Heparan Sulfates from Swiss Mouse 3T3 and SV3T3 Cells: *O*-Sulfate Difference†

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ABSTRACT: A difference in the extent of sulfation between the heparan sulfate isolated from Swiss 3T3 mouse cells and that from Swiss 3T3 cells transformed by the DNA virus SV40 has been reported previously. This variance is manifested by different chromatographic and electrophoretic properties. Heparan sulfates from the two cell types were treated with nitrous acid under conditions that gave selective deaminative cleavage of glucosaminyl residues with sulfated amino groups in order to define the nature of the difference in sulfation further. The *O*-sulfate-containing fragments from the heparan sulfates were compared by gel filtration and ion-exchange chromatography. The results showed that the 3T3 heparan sulfate contains 8% more *O*-sulfate than does the SV3T3 heparan sulfate. Analysis of uronic acids revealed that both types of heparan sulfates contain 45% L-iduronic acid and 55% D-glucuronic acid. These and other observations indicate that the primary difference in sulfation between the 3T3 and SV3T3 heparan sulfates lies in the extent of O-sulfation.

Heparan sulfate has a number of properties which warrant studies of its role in cell–cell interactions. These properties include its structural complexity (Lindahl, 1976; Muir & Hardingham, 1975), its association with the cell surface (Kraemer, 1971; Underhill & Keller, 1975), and qualitative and quantitative changes which correlate with changes in cell behavior resulting from transformation or differentiation (Satoh et al., 1973; Underhill & Keller, 1975, 1977). A structural difference exists between the cell surface, medium and intracellular heparan sulfates isolated from Swiss 3T3 mouse cells, which exhibit density-dependent growth regulation, and those isolated from DNA virus transformed derivatives of Swiss 3T3 mouse cells, which no longer exhibit such regulation (Underhill & Keller, 1975; Johnston et al., 1979). A difference in sulfation between the two types of heparan sulfates was inferred from different elution positions on DEAE-cellulose chromatography (Underhill & Keller, 1975) and electrophoretic mobilities on cellulose acetate at pH 1.0 (Johnston et al., 1979). The difference is not due to dissimilar molecular weights (Keller & Keller, 1976), changes in cell density, a mutation in the parent cell line (Underhill & Keller, 1977), or the action of extracellular enzymes (Johnston et al., 1979).

Recent studies have illuminated the steps involved in the sulfation of heparin and heparan sulfate (Höök et al., 1974a,b; Jacobsson et al., 1979a). A nonsulfated polymer, initially composed of alternating D-glucuronosyl and *N*-acetyl-D-glucosaminyl units, subsequently is modified by several reactions including N-deacetylation, N-sulfation,[1] C_5-epimerization of some of the uronic acid residues, and, finally, O-sulfation in one or two possible positions. Thus, a minimum of five enzymes would appear to be involved. Since a single cell type produces a single type of heparan sulfate, but different cells can produce heparan sulfates of differing sulfate content (Keller et al., 1978), natural variances in either the enzymes and/or the control mechanisms are indicated. For the Swiss mouse 3T3 and SV3T3 cells, the difference in heparan sulfates may reflect a change in the biosynthetic scheme directly or indirectly induced by the virus. Knowledge of the structural basis for the change in sulfation should assist elucidation of the cellular processes leading to such a change. We have, therefore, initially inquired as to whether both *N*- and *O*-sulfates are involved in the observed differences.

Approaches to the structural study of heparin-like polymers have included both enzymatic (Linker & Hovingh, 1977) and chemical degradation with such reagents as nitrous acid (Cifonelli, 1968; Shively & Conrad, 1976). This paper compares the fragments produced by the nitrous acid degradation of 3T3 and SV3T3 heparan sulfates. At low pH (2.5), the glucosaminyl residues with sulfated amino groups are selectively deaminated by nitrous acid (Shively & Conrad, 1976). The accompanying cleavage of the glucosaminidic bond and release of the *N*-sulfate as inorganic sulfate produce oligosaccharide fragments which can be as small as disaccharides. These fragments contain variable amounts of L-iduronic acid and O-sulfated residues (Cifonelli, 1968; Höök et al., 1974a; Jacobsson et al., 1979b). The relative proportions of the different types of fragments are directly related to the arrangement of N-sulfated and N-acetylated glucosaminyl residues within the molecules. Thus, a comparison of the gel filtration and ion-exchange elution profiles of the fragments obtained from two different heparan sulfates should yield information on differences in the distribution of both N- and O-sulfated residues.

Materials and Methods

Materials. Trypsin (1× crystallized) and hyaluronidase (bovine testes, 3000–7000 units/mg) were obtained from Worthington Biochemical Corp. Chondroitin ABC lyase and Pronase were products of Miles Laboratories and Calbiochem, respectively. Heparitinase, free of chondroitinase activity, was prepared from *Flavobacterium heparinum* (ATCC 13 125) by the procedure of Linker & Hovingh (1972). The radioisotopes $H_2{}^{35}SO_4$ (carrier free), D-[6-^{3}H(N)]glucosamine (20 Ci/mmol), D-[1-^{14}C]glucosamine (55 Ci/mol), and D-[1-^{3}H-(N)]galactose (14.2 Ci/mmol) were purchased from New England Nuclear. Unless otherwise indicated, all reagents and chemicals were of the highest grade and purity available. The sources and maintenance of the mouse cells have been described previously (Johnston et al., 1979).

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[1] N-Sulfation = N-sulfamidation.

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Isolation of Radioisotopically Labeled Cell-Surface and Intracellular Heparan Sulfates. Subconfluent cell cultures were grown for 2 days with either $H_2{}^{35}SO_4$ (5 μCi/mL), D-[6-^{3}H]glucosamine (1 μCi/mL), D-[1-^{14}C]glucosamine (0.5 μCi/mL), or D-[1-^{3}H]galactose (20 μCi/dish) in modified media as described previously (Underhill & Keller, 1977). Cell-surface and intracellular heparan sulfates were isolated and characterized as described by Johnston et al. (1979). The heparan sulfate from SV3T3 cells coelectrophoresed at pH 1 with a standard heparan sulfate containing a N-sulfated glucosamine/hexosamine ratio of 0.45 and a sulfate/disaccharide ratio of 0.93 (this reference standard and its specifications were obtained from Dr. J. A. Cifonelli of the University of Chicago). The heparan sulfate from 3T3 cells migrated slightly ahead of both of these. The materials, as isolated and identified above, were radiochemically pure.

Analytical Methods. Analysis for radioactivity was performed as described previously (Underhill & Keller, 1975; Keller et al., 1978). Double isotope counting conditions were established so that no crossover of ^{3}H into the ^{14}C channel occurred. There was an 18% crossover of ^{14}C or ^{35}S into the ^{3}H channel. In all cases, radioactive samples were counted for a period of time adequate to reduce the counting error below 5% (Cooper, 1977).

