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CHLORATE: A REVERSIBLE INHIBITOR OF PROTEOGLYCAN SULFATION

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Bovine aorta endothelial cells were cultured in medium containing [^{3}H]glucosamine, [^{35}S]sulfate, and various concentrations of chlorate. Cell growth was not affected by 10 mM chlorate, while 30 mM chlorate had a slight inhibitory effect. Chlorate concentrations greater than 10 mM resulted in significant undersulfation of chondroitin. With 30 mM chlorate, sulfation of chondroitin was reduced to 10% and heparan to 35% of controls, but [^{3}H]glucosamine incorporation on a per cell basis did not appear to be inhibited. Removal of chlorate from the culture medium of cells resulted in the rapid resumption of sulfation.

The sulfate substituents of proteoglycans make these substances the most negatively charged of animal macromolecules. Moreover, proteoglycan function is probably mediated to a considerable degree by the strong negative charge that these substituents impart. This raises the possibility that variations in sulfation might have considerable biological ramifications. Although several conditions have been described involving deficiencies in proteoglycan sulfation (1-6), the most direct way to examine this would be to establish in vitro conditions that might promote undersulfation.

We, and others, have obtained undersulfation of proteoglycans by incubating cells or tissues in sulfate-depleted medium (7-11). These studies had to be performed in medium that lacked serum, since serum contains appreciable amounts of sulfate. Thus, the cells were not maintained in optimal growth conditions,

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The abbreviations used are: ΔDi-0S, 2-acetamido-2-deoxy-3-0-(B-D-Glc-4-enepyranoslyuronic acid)-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-0- (B-D-Glc-4-enepyranosyluronic acid)-4-0-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-0-(B-D-Glc-4-enepyranosyluronic acid)-6-0-sulfo-D-galactose; PAPS, adenosine 3'-phosphoadenylylphosphosulfate.

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nor could incubations be carried out for protracted periods. An alternative method of achieving proteoglycan undersulfation in cell cultures has also been recently attempted. This consisted of specifically inhibiting 3'-phosphoadenylylphosphosulfate (PAPS, the active form of sulfate) formation with a variety of chemicals (12,13). Chlorate appeared to be the most effective of the substances used in reducing the sulfation of a variety of macromolecules. However, proteoglycans were not isolated, so that sulfation of this class of macromolecule was not examined in any detail, nor were effects on glycosaminoglycan formation described. Moreover, these studies were performed with sulfate-depleted medium so the degree of undersulfation caused by chlorate alone could not be assessed. Furthermore, little or no calf serum was used, thus limiting the length of time for exposures of the cells to chlorate. Thus there was no information concerning the effects of chlorate on cell growth or cell viability in longer incubations. For these reasons, it is unclear how useful the technique would be to limit sulfation of proteoglycans in cells which are growing normally in other respects.

We have now investigated the effects of various concentrations of chlorate on cells cultured in sulfate-containing growth medium with usual amounts of serum. We determined that under these conditions, as much as 30 mM chlorate could be added without significantly affecting cell growth and viability. We have analyzed the proteoglycans to determine their degree of sulfation in the presence of varying amounts of chlorate and have been able to inhibit approximately 90% of chondroitin sulfation and approximately 65% of heparan sulfation. When we used chlorate with sulfate-free, serum-free medium, we were able to decrease sulfation still further.

MATERIALS AND METHODS

Sulfate-free RPMI 1640 medium in which $MgCl_2$ was substituted for $MgSO_4$ was purchased from GIBCO. [^{3}H]Glucosamine (44.8 Ci/mmol) and $H_2{}^{35}SO_4$ (carrier-free) were purchased from New England Nuclear. Chondroitin ABC lyase and the standard disaccharides ΔDi-6S , ΔDi-4S, and ΔDi-0S were obtained from ICN Immunobiologicals. Sodium chlorate, chondroitin 6-sulfate, chondroitin 4-sulfate, and papain were from Sigma. Chondroitin was made by desulfation of chondroitin 4-sulfate as described previously (14).

Endothelial cells, obtained from calf aorta by the standard method of collagenase digestion, were grown in RPMI-1640 media containing 0.4 mM sulfate and 20% fetal calf serum. Cells were grown to confluency and preincubated for 16 h in 4 ml of the same medium containing various concentrations of chlorate. After examining the cells by light microscopy, the medium was removed and replaced with 2 ml of the medium containing the same concentrations of chlorate plus [^{3}H]glucosamine (17×10^6 cpm/ml) and [^{35}S]sulfate (13×10^6 cpm/ml). After 4 h, the medium was removed and an aliquot desalted on Sephadex G-50 with 0.5 M ammonium bicarbonate as eluent. The excluded fractions were pooled, freeze-dried, and redissolved in H_2O. Cells were removed by scraping, and DNA determined fluorometrically (15).[2] In another experiment, cells were grown to confluence in the medium as above with 30 mM chlorate. The cells were then incubated in serum-free, sulfate-free RPMI-1640 containing 2 mM chlorate plus [^{3}H]glucosamine (21×10^6 cpm/ml) and [^{35}S]sulfate (17×10^6 cpm/ml). After 4

h, medium from these cells was treated with papain (5 units, 45° C, 16 h) before desalting on Sephadex G-50.

