Chlorate-induced Inhibition of Tyrosine Sulfation on Bone Sialoprotein Synthesized by a Rat Osteoblast-like Cell Line (UMR 106-01 BSP)*

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Bone sialoprotein (BSP) is a major noncollagenous, RGD-containing glycoprotein found in the extracellular matrix of bone. The RGD sequence is flanked by two tyrosine-rich regions, which fit the established consensus requirements for tyrosine sulfation. Tyrosine sulfation is suggested to be important in the regulation of protein secretion and function. The role of this posttranslational modification on the cell attachment activity and secretion of a highly sulfated form of BSP isolated from a rat osteoblast-like cell line (UMR 106-01 BSP) was investigated by inhibiting sulfation with chlorate. [35S]Sulfate, [3H]glucosamine, and [3H]tyrosine were used as metabolic precursors to monitor biosynthetic products. Chlorate was effective in inhibiting total [35S]sulfate incorporation by 90% without altering overall protein synthesis and secretion in cultures up to 72 h under serum-free conditions. Isolated proteoglycans and purified BSP were analyzed for sulfate incorporation. Proteoglycans isolated from the medium of cells treated with chlorate displayed a difference in the hydrodynamic properties of the molecules as compared with control cultures. An increase in the specific activity of proteoglycans labeled with [3H]glucosamine isolated from chlorate-treated cells was also observed suggesting a change in hexosamine metabolism induced by chlorate. BSP purified from the medium of chlorate-treated cells contained ~7% of the 35S incorporation as compared with control cultures. Sulfation of BSP incorporation into glycoconjugates versus tyrosine sulfation of BSP indicates that the amount of sulfate associated with N- and O-linked oligosaccharides was reduced by ~97%, while that on tyrosine residues was reduced by ~90%. Using normal human bone cells, the cell attachment activity of the reduced sulfate form of BSP was nearly equivalent to that of the fully sulfated product.

Tyrosine sulfation is a widespread modification of secreted proteins (1, 2). Although secretory proteins are the most abundant substrate (1, 3), intracellular and integral membrane proteins can also contain sulfated tyrosine residues (4–7). The covalent addition of sulfate esters to tyrosine occurs in the trans-Golgi network and is catalyzed by a tyrosylprotein sulfotransferase (8–11). This suggests that sulfation may be one of the last modifications of proteins before exiting the cell (10). Sulfation of tyrosine can be inhibited by specific inhibitors of sulfate incorporation (12, 13). Chlorate, a competitive inhibitor of sulfate adenyllytransferase, rapidly and reversibly inhibits the sulfation of both tyrosine residues and glycoconjugates when added to the medium of cells grown in culture (13, 14). This and other specific inhibitors of sulfate incorporation can be used to demonstrate the biological significance of this modification (12, 13). Sulfation has been shown to affect the biologic activity of proteins (15–17) and influence the half-life of proteins secreted into circulation (18) and may act as a targeting signal for the secretion of certain proteins (19–21).

The proteins found in the extracellular matrix of bone undergo extensive posttranslational modifications (22). Bone sialoprotein (BSP) comprises 10–15% of the total noncollagenous proteins associated with the mineral phase of human bone and contains approximately 50% of the mass as N- and O-linked oligosaccharides, some of which are sulfated (23, 24). BSP also contains sulfate esters on approximately half of its tyrosine residues (23). The majority of the tyrosine residues have been mapped in one-fourth of the polypeptide located at the carboxyl-terminal end of the molecule (25). This region of the molecule contains the Arg-Gly-Asp (RGD) sequence that binds to integrins (26, 27) and is therefore important in the cell attachment activity of this protein (28). Acidic amino acids as well as “β turn-inducing” amino acids in this region of the molecule predict that sulfation can occur on some of the tyrosines that flank the RGD sequence (25, 29, 30). Thus, the presence of tyrosine sulfate in BSP may modulate its integrin binding activity or potentiate its ionic interactions with bone mineral (23). We investigated the role of tyrosine sulfation on the synthesis, secretion, and integrin binding activity of BSP by using sodium chlorate to inhibit sulfation of BSP synthesized and secreted by a rat osteoblast-like cell line (UMR 106-01 BSP) (23, 31, 32). In this paper, we demonstrate that a near lack of sulfation of BSP (~7% of control levels) does not significantly modify any of these processes.