Gel filtration chromatography was carried out on columns (1 × 200 cm) of Bio-Gel P2 and Bio-Gel P10 (both 200–400 mesh; Bio-Rad Laboratories, Richmond, CA). Elution was effected with 1 M NaCl in 0.02 M Tris buffer, pH 7.0, at a flow rate of 4 mL/h collected in fractions of 70 drops. Azoprotein was used to ascertain V_0.

Ion-exchange chromatography was performed on columns (1 × 20 cm) of DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) equilibrated with 0.05 M NaCl in 2 mM Tris buffer, pH 7.0, and eluted with a linear gradient of NaCl. The mixing vessel contained 150 mL of 0.05 M NaCl in 2 mM Tris buffer, pH 7.0, and the reservoir contained 150 mL of 1.5 M NaCl in the same buffer at pH 7.0. The elution rate was 24 mL/h collected in fractions of 80 drops. Linearity of the gradients was confirmed by measuring the conductivity of various fractions and converting the obtained values to NaCl concentrations by use of a standard curve. Since conductivity is temperature dependent, and the daily ambient temperature in our laboratory varies significantly, the apparent NaCl concentration at which specific species of glycosaminoglycans elute varies from experiment to experiment, but the relative positions of the peaks are invariant for a given column.

Desalting of pooled fractions was effected with 1 × 60 cm columns of Sephadex G-25 (Pharmacia) or Bio-Gel P2 (Bio-Rad) equilibrated and eluted with distilled water. Recovery of radioactive material from all columns was greater than 95%.

For the analysis of uronic acid, cells were grown in the presence of D-[1-^{3}H]galactose under the conditions described above, and the heparan sulfates were isolated, purified, and characterized as described above. Free hexuronic acids were released from the isolated polymers by using the procedure of Höök et al. (1974a) and analyzed by ion-exchange chromatography on Dowex AG 1-X8 (200–400 mesh) according to Fransson (1978). Elution was effected with 0.3 M formic acid collected at a rate of 9 mL/h in 2.0-mL aliquots which were subsequently assayed for radioactivity as described.

Degradation with Nitrous Acid. Conditions for the reaction of heparan sulfate with nitrous acid have been described (Underhill & Keller, 1975). Completion of the reaction by the end of the 90-min incubation period was verified by subjecting a mixture of ^{35}S-labeled and ^{3}H-labeled purified cell-surface heparan sulfates to two sequential treatments with nitrous acid for 90 min. No additional degradation was observed by the second treatment. Radioisotopically labeled samples were also degraded with nitrous acid according to the room-temperature procedure of Shively & Conrad (1976) and the reaction A conditions of Lindahl et al. (1973). Comparison by cochromatography of the degradation products from all three procedures revealed no differences. Radioisotopically labeled samples subjected to the reaction B conditions of Lindahl et al. (1973) evidenced no degradation when analyzed on Bio-Gel P10, which indicated that these molecules do not contain amounts of unsubstituted amino groups detectable by the analytical methods used in this study.

Results

Comparison of the Heparan Sulfate Deamination Products by Ion-Exchange Chromatography on DEAE-Sephadex A-25

Comparison of ^{3}H- and ^{35}S-Labeled Material. Heparan sulfates labeled with [^{35}S]sulfate and [^{3}H]glucosamine were mixed and degraded with nitrous acid. The radiochromatographic profiles generated by the ^{35}S- and ^{3}H-labeled fragments from 3T3 and SV3T3 heparan sulfates are shown in Figure 1a,b. For convenience, three regions on the graphs are defined as follows. Region I is the earliest eluting material and includes the inorganic sulfate released during the course of the reaction. Region II includes the first well-defined peak emerging after the inorganic sulfate and contains both ^{35}S- and ^{3}H-labeled material. The remaining material, region III, contains ^{3}H-labeled fragments as well as the largest proportion of ^{35}S label. The products from both 3T3 and SV3T3 heparan sulfates yielded similar elution profiles (compare Figure 1, a and b). For both profiles, a small proportion of [^{3}H]glucosamine[2]-containing material with no detectable accompanying ^{35}S-labeled material eluted early in region I before the large, sharp peak of inorganic sulfate. The 3T3 material had proportionately more ^{35}S and ^{3}H label in region III but less ^{35}S label released as inorganic sulfate. Many repeated degradations yielded comparable results. To verify these observations and ensure that the same portion of the elution profile was being used for comparison, we mixed, treated with nitrous acid, and then cochromatographed heparan sulfates from [^{3}H]-glucosamine-labeled SV3T3 cells and [^{35}S]sulfate-labeled 3T3 cells (Figure 1c). This procedure was also performed using [^{35}S]sulfate-labeled SV3T3 and [^{3}H]glucosamine-labeled 3T3 heparan sulfates (Figure 1d). Of the total 3T3 and SV3T3 ^{35}S-labeled material, 31% and 42%, respectively, eluted in region I (Table I). Although a small amount of [^{3}H]-glucosamine[2]-containing material, which could have been O-sulfated, also eluted in region I, the data suggested that the SV3T3 heparan sulfate contained proportionately more *N*-sulfate. Little difference in region II distribution was noted. However, in region III, there was 3% and 12% less ^{3}H- and ^{35}S-labeled SV3T3 material, respectively, than 3T3 material. Since the ^{35}S label associated with regions II and III represented the sulfate present as *O*-sulfate, the data suggested that the 3T3 heparan sulfate contained proportionately more *O*-sulfate than did the SV3T3 heparan sulfate.

Comparison of [^{3}H]Glucosamine- and [^{14}C]Glucosamine-Labeled Material. Because the large peak of released ^{35}S-

[2] The ^{3}H and ^{14}C labels in the nitrous acid derived fragments are located in both the glucosaminyl residues and the 2,5-anhydromannose residues generated from glucosamine by the reaction.

