Preliminary Characterization of the Proteoglycans in the Substrate Adhesion Sites of Normal and Virus-Transformed Murine Cells†

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ABSTRACT: Substrate-attached material consists of the remnants of adhesion sites left firmly adherent to an artificial tissue culture substrate after detachment of normal or SV40-transformed Balb/c 3T3 cells using the Ca^{2+} chelator EGTA. This material is enriched in glycosaminoglycans (GAG's) for which functions in mediating cell–substrate adhesion have been proposed. The GAG's in substrate-attached material have been investigated for their structural connection to protein and their ability to interact with each other. Intact proteoglycan species from this material were resolved by molecular sieve chromatography in $NaDodSO_4$ buffer. Sizing of specific GAG's before and after Pronase treatment revealed that nearly all of the chondroitin and dermatan sulfate species and over 50% of the heparan sulfate species were present as proteoglycans. These species are present in different relative amounts in newly formed footpad adhesion sites as compared to substrate-attached material which contains footpads plus footprints (the latter material being remnants of the footpads left at the trailing end of cells during normal cellular movement). Little, if any, protein was detected in association with hyaluronic acid. Isopycnic density gradient centrifugation of substrate-attached material under associative conditions generated two bands of GAG-containing material at high densities and one band at low density. In the presence of 4.0 M guanidine hydrochloride, nearly all of the material in the higher density bands reversibly dissociated. This material consisted predominantly of heparan sulfate, along with some chondroitinase-digestible GAG and glycopeptide. Competition with hyaluronate oligomers also dissociated part of these denser bands. This suggests the presence in substrate-attached material of heparan sulfate containing aggregates along with the possible presence of cartilage-like proteoglycan–hyaluronate aggregates. These results are discussed in terms of an adhesion model in which proteoglycan interactions with cell surface fibronectin can affect and modify adhesion.

When murine fibroblasts are detached from glass or plastic substrates by using chelating agents (Culp & Black, 1972) or light doses of trypsin (Whur et al., 1977), a significant amount of cellular material is left adherent to the substrate (Culp, 1978). Morphological (Rosen & Culp, 1977) and metabolic (Culp, 1975) evidence indicates that this substrate-attached material represents, in part, the remnants of the focal adhesion sites, or footpads, by which the cell adheres to the substrate (Revel et al., 1974). Chelation of Ca^{2+}, using EGTA,[1] apparently leads to cytoskeletal weakening, allowing the cell body to be sheared away from its footpads which remain tightly attached to the substrate (Rosen & Culp, 1977). Substrate-attached material can then be removed from the substrate by using $NaDodSO_4$ and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Culp, 1976a,b). Such studies have revealed a considerable enrichment in substrate-attached material for cytoskeletal components, as well as for cell surface fibronectin, a glycoprotein commonly implicated in adhesion systems (Keski-Oja et al., 1976; Yamada & Olden, 1978) [also referred to as the LETS glycoprotein (Hynes, 1976)]. This fact plus the resistance of most of this material to extraction by nonionic detergents (Cathcart & Culp, 1979) and its resistance to graded doses of proteases and glycosidases (Culp et al., 1978) has led to the idea that the adhesive site is a differentiated element of the cell surface whose multiple components are distributed in a topographically functional matrix (Culp, 1978).

Initial studies of the carbohydrates in substrate-attached material also revealed enrichment for GAG's (Terry & Culp, 1974; Roblin et al., 1975). Recently, it has been shown that hyaluronic acid, all of the chondroitins, dermatan sulfate, and heparan sulfate are present in substrate-attached material in a distribution considerably different from that on the rest of the cell surface (Rollins & Culp, 1979). Most interestingly, heparan sulfate comprises over 80% of the GAG in newly formed adhesion sites. Since these cells adhere to a layer of serum proteins adsorbed to the substrate (Yaoi & Kanaseki, 1972; Revel & Wolken, 1973; Grinnell, 1974; Culp & Buniel, 1976; Stomatoglou, 1977) and since heparin binds to cold-insoluble globulin (Stathakis & Mosesson, 1977) [the putative adhesion factor in serum (Grinnell & Hays, 1978; B. Murray, R. Haas, and L. Culp, unpublished experiments)], it was proposed that the initial adhesive event involved cell surface heparan sulfate interacting with cell surface fibronectin and substrate-bound CIg (Culp et al., 1978; Rollins & Culp, 1979). Subsequent accumulation of hyaluronate and specific chondroitins in the adhesion site was then postulated to effect "physiological" cell detachment in some fashion, generating footprint material at the trailing end of motile cells.