**EXPERIMENTAL PROCEDURES**

Materials—All reagents were of the highest purity commercially available. Sephadex G-50 (fine), Q-Sepharose, and Superose 6 HR 10/30 were from Pharmacia LKB Biotechnology Inc.; an AminoPac PA1 (4 x 250 mm) column was from Dionex Corp.; Bio-Gel P-10 (200–400 mesh) was from Bio-Rad; [3H]glucosamine HCl (40 Ci/mmol) and [35S]sulfuric acid (carrier free) were from DuPont NEN; [2-3H]tyrosine (51 Ci/mmol) was from Amersham Corp.; Eagle’s minimum essential medium (Earle’s salt) and a 100 x solution of nonessential amino acids were from Mediatech (Herndon, VA); sulfate-free medium 199 and other reagents were of the highest purity commercially available.

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† The abbreviations used are: BSP, bone sialoprotein; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; PAGE, polyacrylamide gel electrophoresis.
culture fluids were from Life Technologies, Inc.; tissue culture flasks and dishes were from Falcon; Centricell 10 ultrafilters (10-kDa cutoff) were from Polysciences Inc.; Centricell 10 microconcentrators (10-kDa cutoff) were from Amicon; Ultrafree-MC ultrafilters (0.2 µm) were from Millipore.

Cell Culture—UMR 106-01 BSP cells were grown from frozen cell stocks stored at \(-80^\circ C\) in 10% (v/v) dimethyl sulfoxide in fetal calf serum. The cells were thawed and grown to confluence in Eagle’s minimum essential medium containing added nonessential amino acids, 20 mM Hepes, pH 7.2, and 10% fetal calf serum in tissue culture flasks (75 cm\(^2\)) at 37 °C in a humidified 5% CO\(_2\) atmosphere. Subcuturing to 9.6 cm\(^2\) wells (35-mm dishes) was accomplished by incubating the confluent monolayers with 0.05% (w/v) trypsin, 0.53 mM disodium EDTA in Hank’s balanced salt solution without Ca\(^{2+}\) and Mg\(^{2+}\) at 37 °C for 5 min. Confluent monolayer cultures were rinsed three times with serum- and sulfate-free medium 199 (necessary for chlorate to be effective below 10 mM). They were then preincubated for 30 min in this medium (with or without added chlorate) to promote the secretion of intracellular stores of BSP (23, 31, 32) and to achieve maximal inhibition of sulfation reactions (13, 14). This preincubation medium was discarded and replaced with an identical medium containing radiolabels (100 µCi/ml [\(^{35}\)S]sulfate and either 100 µCi/ml [\(^3\)H]tyrosine or 50 µCi/ml [\(^3\)H]glucosamine). The cultures were then metabolically labeled for 8, 24, 48, or 72 h. Duplicate cultures were used to monitor cell numbers. The number of cells/culture was determined by counting in a hemacytometer after trypsinization. Cell viability was determined by trypan blue exclusion. Except where noted, chlorate was added to a final concentration of 3 mM to inhibit sulfation of tyrosine and oligosaccharide residues.

Primary cultures of human bone cells were isolated and cultured as described previously (33). Cell attachment assays were performed as outlined in Mintz et al. (28).

Isolation of Bone Sialoprotein—Radioiodinated BSP was isolated from the serum-free culture medium of UMR 106-01 BSP cells as previously described (23). Briefly, medium collected from labeled cultures was made 4 x in guanidine HCl by adding solid guanidine HCl. The labeled macromolecules were separated from unincorporated radioiodocorticosteroid precursors by chromatography on Sephadex G-50 as described (23). A column of Sephadex G-50 was prepared as follows: 50 ml of 4 M sodium acetate, pH 6.0. The peak fractions were applied to columns of Q-Sepharose (4-ml packed column) and eluted with 0.5 M formamide, 0.1 M NaCl, 0.5% Triton X-100, 50 mM sodium acetate, pH 6.0. Labeled macromolecules recovered in the excluded volume fractions were applied to columns of Q-Sepharose (4-nl packed resin/1.5-ml sample) and eluted in the same buffer. In analytical experiments, the labeled macromolecules bound to the Q-Sepharose were eluted with a continuous salt gradient (0–1.2 M NaCl), and eluant fractions were analyzed for radioactivity. Salt gradients were monitored by conductivity measurements of the fractions. For preparative experiments, the columns were extensively washed with 10 x formamide, 0.1 M NaCl, 0.5% Triton X-100, 50 mM sodium acetate, pH 6.0, to elute the excess borohydride and then batch eluted by the addition of 4 x guanidine HCl, 0.5% CHAPS, 50 mM sodium acetate, pH 6.0, to recover the bound molecules. Aliquots of the unbound and bound/guanidine-eluted fractions were assayed for radioactivity. Fractions with labeled macromolecules eluted by the guanidine-HCl step were concentrated by ultrafiltration using Centricell 10 devices. The concentrated samples were applied to a Superose 6 column and eluted with 4 x guanidine HCl, 0.5% CHAPS, 50 mM sodium acetate, pH 6.0. The peak fractions that contained BSP were recovered and concentrated using Centricell 10 microconcentrators.