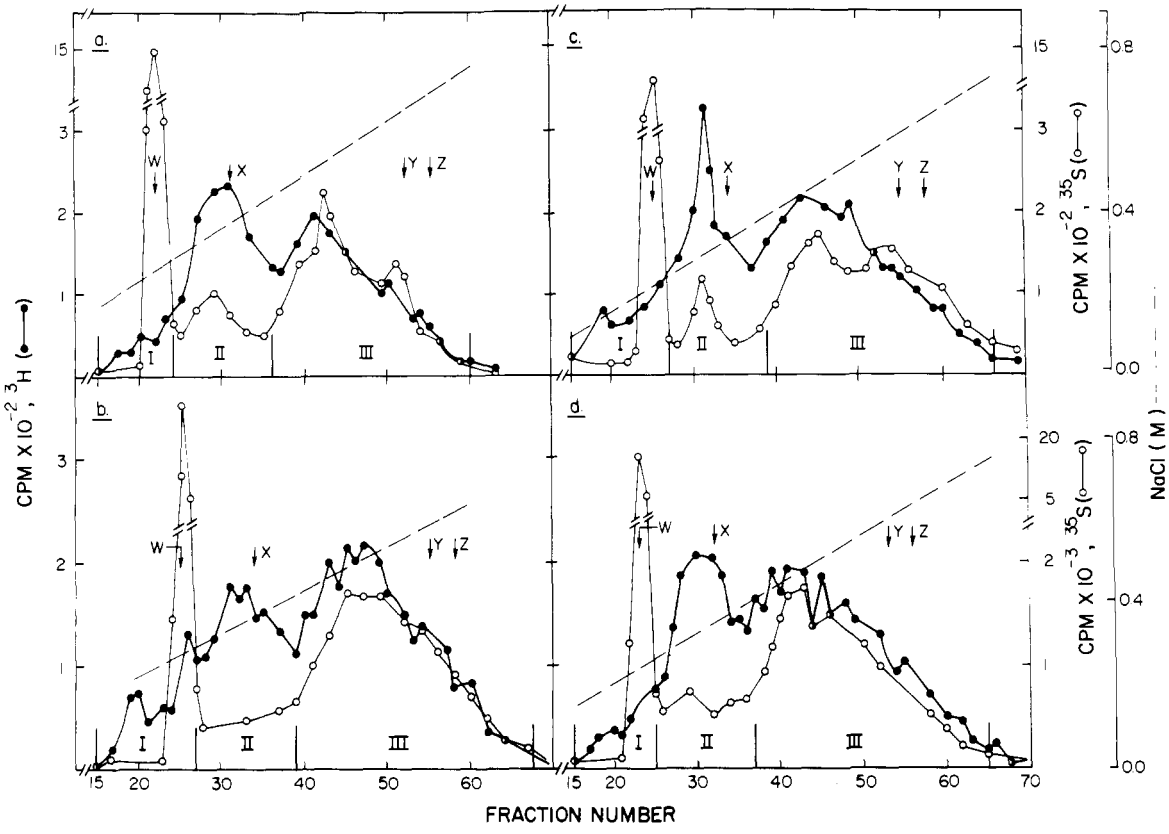

FIGURE 1: Cochromatography on DEAE-Sephadex A-25 of nitrous acid degradation products of [^{35}S]sulfate- or [^{3}H]glucosamine-labeled heparan sulfates isolated from Swiss 3T3 and SV3T3 cells as described under Materials and Methods. (a) ^{3}H- (●) and ^{35}S-labeled (○) material from 3T3 cells, (b) ^{3}H- (●) and ^{35}S-labeled (○) material from SV3T3 cells, (c) ^{3}H-labeled material (●) from SV3T3 cells and ^{35}S-labeled material (○) from 3T3 cells, and (d) ^{3}H-labeled material (●) from 3T3 cells and ^{35}S-labeled material (○) from SV3T3 cells. The dashed line indicates the slope of the salt gradient with only the area of interest drawn in (see Materials and Methods). The three short vertical lines designate fractions included in the regions labeled I, II, and III. The arrows indicate the peak elution positions of inorganic sulfate (W), hyaluronic acid (X), and undegraded heparan sulfate from SV3T3 cells (Y) and 3T3 cells (Z). The position of these reference markers on a given column was independently determined. Experimental details are described under Materials and Methods.

Table I: Distribution of ^{3}H- and ^{35}S-Labeled Nitrous Acid Degradation Products on DEAE-Sephadex A-25[a]

	% radioactivity[b]					
	region I[c]		region II[c]		region III[c]	
cell line	^{3}H	^{35}S	^{3}H	^{35}S	^{3}H	^{35}S
3T3	7	31	35	12	58	57
SV3T3	13	42	32	13	55	45

[a] The data in this table were derived from the data used to construct Figure 1c,d. Data derived from the cochromatography of deamination products from different heparan sulfates were considered the most appropriate for revealing similarities or differences because of the difficulty in accurately comparing, point for point, data derived from two different column analyses. This is due to the fact that the computed salt concentration at which a given peak elutes varies somewhat from day to day as a direct function of the ambient temperature of our laboratory (see Materials and Methods). [b] Percentages were computed as the fraction of total radioactivity recovered from the column. Recovery was greater than 95%. These comparisons have been repeated twice with comparable results. [c] Regions I, II, and III are defined in Figure 1c,d and refer to the fractions included in a particular portion of the total elution profile.

labeled inorganic sulfate complicated interpretation of the chromatographic profile in region I, we compared, by cochromatography, material labeled with [^{3}H]- or [^{14}C]-glucosamine. The elution patterns of 3T3 and SV3T3 materials (Figure 2a,b) were similar, although the SV3T3 material appeared to yield a somewhat larger proportion of fragments eluting in region I. This observation was confirmed by cochromatography of [^{3}H]glucosamine^{2}-labeled heparan sulfate fragments from 3T3 cells and [^{14}C]glucosamine^{2}-labeled heparan sulfate fragments from SV3T3 cells (Figure 2c) and was also shown not to be a radioisotope artifact by repeating the cochromatography with the labels reversed (Figure 2d). Although more SV3T3 heparan sulfate fragments eluted in region I and more 3T3 heparan sulfate fragments eluted in region III, both profiles were qualitatively similar and did not suggest the total absence of any group or groups of fragments.

Evaluation of N- and O-Sulfation

As discussed above, some glucosamine^{2}-containing fragments eluted with the inorganic sulfate released by the reaction with nitrous acid. To determine whether or not these fragments contained *O*-sulfate, we employed sequential gel filtration and ion-exchange chromatography to effect separation of the inorganic sulfate.

Gel Filtration on Bio-Gel P2. Figure 3a shows the distribution, on Bio-Gel P2, of the deamination products of [^{35}S]-sulfate- and [^{3}H]glucosamine-labeled heparan sulfates from SV3T3 cells. A similar profile is generated by equivalent material from 3T3 heparan sulfate (Figure 3b). In both cases, the inorganic sulfate (fractions 38–47) was separated from the bulk of the [^{3}H]glucosamine^{2}-labeled deamination products. The proportions of total ^{35}S label in this fraction were 43% and 47% for 3T3 and SV3T3 materials, respectively. However, 5% of the 3T3 glucosamine^{2}-containing material and 12% of the SV3T3 glucosamine^{2}-containing material still coeluted with the inorganic sulfate. Therefore, this pooled fraction (henceforth designated as a′ for SV3T3 and b′ for 3T3) was