Documentation of these interactions requires a knowledge of the molecular structure of the GAG-containing species found in substrate-attached material. This communication provides evidence for the proteoglycan nature of several of these carbohydrate species and presents preliminary indications

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[1] Abbreviations used: CIg, cold-insoluble globulin, the properties of which are very similar to fibronectin (Yamada & Olden, 1978); ΔDi-OHA, Δ^4-unsaturated disaccharide liberated by chondroitinase digestion of hyaluronic acid; EDTA, ethylenediaminetetraacetic acid: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GAG, glycosaminoglycan; LETS, large external transformation sensitive glycoprotein (Hynes, 1976); MEM × 4, Eagle's minimal essential medium supplemented with a fourfold concentration of vitamins and essential amino acids; $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline without divalent cations; PMSF, phenylmethanesulfonyl fluoride; SV40, simian virus 40.

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of their ability to reversibly interact with each other and perhaps with other elements in substrate-attached material.

Materials and Methods

Cell Growth. Mouse Balb/c 3T3 cells (clone A31) and SV40-transformed 3T3 cells (clone SVT2) were used between their 12th and 25th passages (~48–100 generations). Cells were grown in MEM × 4 supplemented with 10% donor calf serum, penicillin (250 units/mL), and streptomycin (0.25 mg/mL) and were incubated in humidified 5% CO_2–95% air at 37 °C. For experimental purposes, cells were routinely grown in 100-mm diameter plastic tissue culture dishes and were free of *Mycoplasma* according to the radiolabeling assay of Culp & Black (1972).

Radiolabeling Procedures. For preparation of *long-term radiolabeled* material, which contains both footpad and footprint substrate-attached material (Culp, 1978), cells (0.75×10^6 Balb/c 3T3 cells or 2.0×10^6 SVT2 cells) were inoculated into each of 64 or 128 100-mm plastic tissue culture dishes containing 10 mL of medium. After overnight incubation, the medium was removed and replaced with 10 mL of medium containing one of the following: 5.0 μCi/mL D-[6-^{3}H]glucosamine (sp act. 29.0 Ci/mmol), 0.5 μCi/mL D-[1-^{14}C]glucosamine (sp act. 55 mCi/mmol), 50 μCi/mL $Na_2{}^{35}SO_4$ (sp act. 800 mCi/mmol), or 0.5 μCi/mL L-[U-^{14}C]leucine (sp act. 324 mCi/mmol). Cells were radiolabeled by incubation in this medium for 72 h, after which 70–80% of the dish surface was covered by cells.

For examination of substrate-attached material from *reattaching* cells, material which contains only footpads and not footprints, long-term radiolabeled cells were EGTA-detached, pelleted by centrifugation, washed once in cold PBS, and resuspended in fresh medium. Cells (3.0×10^6 3T3 or 11.0×10^6 SVT2 cells) were inoculated into each of 32 100-mm dishes containing 5 mL of medium and allowed to attach for 1 h before fractionation. $Na_2{}^{35}SO_4$ (50 μCi/mL) was added when the reattaching cells had been preradiolabeled with $^{35}SO_4$. A minimal amount of chasing of glucosamine-radiolabeled material occurs under these conditions due to the extremely large endogenous pool of radiolabeled glucosamine and GAG's present in these cells which were preradiolabeled for 72 h (Kornfeld & Ginsburg, 1966). This is confirmed by the fact that the amount of radiolabeled cell-associated polysaccharide per reattaching cell is the same as the amount in the long-term radiolabeled cells from which the reattaching cells were taken (~250 000 cpm/10^6 cells). None of the radiolabeling procedures used in these studies were deleterious to cell growth.