Cell layers from the experiments were extracted with a solution of 4 x guanidine HCl containing 2% Triton X-100 and protease inhibitors as described elsewhere (34). The radioiodinated macromolecules were separated from the unincorporated precursors by gel filtration on Sephadex G-50 columns as described above, and aliquots of the excluded volume fractions were analyzed for radioactivity. Cell layer samples were further analyzed because their BSP contents were very low. Radioactivities in samples were determined by liquid scintillation spectrometry in an LS 5801 spectrometer (Beckman) using High Safe 3 (Wallac) as the scintillant. BSP samples for the cell attachment studies were purified as described above from the sulfate- and serum-free medium of 72-h cultures of UMR 106-01 BSP cells.

SDS-PAGE Analysis of BSP—Aliquots of the concentrated BSP fractions isolated from Superose 6 were exchanged into 10 x formamide, 50 mM NaCl, 50 mM sodium acetate, pH 6.0, by several cycles of adding buffer followed by concentration by ultrafiltration on Centricell 10 devices. Portions of these samples were analyzed on 4–20% linear gradient polyacrylamide gels under reducing conditions and in the presence of SDS as described previously (24). Gels were prepared for fluorography according to the method of Bonner and Laskey (35).

Oligosaccharide Analyses—After fluorography, a dried SDS-PAGE gels was aligned with the exposed x-ray film, and the band corresponding to BSP was excised. A gel slice (lacking any bands) of approximately the same size and position in the polyacrylamide gradient was used as a control. The gel slices were incubated in 50 mM NaOH in the presence of 1 x NaBH\(_4\) at 45 °C overnight (23). The reaction solution was then removed and clarified by low speed centrifugation. The excess borohydride was neutralized by the dropwise addition of 5 M acetic acid at 4 °C. Borate in the samples was then methylated by the addition of methanol and removed by vacuum evaporation. These additions and evaporation were repeated 3–4 times. The resulting white powder was resuspended in water, filtered by centrifugation on Ultrafree-MC devices, and applied to a Bio-Gel P-10 column (0.65 x 95 cm) preequilibrated with 0.5 M pyridinium acetate, pH 5.0 (23). Eluant fractions were analyzed for radioactivity. Recovery of radioactivity from the gel slices was >95%.

Tyrosine O-Sulfate Analysis—Other samples of BSP bands were excised from the dried gels, minced into small pieces (to enhance sample recovery), and then hydrolyzed in 1 x NaOH at 110 °C for 24 h under nitrogen. Sample recoveries were typically >90%. The hydrolysates were cooled on ice and filtered by centrifugation on Ultrafree-MC filters. The filtrates were applied (1 ml/min) to an Aminopac PA1 column and equilibrated with 0.38 M sodium acetate in 10 mM NaOH (solution A). The material bound to the column was eluted with solution A and then with 1.5 M sodium acetate, pH 6.0 (solution B) as follows: 0–7 min in solution A, 7–20 min in solution B, and after 20 min, back to solution A. Fractions (15 µl) were collected and analyzed for radioactivity.

RESULTS

Effect of Chlorate on Overall Sulfation and Cell Number in UMR 106-01 BSP Cultures—The addition of increasing concentrations of sodium chloride to the medium of the UMR cultures decreased the incorporation of [\(^{35}\)S]sulfate into macromolecules sequestered into the medium (Fig. 1). The incorporation of [\(^3\)H]tyrosine into secreted macromolecules was not changed up to 3 mM chlorate. As previously observed for aortic endothelial cell cultures (14), the lowest concentration of chloride in serum- and sulfate-free medium that produced the greatest inhibition of sulfation in UMR cultures was 3 mM; this concentration was used for all the remaining experiments.