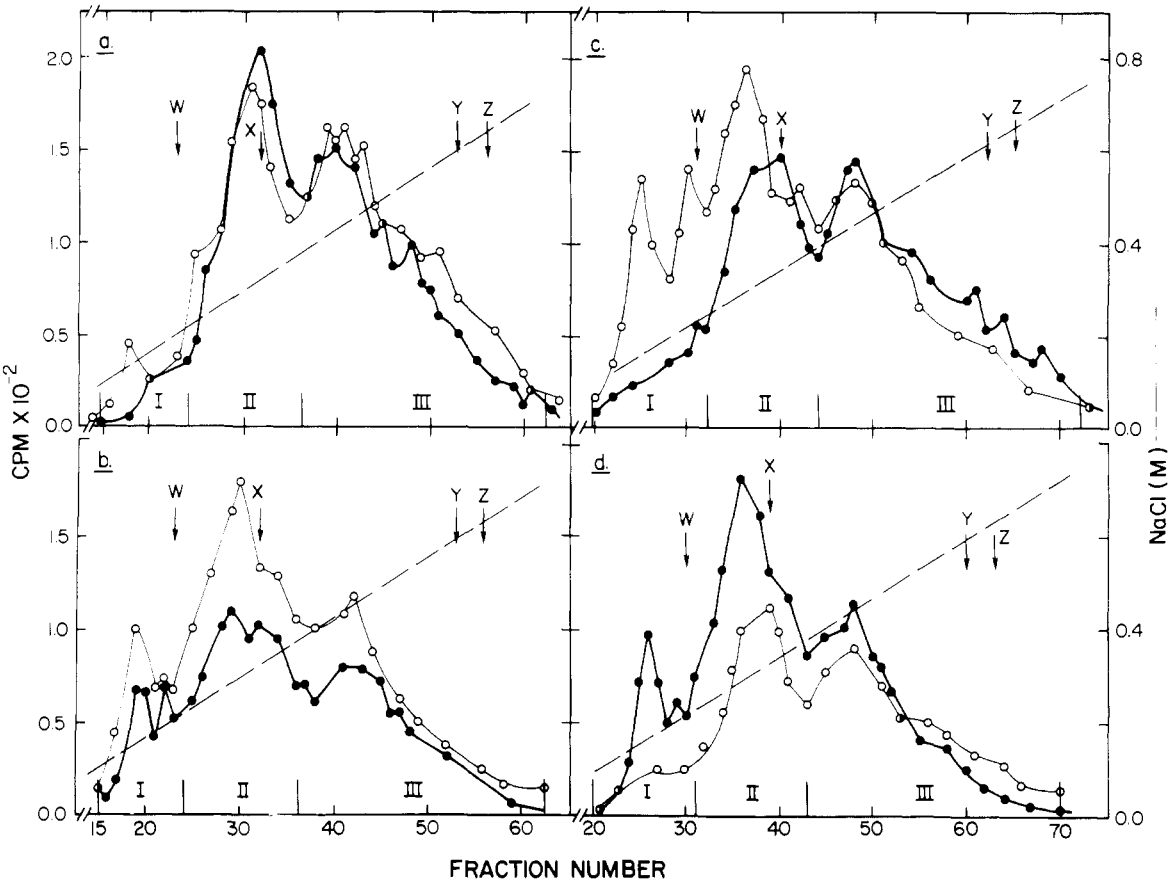

FIGURE 2: Cochromatography on DEAE-Sephadex A-25 of nitrous acid degradation products of [^{3}H]- or [^{14}C]glucosamine-labeled heparan sulfates from Swiss 3T3 or SV3T3 cells. (a) ^{3}H- (●) and ^{14}C-labeled (○) 3T3 heparan sulfates, (b) ^{3}H- (●) and ^{14}C-labeled (○) SV3T3 heparan sulfates, (c) ^{3}H-labeled (●) 3T3 and ^{14}C-labeled (○) SV3T3 heparan sulfates, and (d) ^{3}H-labeled (●) SV3T3 and ^{14}C-labeled (○) 3T3 heparan sulfates. The other symbols are described in the legend to Figure 1.

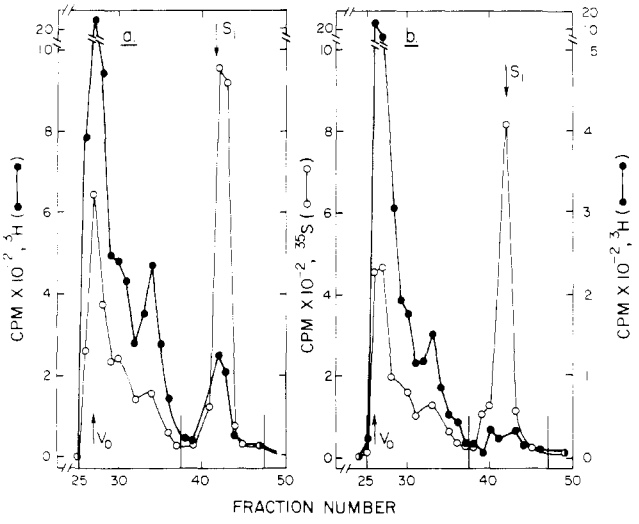

FIGURE 3: Cochromatography on Bio-Gel P2 of nitrous acid degradation products labeled with [^{3}H]glucosamine (●) and [^{35}S]sulfate (○) from (a) SV3T3 heparan sulfate and (b) 3T3 heparan sulfate. Samples were applied to a column (1 × 200 cm) which was eluted with 1 M NaCl in 2 mM Tris, pH 7.0. Fractions of 70 drops were collected, and 25 μL of each fraction was analyzed for radioactivity. S_i designates the elution position of the inorganic sulfate independently determined. Fractions were pooled as indicated by the vertical lines. Experimental details are described under Materials and Methods.

subjected to further analysis by ion-exchange chromatography.

Analysis of Fractions a' and b' from Bio-Gel P2 by Ion-Exchange Chromatography on DEAE-Sephadex A-25. Fractions a' and b' (see above) were independently pooled, diluted to less than 0.05 M NaCl with distilled water, and analyzed by ion-exchange chromatography on DEAE-Sephadex A-25. Both 3T3 and SV3T3 [^{3}H]glucosamine^2-labeled material resolved into two distinct peaks (Figure 4a,b). The first of these, in order of elution, appeared at 0.2 M NaCl and was designated peak A. This peak was distinct from that of the inorganic sulfate (S_i) which emerged from the column at 0.12 M NaCl. The second peak of [^{3}H]glucosamine^2-labeled material, designated peak B, eluted at 0.42–0.45 M NaCl. In both cases, peak B included both ^{3}H-labeled material and ^{35}S-labeled material. Furthermore, in both cases the distribution of ^{3}H-labeled material between peaks A and B was the same, 54% and 46%, respectively. However, of the total amount of ^{35}S label applied to the column, 17% of the 3T3 radioactivity eluted with peak B, but only 5% of the SV3T3 radioactivity eluted in that position. In order to directly compare peaks A and B from both cell types, heparan sulfates from [^{3}H]glucosamine-labeled SV3T3 cells and [^{14}C]glucosamine-labeled 3T3 cells were treated with nitrous acid, and the deamination products were subjected to gel filtration on Bio-Gel P2 (data not shown). The material emerging in fractions 38–47 was pooled, treated as described above, and subjected to ion-exchange chromatography on DEAE-Sephadex A-25. Again, two distinct, well-separated peaks emerged (Figure 4c), with the ^{3}H-labeled material coeluting with the ^{14}C-labeled material. This separation of low molecular weight material, containing both glucosamine^2 and sulfate, from the inorganic sulfate and from an apparently sulfate-free glucosamine^2-containing fragment permitted a reassessment of the proportion of *N*- and *O*-sulfates present in each of the heparan