Isolation of Substrate-Attached Material. After radiolabeling, the medium was decanted and the cell layer gently washed 3 times with PBS. Cells were detached from the substrate by incubation in 0.5 mM EGTA in PBS containing 1 mM PMSF on a gyratory shaker at 37 °C for 30 min. The cell suspension was gently pipetted over the dish surface to ensure detachment of all cells, and the cell suspension was then removed from the dish. Dishes were washed once with PBS and then 2 times with distilled water at 37 °C, and the *substrate-attached material* was quantitatively removed by incubating dishes in 0.2% $NaDodSO_4$ (w/v in H_2O) with 1 mM PMSF with shaking at 37 °C for 30 min. This material was concentrated by vacuum dialysis at room temperature.

Polysaccharide Preparation. Radiolabeled polysaccharides were isolated from substrate-attached material by techniques described previously (Rollins & Culp, 1979; Cohn et al., 1976). Briefly, after addition of carrier GAG, substrate-attached material was extensively digested with Pronase and then dialyzed against 0.2 mM glucosamine hydrochloride (with 0.2 mM Na_2SO_4 when using $^{35}SO_4$-radiolabeled samples) for 24 h and distilled water for an additional 24 h to reduce the concentration of $NaDodSO_4$. Polysaccharides were purified by three rounds of precipitation using cold 95% ethanol–1% potassium acetate.

Determination of GAG and Glycopeptide. Techniques for identifying the various GAG's and glycopeptide have been described (Rollins & Culp, 1979; Saito et al., 1968). Purified radiolabeled polysaccharides were digested with chondroitinases ABC or AC, and an aliquot of the digests was subjected to paper chromatography in order to separate the unsaturated disaccharide digestion products. These products were identified by their UV absorbance, and the amount of radioactivity associated with each digestion product was determined by scintillation counting of material eluted from the paper.

A second aliquot of the chondroitinase ABC digest was chromatographed on Sepharose CL-6B before and after nitrous acid degradation (Cifonelli, 1968; Lindahl et al., 1973; Rollins & Culp, 1979). This technique separates heparan sulfate, glycopeptide, and the chondroitinase digestion products (Rollins & Culp, 1979). Recovery of the radioactivity from paper and column chromatography was routinely 80–100%.

Column Chromatography. $NaDodSO_4$-extracted substrate-attached material was boiled for 2 min, cooled to room temperature, and then chromatographed on 1 × 120 cm columns of Sepharose CL-2B, CL-4B, and CL-6B or a 1 × 75 cm column of Sephadex G-100. When Pronase-digested material was examined, the digest was boiled for 2 min, cooled to room temperature, and chromatographed directly, without dialysis, so that control and Pronase-digested profiles could be compared directly. In all cases, the columns were eluted with 150 mM Tris-HCl (pH 7.4) with 0.2% $NaDodSO_4$ at flow rates of ~0.06–0.08 mL/min. An aliquot of each of the eluted fractions was used for determination of radioactive material by scintillation counting. Recoveries were 85–100%. Individual peaks of radioactive material were then analyzed for their carbohydrate composition after concentration by vacuum dialysis. (Thus, dialyzable radioactive material was lost from Pronase-digested samples during this step.)

Reduction and Alkylation. $NaDodSO_4$-extracted substrate-attached material was reduced and alkylated by the technique described by Hascall & Sajdera (1969). The extract was made 10 mM in dithiothreitol and was then deoxygenated by repeated flushing with N_2. The mixture was kept at 37 °C for 2 h and then alkylated by adding *N*-ethylmaleimide to 40 mM and gently agitating at room temperature for 1 h. It was then dialyzed against 150 mM Tris-HCl (pH 7.4) with 0.2% $NaDodSO_4$.

Guanidine Hydrochloride Extraction of Substrate-Attached Material. Cells were radiolabeled and detached by using EGTA as described. Substrate-attached material was washed once with PBS and then twice with distilled water. The substrate-attached material in each 100-mm dish was extracted with 5 mL of 4.0 M guanidine hydrochloride in 0.05 M sodium acetate buffer (pH 5.8) at 4 °C on a rocker platform for 48 h. The extract was pipetted against the surface of the dish and then removed and vacuum dialyzed at 4 °C to a small volume (generally 5–10 mL). This material was placed into fresh dialysis tubing and then dialyzed against 0.05 M sodium acetate (pH 5.8) at 4 °C overnight. The retentate was then either (1) dialyzed against 150 mM Tris-HCl (pH 8.2)–1.5 mM $CaCl_2$ for Pronase digestion and polysaccharide analysis as described or (2) prepared for equilibrium density gradient sedimentation analysis as described below.