Confluent UMR cultures were switched to serum- and sulfate-free medium in the absence and presence of 3 mM chloride and allowed to incubate for an additional 72 h (Fig. 2). Samples were analyzed for adherent and nonadherent cell numbers at 0, 24, 48, and 72 h. As shown in Fig. 2, the total cell number (adherent plus nonadherent) increased by ~20% over the initial 24 h of incubation, then remained at this level through 72 h. The proportion of nonadherent cells was 5–10% at 48 h and increased to 25–30% by 72 h. In all cases, the majority of the cells (>85%) were viable as determined by trypan blue exclusion. The pres-
ence of 3 mM chlorate did not significantly change the total cell number or the proportion of nonadherent cells/culture.

Effect of Chlorate on the Kinetics of Radiosotope Incorporation into Macromolecules—The time course of incorporation of radiolabeled precursors into macromolecules in the medium and cell layer compartments was determined over 72 h in the presence or absence of 3 mM chlorate (Figs. 3 and 4). After an initial lag period, the incorporation of all precursors into macromolecules secreted in the medium was essentially linear from 24–72 h of labeling, while incorporation into macromolecules retained in the cell layer essentially reached plateau values by 30–40 h of incubation. Analyses of the macromolecules in the cell layer by methods outlined below indicated that this compartment contained very little BSP (data not shown), consistent with our previous results (23). Therefore, cell layer fractions were not analyzed further.

An 8-h exposure to chlorate was as effective as all longer exposures in inhibiting sulfation (Figs. 3 and 4). In the presence of chlorate, macromolecules in the medium contained ~10% as much incorporated [35S]sulfate, approximately the same amount of incorporated [3H] when [3H]tyrosine was the precursor, and an increase of ~40% of incorporated [3H] when [3H]glucosamine was the precursor compared with controls (Figs. 3 and 4). Data from the 48-h time point are summarized in Table I. Analyses of sulfated disaccharides recovered from chondroitin ABC lyase digests of proteoglycans (isolated as outlined below) from cultures labeled with [3H]glucosamine and [35S]sulfate showed that the ratio of [3H]/[35S] in the monosulfated disaccharides from chlorate-treated cultures was ~35% greater than that for disaccharides from controls (data not shown). This indicates that the specific activity of the UDP-hexosamine pool increases in cells exposed to chlorate, most likely as the result of inhibition of the metabolic pathways that synthesize glucosamine from glucose with a compensatory greater use of the exogenous labeled glucosamine (36).

Purification of BSP from the Medium of Chlorate-treated and Untreated Cells—BSP was purified from the medium from the 48- and 72-h time points. The results were essentially identical. Therefore, only data from the 48-h time point will be presented.

Fig. 5 shows analyses in which aliquots from chlorate-treated and control samples labeled with [3H]tyrosine and [3H]tyrosine were applied to Q-Sepharose columns and the bound material subsequently eluted with a linear NaCl gradient (0.1–1.2 M). In both cases essentially all of the [3H] activity and ~45% of the [3H] activity bound to the column. For the control sample, the [3H] activity eluted during the NaCl gradient as a broad peak centered around 0.6 M NaCl, while the [3H] activity eluted with a major peak centered around 0.5 M NaCl. For the chlorate-treated sample, both radioisotopes eluted earlier in the gradient, with a peak at ~0.2 M NaCl, indicating that sulfation contributes significantly to the ionic properties of the bound macromolecules. The results suggest that nearly all of the macromolecules in the chlorate-treated sample were undersulfated because the total population of bound material had a reduced affinity for the anion exchange column.

Proteoglycans and BSP do not separate well on the Q-Sepharose columns using NaCl gradients. For this reason, step elution with 4 M guanidine HCl was used to elute the columns in subsequent preparative experiments. Data from a representative experiment are shown in Table II. The unbound fractions...
Inhibition of Sulfation on Bone Sialoprotein

**FIG. 4.** Effect of chlorate on the kinetics of \(^{35}\)Slsulfate and \(^{3}\)Hglucosamine incorporation into macromolecules. See the Fig. 3 legend for details.