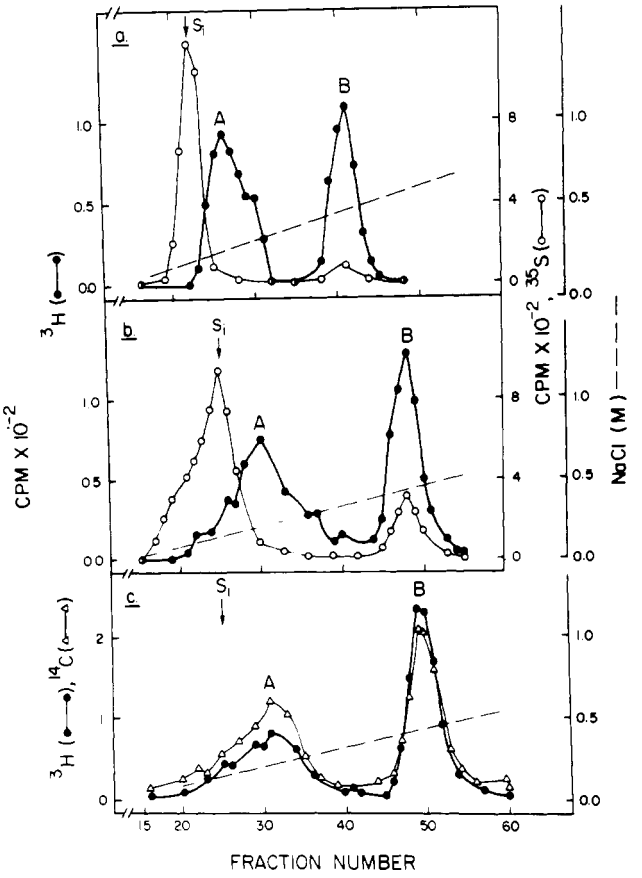

FIGURE 4: Ion-exchange chromatography on DEAE-Sephadex A-25 of the nitrous acid degradation products in fractions 38–47 from Bio-Gel P2 (as defined in Figure 3). ^{3}H- (●) and ^{35}S-labeled (○) material derived from (a) SV3T3 and (b) 3T3 heparan sulfates and (c) [^{3}H]glucosamine^2-labeled SV3T3 (●) and [^{14}C]glucosamine^2-labeled 3T3 (△) fragments. The dashed line describes the slope of the salt gradient with only the area of interest illustrated. Experimental details are described under Materials and Methods.

sulfates. By use of the data discussed for Figures 3 and 4, it was calculated[3] that the 3T3 heparan sulfate contained 36% *N*-sulfate and 64% *O*-sulfate. Of the total *O*-sulfate-containing fragments generated by nitrous acid treatment of the intact polymer, 11% were included in the Bio-Gel P2 column with the remaining 89% essentially excluded. For the SV3T3 heparan sulfate, the extent of N-sulfation and O-sulfation was computed[3] to be 45% and 55%, respectively, with only 4% of the nitrous acid generated O-sulfated fragments included in the Bio-Gel P2 column.

[3] Computation of the extent of N- and O-sulfation is based on data derived from Figures 3 and 4 for the distribution of radioactive sulfate on Bio-Gel P2 and DEAE-Sephadex A-25, respectively. When 3T3 heparan sulfate was subjected to nitrous acid, 43% of the total radioactive sulfate emerged in a peak whose position was identical with that of inorganic sulfate. However, examination of this peak on DEAE-Sephadex A-25 showed that only 83% is inorganic sulfate. The remaining 17% of the label is present as *O*-sulfate. Thus, 36% [100(0.43 × 0.83)] of the total ^{35}S label was released as inorganic sulfate and represents the total proportion of label originally present in the intact polymer as *N*-sulfate. By subtraction, 64% (100 − 36) of the total radioactive sulfate is present as *O*-sulfate. Another means to compute the extent of O-sulfation is the following: 57% of the total 3T3 ^{35}S label is present in the Bio-Gel P2 fraction b and represents *O*-sulfate. However, 7% [100(0.43 × 0.17)] of the label which eluted in the Bio-Gel fraction b′ also represents *O*-sulfate. Thus, the total extent of O-sulfation in the 3T3 heparan sulfate is 64% (57% + 7%). Of this 64%, 11% (7%/64%) eluted with the released inorganic sulfate (fraction b′). The same type of computation was made to determine the extent of *N*- and *O*-sulfates in the SV3T3 heparan sulfate.

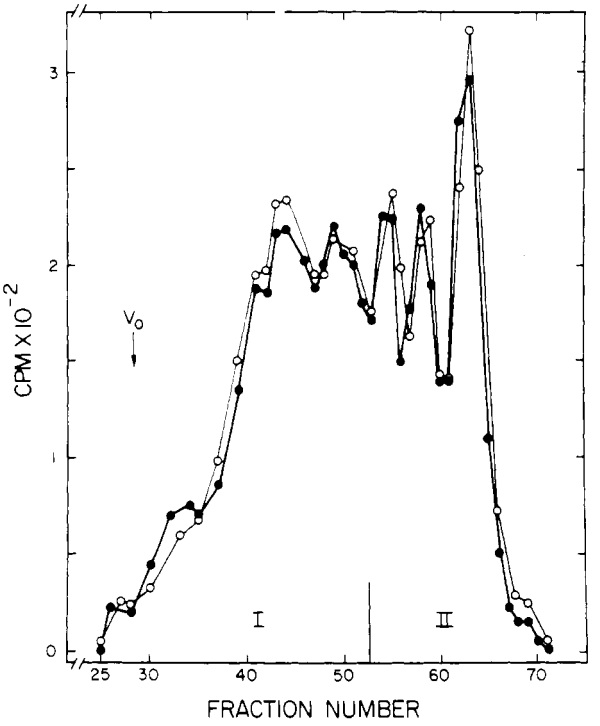

FIGURE 5: Chromatography on Bio-Gel P10 of the *O*-sulfate-containing fragments. For these experiments, [^{14}C]- and [^{3}H]glucosamine-labeled heparan sulfates from SV3T3 and 3T3 cells were prepared and degraded with nitrous acid (see Materials and Methods). This material was fractionated on Bio-Gel P2 as described in the legend of Figure 3. Fractions 25–37 (defined in Figure 3) were pooled, concentrated, and then analyzed on a column (1 × 200 cm) of Bio-Gel P10. (●) ^{3}H-Labeled material from 3T3 material; (○) ^{14}C-labeled material from SV3T3 material. The short vertical lines designate the fractions included in regions I and II.