Amounts and degree of sulfation of [^{3}H]chondroitin [^{35}S]sulfate and [^{3}H]heparan [^{35}S]sulfate were determined by treatment of the isolated [^{3}H,^{35}S]glycosaminoglycans with chondroitin ABC lyase (16) (0.15 unit, 1 h, 37° C) followed by chromatography on Whatman No. 1 paper with a descending system of 1-butanol:acetic acid:1 M ammonium hydroxide (2:3:1) (16). This separated the undegraded [^{3}H]heparan [^{35}S]sulfate, which remained at the origin, from the [^{3}H]chondroitin [^{35}S]sulfate disaccharides, ΔDi-6S, ΔDi-4S, and ΔDi-0S. Standard disaccharides were localized under ultraviolet light. The origins and the disaccharides were counted by liquid scintillation spectrometry.

RESULTS AND DISCUSSION

Cell cultures incubated for the total 20 h in serum-containing medium with 10 mM chlorate appeared unaffected in number of attached cells as measured by DNA (Table 1). However, at 30 mM chlorate there appeared to be some loss of cells. Visual appearance (light microscopy) of the cultures was consistent with this. Incubations containing 100 mM chlorate resulted in detachment of most of the cells and a more rounded appearance of the remainder. This probably was due to the excess salt concentration. When [^{3}H]glucosamine was present for the final 4 h of the incubation, the ratio of total ^{3}H incorporated to DNA remained relatively constant, suggesting that any decreases in cpm with increasing concentration of chlorate was due to the loss of cells and not to a specific inhibition of ^{3}H incorporation. The ratio of total ^{3}H incorporation to the formation of [^{3}H]chondroitin also remained constant, indicating that there was no specific change in glycosaminoglycan synthesis when up to 30 mM chlorate was used.

Aliquots of the medium were digested with chondroitin ABC lyase in order to examine the sulfation of the [^{3}H]chondroitin. The disaccharide degradation products, as analyzed by paper chromatography (Fig. 1), indicated no loss of sulfation up to 3 mM chlorate. However, at 10 mM chlorate there was less [^{3}H]ΔDi-6S and [^{3}H]ΔDi-4S and approximately 60% [^{3}H]ΔDi-0S. At 30 mM chlorate there was less than 10% chondroitin sulfation, since most of the disaccharide products of the chondroitin ABC lyase digestion were ΔDi-0S. The ratio of

Table 1

Effect of chlorate on the incorporation of [^{3}H]glucosamine into macromolecules produced by cultured endothelial cells

Chlorate mM	DNA μg	^{3}H cpm	^{3}H cpm per μg DNA	[^{3}H]chondroitin cpm
0	23	240,000	10,400	37,000
3	23	213,000	9,300	33,000
10	22	223,000	10,000	36,000
30	18	172,000	9,600	28,000

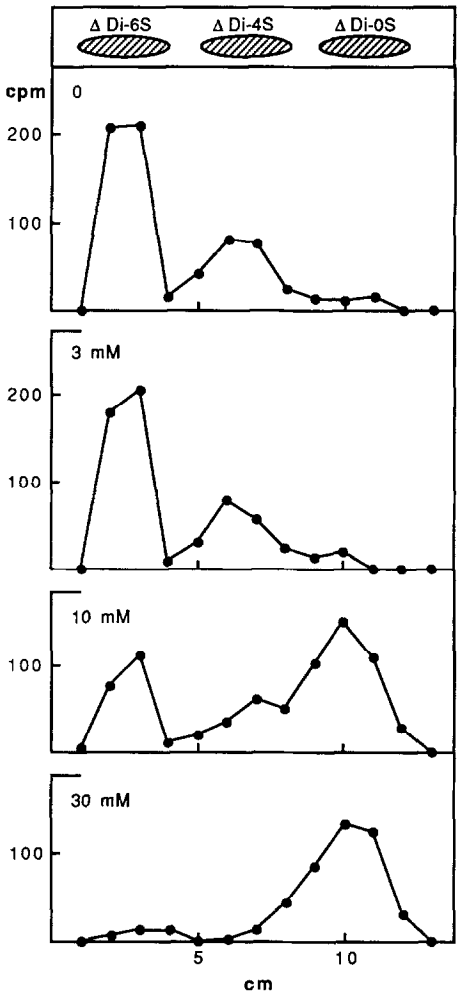

Fig. 1. Paper chromatography of disaccharides from [^{3}H]chondroitin sulfate. Labeled glycosaminoglycans formed by cells incubated in medium containing [^{3}H]glucosamine and varying concentrations of sodium chlorate were mixed with chondroitin, chondroitin 4-sulfate, and chondroitin 6-sulfate and degraded with chondroitin ABC lyase prior to chromatography on paper. Strips of 1 cm were eluted and analyzed for [^{3}H]hexosamine. Migration of standard degradation products, ΔDi-6S, ΔDi-4S, and ΔDi-0S, is shown at the top.