### TABLE I

|                | Control        | +Chlorate      |
|----------------|----------------|----------------|
|                | Sulfate Tyr GlcN | Sulfate Tyr GlcN | Sulfate Tyr GlcN |
| Cell layer     | 46 (14)         | 88 (114)       | 55 (145)        |
| Medium         | 54 (13)         | 12 (107)       | 45 (141)        |

\(^{35}\)S Radioactivity \(\times 10^5\) cpn/million cells

\(^{3}\)H Radioactivity \(\times 10^5\) cpn/million cells

Summary of the data for the 48-h time points from the Sephadex G-50 analyses (Figs. 3 and 4). Values are represented as percentage of the average total incorporated radioactivity from duplicate samples. The 100% values (counts/min/million cells) for \(^{35}\)Sulfate, \(^{3}\)HTyr, and \(^{3}\)Hglucosamine (glcN), respectively, are as follows: 1.52 \(\times 10^7\), 6.69 \(\times 10^6\), and 5.52 \(\times 10^6\) for control cultures; 1.64 \(\times 10^7\), 7.17 \(\times 10^6\), and 7.81 \(\times 10^6\) for cultures treated with 3 mM chlorate. Standard error of the mean values were \(<1\% of their respective means.

**FIG. 5.** Analytical Q-Sepharose chromatography of Sephadex G-50 samples labeled with \(^{35}\)Sulfate and \(^{3}\)Htyrosine. Aliquots from medium samples labeled for 48 h (Fig. 3) were applied to Q-Sepharose columns eluted with a linear NaCl gradient (0.1–1.2 M, solid line in the lower panel).

**TABLE II**

| Preparative Q-Sepharose chromatography |
|---------------------------------------|
| Macromolecules from the void volume of Sephadex G-50 were applied to Q-Sepharose as described under "Experimental Procedures." Samples represent pooled duplicates of the medium compartment from cultures labeled with the indicated radioactive precursors for 48 h. Proteoglycans (PGs) and BSP bound to the resin were recovered by step elution with 4 M guanidine HCl, 0.5% CHAPS, and 50 mM sodium acetate, pH 6.0. The data shown below are percentages of the pooled samples before analysis. The 100% values (counts/min) for \(^{35}\)Sulfate, \(^{3}\)Htyrosine, and \(^{3}\)Hglucosamine, respectively, are as follows: 6.8 \(\times 10^7\), 5.8 \(\times 10^6\), and 1.8 \(\times 10^7\) for control cultures; 6.5 \(\times 10^6\), 6.3 \(\times 10^6\), and 2.6 \(\times 10^7\) for cultures treated with 3 mM chlorate. S.E. values were \(<1\% of their respective means.

|                | Control        | +Chlorate      |
|----------------|----------------|----------------|
|                | Sulfate Tyr GlcN | Sulfate Tyr GlcN | Sulfate Tyr GlcN |
| Unbound        | 2 47 14         | 2 51 13        |
| PGs + BSP      | 84 42 71        | 83 40 67       |
| Unrecovered*   | 14 11 10        | 15 9 20        |

* Represents residual radioactivity still associated with the Q-Sepharose resin after extensive washing with the 4 M guanidine HCl solution. Determined by resuspending the gel and counting an aliquot of the slurry.

\(^{3}\)Hglucosamine as a precursor independent of whether or not the cultures were treated with chlorate. Recoveries from the columns ranged between 80–90%. A residual 10–20% of the samples remained bound to the Q-Sepharose resin even after prolonged incubation in 4 M guanidine HCl.

The macromolecules eluted from Q-Sepharose were concentrated by ultrafiltration, and the retentates eluted on Superose 6 in a 4 M guanidine HCl solvent (Fig. 6). Labeled proteoglycans in the control samples eluted as 2 peaks, the first in the excluded volume and the second partially included \((K_v = 0.18)\) (23). In the chlorate-treated samples, the \(^{35}\)S activity associated with the proteoglycans eluted as a single, broad peak with
The BSP peaks were recovered and concentrated by Centricon cultures gave essentially identical, broad bands at -80 kDa of parent molecular size. When similar gels were sliced (1-mm apparent molecular mass) and counted, the BSP peaks accounted for 80% of the total radioactivity in each gel lane (data not shown).

Repetitive Centricon 10 ultrafiltration and then analyzed by SDS-PAGE and fluorography (Fig. 7) as described under "Experimental Procedures." BSP from control and chlorate-treated untreated controls (Table 111).

Fractions having 3H (3H tyrosine as precursor) activity as their respective means. S.E. values were <10% of their respective means.