Comparison of the O-Sulfated Fragments

Comparison of the small molecular weight *O*-sulfate-containing fragments included in Bio-Gel P2 has been discussed above. Analysis of the remaining *O*-sulfate-containing fragments utilized 3T3 and SV3T3 heparan sulfates labeled with [^{14}C]- or [^{3}H]glucosamine or [^{35}S]sulfate which had been degraded with nitrous acid, fractionated on Bio-Gel P2, and pooled as fractions 25–37 (Figure 3).

Analysis by Gel Filtration on Bio-Gel P10. The [^{14}C]-glucosamine^2-labeled SV3T3 fragments and [^{3}H]glucosamine^2-labeled 3T3 fragments described above were desalted, concentrated, and analyzed by gel filtration on Bio-Gel P10 in 1 M NaCl. No qualitative differences in the distribution of the O-sulfated deamination fragments were observed (Figure 5). Fractions 25–52, region I, included the higher molecular weight fragments probably of more complex nature since they were poorly resolved. Fractions 53–75, region II, included the lower molecular weight fragments, which were resolved into three distant fractions. For both 3T3 and SV3T3 material, 55% of the total label eluted in region I and 45% in region II, indicating no detectable quantitative differences.

The distribution of sulfate with respect to glucosamine^2 was examined by cochromatographing on Bio-Gel P10 the *O*-sulfate-containing material from each cell type which was labeled with [^{35}S]sulfate and [^{3}H]glucosamine^2 (Figure 6a,b). Over 65% of the total sulfate label eluted in the three peaks of region II. The ^{35}S/^{3}H distribution (Figure 6c) indicated that the small fragments had a greater relative sulfate content than the large fragments. However, the 3T3 ^{35}S/^{3}H ratios for the earliest eluting fractions (30–35) and the most retarded fractions (55–80) were higher than those for the comparable

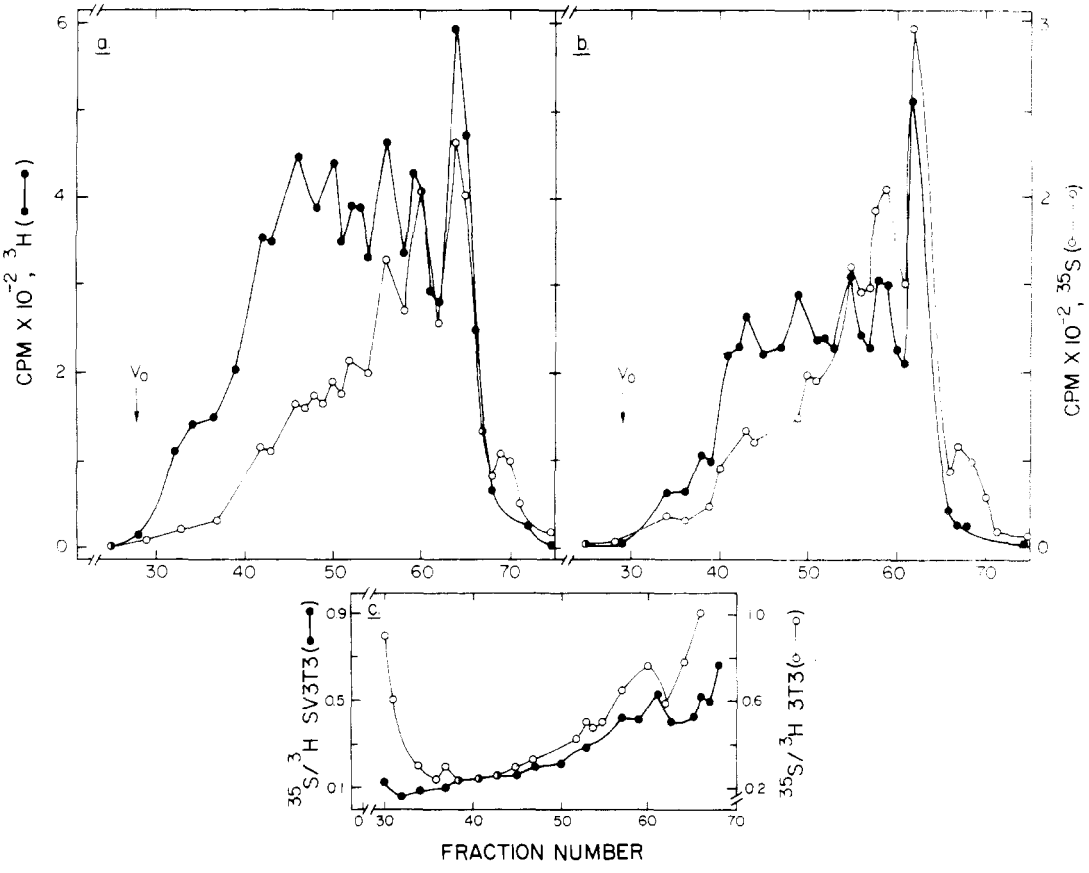

FIGURE 6: Chromatography on Bio-Gel P10 of [^{3}H]glucosamine^2-labeled (●) and [^{35}S]sulfate-labeled (○) *O*-sulfate-containing fragments from (a) SV3T3 material and (b) 3T3 material. The material used for this analysis is defined in Figure 3 as fractions 25–37 from the Bio-Gel P2 column. (c) The ^{35}S/^{3}H ratios for SV3T3 (●) and 3T3 (○) material were calculated from the data used to construct Figure 6, a and b, respectively.

SV3T3 fractions, indicating that the 3T3 material had a greater relative sulfate content in these two regions. These studies have been repeated with comparable results. Additionally, each of the resolved peaks (Figure 5) was, in turn, analyzed by ion-exchange chromatography on DEAE-Sephadex A-25 (data not shown). No qualitative differences in the resulting cochromatograms were observed. Furthermore, the material in each peak (Figure 5) was found to be heterogeneous with respect to charge and of variable complexity. However, with the exception of the material in the void volume, the 3T3 material was, relative to the SV3T3 material, slightly more retarded.