ΔDi-6S to ΔDi-4S remained constant, indicating that the decrease in sulfation was equal for chondroitin 6-sulfate and chondroitin 4-sulfate. This consistent ratio is similar to results we described for undersulfation of cells when they were grown in sulfate-depleted medium (9). Examination of the origins for [^{3}H]heparan ^{35}S-sulfation indicated a lesser inhibition of sulfation than that of chondroitin. At 30 mM chlorate the heparan sulfate was 35% as sulfated as heparan sulfate formed in the absence of chlorate.

In an attempt to undersulfate the glycosaminoglycans further, a modification of the growth conditions was used. Sulfate-free (rather than 0.4 mM sulfate) RPMI-1640 was used together with 20% fetal calf serum and 30 mM

Table 2
Effect of chlorate on the production of
[^{3}H]chondroitin [^{35}S]sulfate and [^{3}H]heparan [^{35}S]sulfate

Treatment	^{3}H	^{35}S	^{35}S/^{3}H	SO_4/hexosamine
	cpm			
Chondroitin Sulfate				
None	4500	1700	0.39	1*
Chlorate	3500	30	0.01	0.02
Heparan Sulfate				
None	1400	370	0.26	0.68
Chlorate	940	60	0.07	0.17

*The chondroitin sulfate was essentially fully sulfated (Fig. 1, 0 chlorate), providing a 1/1 ratio of SO_4/hexosamine.

chlorate. Under these conditions, cells grew well and were passed. At confluence the cells were incubated in sulfate-free (rather than 0.4 mM sulfate) and serum-free (rather than 20% serum) medium containing [^{3}H]glucosamine, [^{35}S]sulfate, and 2 mM chlorate for 4 hours. (The cells were not viable in higher concentrations of chlorate when serum-free medium was used.) [^{3}H,^{35}S]Glycosaminoglycans were isolated and [^{3}H]hexosamine and [^{35}S]sulfate incorporated into [^{3}H]heparan [^{35}S]sulfate and [^{3}H]chondroitin [^{35}S]sulfate determined. As shown in Table 2, the chondroitin sulfate formed by cells exposed to chlorate had less than 3% as much sulfate as control cells. Heparan sulfate formed by these same cultures contained 25% as much sulfate as heparan sulfate formed by cells grown without chlorate. Thus the sulfation of chondroitin sulfate was much more sensitive to chlorate than was the sulfation of heparan sulfate. This is similar to our previous results concerning under-sulfation in cultured cells grown under low sulfate conditions (9).

Cells were also grown in normal media rather than chlorate-containing media followed by incubation for 2 h in medium containing [^{3}H]glucosamine and 30 mM chlorate (Fig. 2A). Under these conditions, sulfation was limited to the same extent as with cells that had been exposed to chlorate for a full 16 h before incubation with the [^{3}H]glucosamine. Thus the effect of chlorate on sulfation appeared to be rapid. Cells incubated in 30 mM chlorate overnight were then incubated with [^{3}H]glucosamine in normal chlorate-free medium for 2 h (Fig. 2B). They produced completely sulfated glycosaminoglycans. Thus the chlorate effect appeared to be rapidly reversible. These findings also indicated that there was no substantial intracellular reservoir of PAPS. The cells cultured in serum-containing medium with 30 mM chlorate looked like and grew similarly to cells incubated without chlorate, suggesting that the full sulfation of chondroitin and heparan was not important for the growth of endothelial cells.

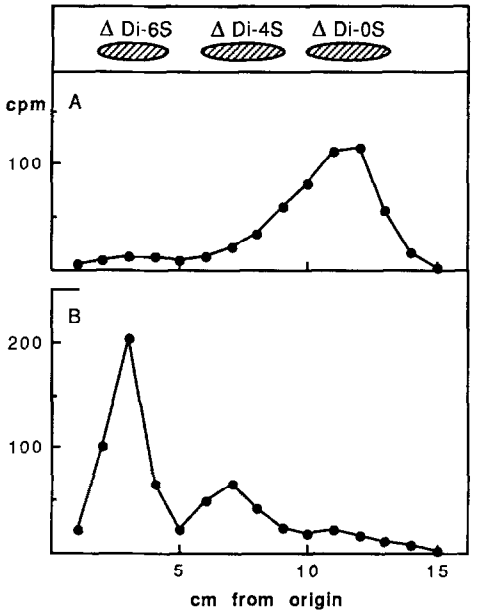

Fig. 2. Paper chromatography of disaccharides from [^{3}H]chondroitin sulfate. Labeled glycosaminoglycans formed by cells A) grown in chlorate-free medium, then incubated with [^{3}H]glucosamine for 2 h in medium containing chlorate, or B) grown in medium containing chlorate, then incubated with [^{3}H]glucosamine for 2 h in chlorate-free medium. [^{3}H]Chondroitin sulfate was degraded and analyzed as described in Fig. 1.

This is of particular note since heparan sulfate has been implicated in cell attachment and spreading (17-19). Our results also indicate that treatment of cells with chlorate in order to reduce the sulfation of proteoglycans might be an ideal method for examining possible functions of the sulfate groups.

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