BSP samples were equilibrated with a 10 fold formamide solvent. In a typical experiment, the BSP recovered from chlorate-treated cultures contained ~7, ~96, and ~15% as much incorporated 35S, 3H (3H tyrosine as precursor), and 3H (3H glucosamine as precursor) activity as their respective untreated controls (Table III).

SDS-PAGE Analysis of Purified BSP—Aliquots of the purified BSP samples were equilibrated with a 10 x formamide solvent by repeated Centricon 10 ultrafiltration and then analyzed by SDS-PAGE and fluorography (Fig. 7) as described under "Experimental Procedures." BSP from control and chlorate-treated cultures gave essentially identical, broad bands at ~80 kDa of apparent molecular mass. This provides additional support that a lack of sulfation of BSP does not significantly affect its apparent molecular size. When similar gels were sliced (1-mm pieces) and counted, the BSP peaks accounted for 50% or more of the total radioactivity in each gel lane (data not shown).

Oligosaccharide and Tyrosine O-Sulfate Analyses—Bands corresponding to the purified BSP (Fig. 7) were excised from dried gels following fluorography and used to determine either oligosaccharide or tyrosine sulfate contents. Recovery of radioactivity from the gel slices after either alkaline borohydride treatment (oligosaccharide analysis) or NaOH hydrolysis (tyrosine sulfate analysis) was 85–95%.

Oligosaccharides and N-linked glycopeptides released by alkaline borohydride treatment of BSP samples labeled with [35S]sulfate and [3H]glucosamine as precursors were analyzed on a Bio-Gel P-10 column (Fig. 8). The profiles of 3H activity for BSP from control and chlorate-treated cultures were essentially identical with the N-linked glycopeptides eluting as a broad peak at a Kd of 0.22 (~58%), the O-linked oligosaccharides as a series of three narrower peaks from a Kd of 0.5–0.8 (~35%), and a peak of di- and monosaccharides at a Kd of 0.9 (~5%) (23). Thus, chlorate treatment did not appear to alter the relative amounts of BSP's glycoconjugates. Most of the 35S activity in both samples (~65 and ~85% for control and chlorate-treated samples, respectively) was present as tyrosine sulfate and tyrosine sulfate-containing peptides, which elute as a split peak from a Kd of 0.85–1.2 on this column, while the remainder elutes with the glycoconjugate peaks. The proportion of 3H activity in glycoconjugates was less (~15% of the total) in the BSP from chlorate-treated cultures than in the control (~35%). This suggests that sultation of oligosaccharides is somewhat more sensitive to chlorate treatment than sulfation of tyrosine.

Based on the Bio-Gel P-10 data in Fig. 8, the calculations to determine the ratio of tyrosine sulfate in chlorate-treated samples compared with control samples are as follows. (35S cpm in tyrosine sulfate/total 3H cpm in glycoconjugates) x 100/35S cpm in tyrosine sulfate = 85.6. The increase in 3H specific activity for the glucosamine precursor in chlorate-treated cultures was assayed by measuring the 35S and 3H incorporation into chondroitin-4-sulfate disaccharide as described by Yanagishita et al. (36) and determined to be ~35% higher than that for control cultures.

The amount of 3H proton exchange is reduced as compared with the solvent. When the amounts of 35S in the tyrosine sulfate peaks are normalized to the amounts of 3H in glycoconjugates and corrected for the difference in specific activity of the UDP-hexosamine pools (~35% increase for the chlorate-treated samples), the sample from the chlorate-treated cultures contained ~9% as much tyrosine sulfate as the control.

The relative amounts of tyrosine and tyrosine sulfate in samples were assessed by analysis of NaOH hydrolysates of BSP from [35S]sulfate- and [3H]tyrosine-labeled cultures on an AminoPac PA1 column (Fig. 9). Three characteristic 3H peaks were observed in the control. The earliest eluting, small peak (1.8 min) contains 3H that has exchanged from unsubstituted tyrosines in BSP into the solvent during hydrolysis (23). The second eluting, large peak (3.3 min) contains [3H]tyrosine, and the relative amounts of tyrosine and tyrosine sulfate in the sample from the chlorate-treated cultures were determined (~9% as much tyrosine sulfate as the control).
Inhibition of Sulfation on Bone Sialoprotein

