be isolated immunologically, the influence of chlorate on the structure of cell-associated chondroitin sulfate/dermatan sulfate and heparan sulfate, respectively, was also investigated. Concerning the galactosaminoglycan, 6-sulfation of the amino sugar was more resistant to chlorate than 4-sulfation. Such an effect would be expected if the 6-sulfotransferase has a lower K_m than the 4-sulfotransferase for 3′-phosphoadenylylsulfate. However, the two sulfotransferase activities in microsomal preparations from chick cartilage and mouse mastocytoma, respectively, exhibited similar apparent K_m values for 3′-phosphoadenylylsulfate (41–43). It cannot yet be decided whether this discrepancy results from the use of either living cells or microsomes, or whether the fibroblast sulfotransferases differ in their kinetic properties from cartilage and mastocytoma enzymes.

Of special interest was the observation that chlorate treatment led to a greater reduction of sulfate incorporation into galactosaminoglycans than into heparan sulfate. Within the heparan sulfate molecule, the formation of sulfamate groups was less affected than that of ester sulfate groups. The K_m for *O*-sulfation of heparin has been reported to be higher than the K_m for *N*-sulfation (44). Nevertheless, an uncoupling of glucosamine deacetylation and *N*-sulfation was shown unambiguously. The glycosaminoglycan chains of heparan sulfate proteoglycans bind to fibronectin and laminin, and specific sulfate-ester-containing structures may exhibit anticoagulant and antiproliferative properties (see 45–47 for review). No such specific functions have been ascribed so far to the polysaccharide moiety of CS/DS-PG. Since cultured fibroblasts tolerate chlorate treatment for at least 1 week, it appears possible to isolate sufficient quantities of native CS/DS-PG of different sulfation degrees for functional investigations.

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