Analysis by DEAE-Sephadex A-25 Ion-Exchange Chromatography. The elution profiles displayed when the [^{35}S]-sulfate- and [^{3}H]glucosamine^2-labeled *O*-sulfate-containing fragments were subjected to ion-exchange chromatography on DEAE-Sephadex A-25 were similar (Figure 7a,b). The greater proportion of [^{35}S]sulfate label was associated with the glucosamine^2-containing material that eluted after fraction 40. The removal of inorganic sulfate (discussed above) clarified the distribution profile of the early eluting glucosamine^2-containing material and confirmed its presence in both 3T3 and SV3T3 fractions. In addition, it was evident that little, if any, ^{35}S label was associated with the early eluting material. Although general similarities in the distribution profile of the *O*-sulfate-containing fragments from both 3T3 and SV3T3 materials were observed, marked differences were revealed when [^{3}H]glucosamine^2-labeled fragments from 3T3 cells were cochromatographed with [^{14}C]glucosamine^2-labeled SV3T3 fragments (Figure 7c). The earliest eluting fragments (region I) represented 12% of the SV3T3 material but only 3% of the 3T3 material. Region II contained 40% of the SV3T3 material but only 28% of the 3T3 material. Region III contained 69% of the 3T3 fragments but only 48% of the SV3T3 fragments. Two repeated analyses yielded similar cochromatographic profiles and distribution figures within ±3% for regions I, II, and III. The fragments contained in each region, I–III (Figure 7c), were further analyzed by gel filtration on Bio-Gel P10 (data not shown). In general, individual profiles were complex, but no qualitative differences were observed.

Analysis of Uronic Acids

The basis for the difference in sulfation between the 3T3 and SV3T3 heparan sulfates could reside in the composition of the uronic acids (see Discussion). In order to examine this possibility, we prepared [^{3}H]galactose-labeled heparan sulfate from each of the cell types. The radioisotopically labeled heparan sulfates were then hydrolyzed and analyzed for uronic acid composition as described under Materials and Methods. The results of three separate analyses (using a new preparation of heparan sulfate each time) for each cell type showed no detectable differences in uronic acid composition. For both cell types, D-glucuronic acid comprised 55 ± 3% of the total uronic acid while L-iduronic acid comprised 45 ± 3% of the total.

Deamination Products of Intracellular Heparan Sulfate

In a previous report (Johnston et al., 1979), we showed that although the intracellular heparan sulfate isolated from SV3T3 cells was of smaller molecular weight than the cell-surface heparan sulfate, 6×10^3 and 2.4×10^5, respectively, the intracellular heparan sulfate still exhibited the transforma-

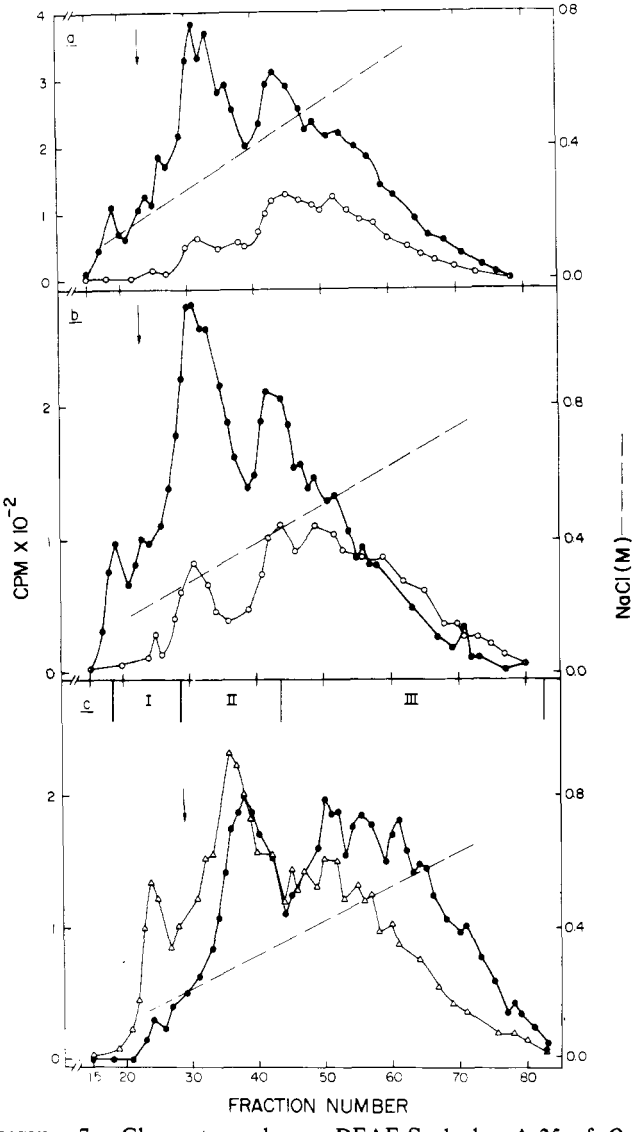

FIGURE 7: Chromatography on DEAE-Sephadex A-25 of *O*-sulfate-containing fragments labeled with [^{3}H]glucosamine^2 (●), [^{35}S]sulfate (○), or [^{14}C]glucosamine^2 (△): (a) SV3T3, (b) 3T3, and (c) SV3T3 (△) and 3T3 (●). The short vertical lines in (c) delineate regions I, II, and III. The arrow indicates the elution position of inorganic sulfate. The dashed line indicates the slope of the salt gradient with only the area of interest shown. For these studies, radioisotopically labeled heparan sulfate from both cell types was prepared and degraded with nitrous acid as described under Materials and Methods. The degradation products were fractionated on Bio-Gel P2 as described in the legend to Figure 3. Fractions 25–37 were pooled, desalted, and analyzed on DEAE-Sephadex A-25.

tion-associated change in chromatographic properties seen in the cell-surface polymer. It was of interest, then, to compare the deamination products of intracellular and cell-surface heparan sulfates. Because of the extremely small quantities of intracellular heparan sulfate that can be isolated from 3T3 cells, only the SV3T3 materials could be compared. As can be seen in Figure 8, the nitrous acid degradation products from [^{3}H]glucosamine-labeled cell-surface heparan sulfate coeluted with those from [^{14}C]glucosamine-labeled intracellular heparan sulfate.

Discussion

The susceptibility of the glycosidic bond adjacent to an N-sulfated[1] residue to cleavage by nitrous acid (Cifonelli, 1968; Shively & Conrad, 1976) has been used to compare the distribution of N- and O-sulfated residues in the heparan sulfates

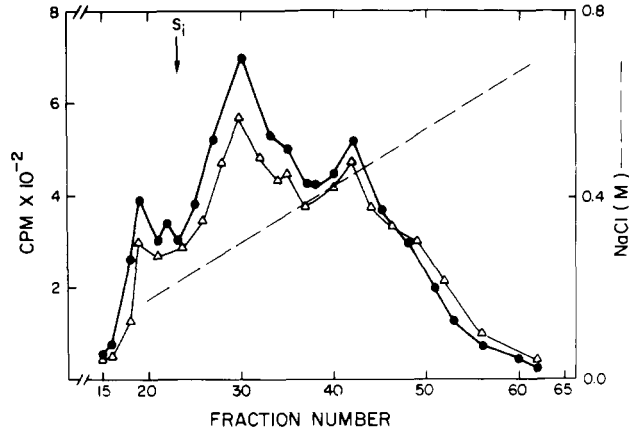

FIGURE 8: Cochromatography on DEAE-Sephadex A-25 of nitrous acid treated cell-surface [^{3}H]glucosamine-labeled heparan sulfate (●) and [^{14}C]glucosamine-labeled intracellular heparan sulfate (△) from Swiss SV3T3 cells. The dashed diagonal line indicates the slope of the NaCl gradient with only the position of interest shown. S_i indicates the peak elution of inorganic sulfate.