While the third peak eluting at 15 min contains $[^3]$H-tyrosine sulfate. Nearly equal proportions of $[^3]$H activity in tyrosine sulfate (~44%) and tyrosine (~46%; sum of the two relevant peaks) were observed for BSP from control cultures in agreement with previous studies (23). The amount of $[^3]$H in the tyrosine sulfate peak for BSP from the chlorate-treated cultures was small and difficult to quantify accurately because of the high proportion of $[^35]$S activity in this region and low peak to baseline levels. However, a conservative estimate suggests ~5% of the $[^3]$H activity in tyrosine sulfate for BSP isolated from chlorate-treated cultures. When compared with the value for control cultures (~49%), this estimate suggests that 3 mM chlorate inhibited the sulfation of tyrosine residues by ~90% in general agreement with the estimate determined from the Bio-Gel P-10 data above. In addition to a major $[^35]$S-labeled peak in tyrosine sulfate (~60% for control BSP and ~84% for chlorate-treated BSP), an early eluting peak of free sulfate is present in the analyses that results primarily from hydrolysis of sulfate esters on glycoconjugates. Consistent with the results described above, the relative amount of $[^35]$S in the free sulfate peak (equivalent to sulfate esters on glycoconjugates) is less for the BSP from chlorate-treated cultures (~12%) than that for control cultures (~34%).

**Cell Attachment Activity**—The ability of primary human bone cells (33) to attach to the two forms of BSP under conditions that promote maximal cell adhesion (28) is shown in Table IV. When saturating amounts of BSP (0.2 $\mu$m) purified from chlorate-treated and control cultures were used to coat the dishes, nearly the same number of cells attached indicating no statistical difference between the effectiveness of the two forms of the protein. Cell attachment assays performed at a lower concentration of both forms of the protein (0.1 $\mu$m) yielded similar results (data not shown). The presence of the soluble peptide, GRGDS, effectively blocked a majority of the cells from binding, indicating that most of the attachment is mediated by cell surface integrins. These data suggest that the extent of sulfation of BSP may not have an important role in modulating the attachment activity of these bone cells to this protein.

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* The peak eluting at 9–10 min elutes with the salt front that results from the steep increase in solvent ionic strength during the chromatography and mainly consists of incomplete hydrolysis products.
TABLE IV

| Type of BSP | Percentage of cells attached to coated dish |
|-------------|-------------------------------------------|
|             | -GRGDS peptide | +GRGDS peptide |
| Control     | 100 ± 2        | 10 ± 2         |
| Chlorate-treated | 90 ± 3     | 6 ± 1          |

**DISCUSSION**

Cultures of the rat osteoblast-like cell line (UMR 106-01 BSP) synthesize and secrete large amounts of BSP, which has a high content of tyrosine sulfate (23). Several of these tyrosine sulfate residues are likely to surround an RGD sequence in this protein, which is critical for integrin binding and cell attachment. We used chlorate in the culture medium to inhibit sulfation of tyrosine residues in BSP to determine if sulfated tyrosine is important for synthesis, secretion, and/or cell attachment properties of BSP. Metabolic precursors were used to assess the effects of chlorate on sulfation ([35S]sulfate, protein synthesis ([3H]tyrosine), and glycoconjugate synthesis ([3H]glucosamine). During the radiolabeling period, the cells were cultured in medium without serum and with reduced levels of sulfate (~50 μM). This level of sulfate is sufficient to support normal levels of sulfation of proteoglycans and BSP while allowing low levels of chlorate to be effective in inhibiting sulfation metabolic pathways. This study showed that 3 mM chlorate rapidly inhibited the sulfation of proteoglycans and BSP (~90% inhibition for an 8-h exposure) without significantly altering the incorporation levels of [3H]tyrosine. Further, the cultures with or without 3 mM chlorate showed the same viable cell numbers as well as the same kinetics of protein synthesis and secretion over the 72-h culture period. Thus, this level of chlorate did not appear to have any adverse effects on these cells.

Analyses of BSP labeled with [3H]tyrosine showed that almost all of the BSP is secreted into the medium whether chlorate is present or not. When BSP was purified from the medium of 48-h labeled samples, nearly identical proportions of [3H] (21–24% of the total in the medium) were recovered in the BSP fraction for both culture conditions. Analyses of BSP labeled with [35S]sulfate revealed that the purified BSP from chlorate-treated cultures contained only ~10% as much tyrosine sulfate and ~3% as much sulfated glycoconjugates as did BSP from control cultures. These results indicate that this level of undersulfation did not significantly alter either synthesis or secretion of BSP by these cells.

When [3H]glucosamine was used as a precursor, the amount of [3H] recovered in the BSP from cultures treated with chlorate was ~1.5 times that for BSP from control cultures. Most of this increase was the result of an increase in the specific activity of the [3H] label in the UDP-N-acetylhexosamine precursor pool, presumably from a slightly reduced efficiency of endogenous metabolic pathways for synthesis of hexosamines from glucose.

BSP and proteoglycans synthesized by chlorate-treated cultures bind less tightly to an anion exchange column than do these macromolecules from control cultures. This is consistent with their net decreases in negative charges from loss of the sulfates normally present. Molecular sieve and SDS-PAGE analyses indicated that the loss of sulfate esters in BSP did not alter its hydrodynamic size significantly. Conversely, undersulfation seems to have changed the hydrodynamic size distribution of the proteoglycans. However, analyses of glycosaminoglycan chain sizes would be necessary to rule out significant changes in chain length as a contributing factor.

BSP bands were excised from SDS-PAGE gels after fluorography and analyzed for the content of sulfated tyrosine residues (after NaOH hydrolysis) and glycoconjugates (after alkaline borohydride treatment). In each treatment protocol, 85–95% of the radioactivity was recovered for analysis, demonstrating the utility of this approach. The procedure is particularly useful for analyzing macromolecules at stages before final purification is achieved. Although BSP was purified to near homogeneity, minor contaminants, which accounted for ~20% in the radioactive, were resolved from BSP by the SDS-PAGE gel in this study. Molar mass analyses of the glycoconjugates recovered from the BSP bands revealed that the relative distributions of N- and O-linked species were not appreciably affected by the chlorate treatment. However, the relative sulfation of the glycoconjugates appeared to be more sensitive to chlorate treatment than tyrosine sulfation. Comparison of the relative amounts of [35S] in sulfated tyrosine and [3H] in glycoconjugates indicated that the level of tyrosine sulfation in BSP from chlorate-treated cultures was only 9–10% of that in BSP from controls, and the tyrosine/tyrosine sulfate analyses were in good agreement with this estimate. This suggests that BSP isolated from the medium of chlorate-treated cultures contains an average of about 1 sulfated tyrosine residue out of the 12 tyrosine residues estimated to be normally sulfated on BSP synthesized by UMR cells (23). Given that the cultures were preincubated in chlorate-containing medium before the labeling period and that chlorate rapidly inhibits sulfation reactions, it is likely that the assessment of tyrosine and glycoconjugate sulfation on metabolically labeled BSP in chlorate-treated cultures accurately reflects the extent of sulfation on the total amount of BSP accumulated in the medium of these cultures during the 72-h incubation period.

The amino acids surrounding the RGD sequence in fibronectin and vitronectin have been shown to modulate receptor specificity for this tripeptide sequence (26, 27). BSP has been shown to support cell attachment via the RGD sequence found in the carboxyl-terminal region of the molecule (28, 37). This tripeptide sequence is flanked by several tyrosine residues that conform with the consensus sequence for sulfation (25, 29, 30), and we assume that many of these tyrosines are sulfated, though the exact sites are unknown at present. We compared the cell attachment activity of the normally sulfated and the undersulfated BSP and found no significant differences in their ability to support RGD-mediated attachment of normal human bone cells. Possible explanations for this observation are that (i) tyrosine sulfation does not significantly alter BSP's interactions with integrin receptors, (ii) the human bone cells used in this study express more than one integrin type (38) and that one form might avidly bind to highly sulfated BSP while the other might selectively bind to undersulfated BSP, or (iii) the residual tyrosine sulfate(s) on undersulfated BSP might still affect the integrin-mediated attachment of bone cells to this protein. Mapping the sites of tyrosine sulfation on BSP might reveal some minor differences between normally sulfated and undersulfated BSP with respect to their bone cell attachment activities.

BSP has been shown to interact with the mineral phase of
bone in vivo (24) and in vitro (39, 40). Recently, several investigators have demonstrated the presence of sulfate groups on BSP (23, 39, 40), though the extent of sulfation of BSP from normal bone has not yet been determined as for BSP from UMR cultures. Based on its elution properties on anion exchange columns (24, 41), BSP from normal bone would appear to be less sulfated than that from UMR cultures. Perhaps the extent of sulfation of BSP may be more important for affecting the binding of this bone glycoprotein to hydroxyapatite than it is for its synthesis, secretion, or cell attachment properties.

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