from 3T3 and SV3T3 cells which have previously been shown to differ in sulfation but not molecular weight (Johnston et al., 1979). Because the size of the nitrous acid generated fragment varies with the number of consecutive N-acetylated and N-sulfated disaccharide repeating units, the similarity or dissimilarity of the general arrangement of such units within different heparan sulfate molecules can be ascertained from a molecular weight comparison of the products obtained after treatment with nitrous acid (Cifonelli, 1968). In addition, because the extent of O-sulfation and the type of uronic acid present vary among the fragments (Höök et al., 1974b), comparison of fragments by ion-exchange chromatography should reveal similarities and dissimilarities resulting from the net charge present on the fragments.

The amount of heparan sulfate, which in practice can be obtained from these cells, is not adequate for extensive quantitative chemical analysis. We have therefore relied solely upon the use of radiochemistry to study both the intact polymers and their degradation products. Earlier attempts (Underhill, 1975; unpublished results) to analyze the sulfated degradation products by employing ^{35}S-labeled heparan sulfate were complicated because the inorganic sulfate generated during the course of the reaction with nitrous acid coeluted with some of the small molecular weight saccharide products on both gel filtration and ion-exchange chromatography. In the present study, the inorganic sulfate was totally separated from the other sulfate-containing degradation products by sequential use of gel filtration on Bio-Gel P2 and ion-exchange chromatography on DEAE-Sephadex A-25.

From electrophoretic data, we had previously estimated the extent of difference in sulfation between the 3T3 and SV3T3 heparan sulfates to be approximately 10% (Underhill, 1975). Our current data demonstrate that the relative levels of *N*- and *O*-sulfates are 37% and 63%, respectively, for 3T3 heparan sulfate and 45% and 55%, respectively, for SV3T3 heparan sulfate, with a reproducibility of ±3%. Clearly the 3T3 heparan sulfate has proportionately more, about 8%, *O*-sulfate than does the SV3T3 heparan sulfate. This difference in O-sulfation is further evidenced by the gel filtration and ion-exchange chromatographic behavior of the nitrous acid generated fragments.

First, no qualitative differences are observed in the Bio-Gel P10 cochromatographic profile of the 3T3 or SV3T3 fragments (Figures 5 and 6). For both fragments, the polydisperse distribution of the [^{3}H]glucosamine^2-containing fragments and

the association of a greater proportion of ^{35}S label with the smaller molecular weight fragments indicate that there is clustering of N- and O-sulfated residues within the molecules as reported for other heparin-like polymers (Cifonelli, 1968; Höök et al., 1974a). However, comparison of the $^{35}S/^{3}H$ ratio plots (Figure 6c) shows that the largest and smallest 3T3 fragments have a greater relative sulfate content than do the comparable SV3T3 fragments. This difference in $^{35}S/^{3}H$ ratios is further substantiated by the observation, using DEAE-Sephadex A-25 ion-exchange chromatography, that the 3T3 and SV3T3 fragments contained in each of the Bio-Gel P10 peaks (Figure 5) are not strictly identical with respect to charge since the labeled 3T3 fragments collectively elute later than the SV3T3 fragments (data not shown). The second line of evidence supporting a difference in O-sulfation between the 3T3 and SV3T3 heparan sulfates is the demonstration that the *O*-sulfate-containing fragments generated by nitrous acid degradation of both polymers distribute differently on DEAE-Sephadex A-25 (Figure 7). For both types of fragments, most of the ^{35}S label is associated with [^{3}H]glucosamine[2]-containing material that is retarded on the column, but the 3T3 material has proportionately more fragments eluting in this region.

Although the gel filtration patterns are qualitatively similar and it has been shown that an 8% difference in the extent of O-sulfation exists between the two polymers, a small difference in N-sulfation cannot be ruled out with the present data. When all fragments (^{14}C and ^{3}H labeled) are compared by cochromatography on Bio-Gel P10, the 3T3 material consistently has 2–4% more material which elutes in the smaller molecular weight regions. The analysis on Bio-Gel P2 (Figure 3) also shows a disproportionate distribution, particularly of the small molecular weight pieces. Clarification of these data will require other approaches. For example, Jacobsson et al. (1979b) have shown that nitrous acid degradation of heparan sulfate can yield six different disaccharides of varying sulfate and uronic acid content. Furthermore, heparan sulfates with small differences in total sulfation yielded not only different proportions of total disaccharides but also different proportions of individual disaccharides. Since the quantity of disaccharides produced upon nitrous acid degradation is a function of the arrangement of the N-sulfated residues, knowledge of which gel filtration and ion-exchange fractions are disaccharides and how these disaccharides are distributed for the 3T3 and SV3T3 heparan sulfates would be useful.

A correlation between the sulfate and L-iduronic acid content of heparin-like polymers has been reported; this indicates that the content of L-iduronic acid increases as the extent of sulfation increases (Höök et al., 1974a). Since the 3T3 and SV3T3 heparan sulfates differ in sulfation by approximately 10%, it was anticipated that the two polymers might also differ by a comparable amount in their uronic acid distribution. However, both polymers were shown to contain 55% D-glucuronic acid and 45% L-iduronic acid (±3%). Although a small difference (3% or less) cannot be ruled out, it appears that both polymers contain the same amount of L-iduronic acid. This is of interest in view of the recent proposal (Jacobsson et al., 1979a) that the late polymer modification reactions occur consecutively, that is, glucuronosyl C_5-epimerization and then L-iduronosyl 2-O-sulfation, followed by D-glucosaminyl 6-O-sulfation. Thus, the similarity of uronic acid distribution in the 3T3 and SV3T3 heparan sulfates supports the data showing that the primary difference is in the extent of O-sulfation.

Finally, the demonstration that the nitrous acid generated fragments from the SV3T3 cell-surface and intracellular heparan sulfates are qualitatively identical (Figure 8) is of interest because the latter, although only $^{1}/_{40}$ the molecular weight of the cell-surface material, still exhibits the transformation-associated chromatographic difference defined for the 3T3 and SV3T3 medium and cell-surface heparan sulfates (Johnson et al., 1979). However, unlike the cell-surface heparan sulfate, the intracellular heparan sulfate is not believed to contain a protein core (Johnston et al., 1979). The comparative data presented in this paper strengthen the possibility that the intracellular heparan sulfate represents some basic unit structure. However, whether the origin of this entity arises by biosynthetic or degradative processes and how it is arranged in the heparan sulfate proteoglycan are not known.

Acknowledgments

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