The extracted dishes were washed thoroughly with distilled water and then extracted with 0.2% $NaDodSO_4$ at 37 °C for 30 min with shaking. This extract was prepared as usual for polysaccharide analysis.

Isopycnic Centrifugation. The 4.0 M guanidine hydrochloride extract of substrate-attached material was brought to associative conditions [0.4 M in guanidine hydrochloride (Hascall & Sajdera, 1969)] by dialysis against 9 volumes of 0.05 M sodium acetate buffer (pH 5.8). Cesium chloride was added to the associative extracts to an initial density of 1.63 g/mL. These mixtures were centrifuged in either cellulose nitrate or polyallomer tubes in a Beckman 50 Ti angle rotor at 34000 rpm at 18 °C for 48 h. After centrifugation, ~1-mL samples were collected by piercing the bottoms of the centrifuge tubes. Densities were determined either by weighing 100-μL samples of each fraction or by comparing the refractive index of each fraction to a standard curve of various concentrations of cesium chloride in 0.4 M guanidine hydrochloride and 0.05 M sodium acetate (pH 5.8). All fractions were dialyzed against 0.05 M sodium acetate (pH 5.8) for subsequent analysis.

The lower two-thirds of the associative gradients (see Results) were brought to dissociative conditions (4.0 M in guanidine hydrochloride) by adding an equal volume of 8.0 M guanidine hydrochloride in 0.05 M sodium acetate (pH 5.8). Cesium chloride was added to an initial density of 1.54 g/mL, and centrifugation was performed as described for the associative gradients. Densities were determined on fractions from dissociative gradients by weighing 100-μL samples.

Materials. Materials were purchased from the following sources: D-[1-^{14}C]glucosamine hydrochloride and L-[U-^{14}C]leucine from Amersham/Searle Corp.; D-[6-^{3}H]glucosamine hydrochloride, $Na_2{}^{35}SO_4$, and NEF-963 aqueous counting cocktail from New England Nuclear Corp.; Pronase (grade B) from Calbiochem Corp.; hyaluronic acid (grade III), chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, *N*-ethylmaleimide, and cesium chloride from Sigma Chemical Co.; dithiothreitol from Aldrich Chemical Co.; guanidine hydrochloride (ultrapure) from Schwarz/Mann; sodium heparin (injectable) from Upjohn Co.; Sephadex G-100 and Sepharose CL-2B, CL-4B, and CL-6B from Pharmacia Fine Chemicals, Inc.; EDTA and EGTA from Eastman Organic Chemicals; sodium dodecyl sulfate from Bio-Rad Laboratories; chondroitinase ABC, chondroitinase AC, ΔDi-6S, ΔDi-4S, and ΔDi-OS from Miles Laboratories, Inc.; MEM × 4 from Grand Island Biologicals Co.; donor calf serum from K. C. Biological, Inc.; plastic tissue culture dishes from Lux Scientific Co. ΔDi-OHA was prepared by the methods of Cohn et al. (1976). Acid mucopolysaccharide reference standards were generously provided by Dr. M. B. Mathews, University of Chicago. HA-80 was the generous gift of Dr. Vincent C. Hascall, National Institutes of Health.

Results

Gel Filtration of Proteoglycans from Long-Term Radiolabeled Substrate-Attached Material. The carbohydrate-containing species of substrate-attached material were initially separated by molecular sieve chromatography. Figure 1 shows the profile obtained when substrate-attached material from long-term [^{3}H]glucosamine–[^{35}S]O_4 doubly radiolabeled 3T3 cells is chromatographed on Sepharose CL-2B in a $NaDodSO_4$-containing buffer. Three areas of tritium and two of ^{35}S radioactivity can be discerned. Area I contains 2%, area II 30%, and area III 68% of the tritium radioactivity. Area I had no ^{35}S radioactivity, while area II had 64% and area III 36%. Separation of these last two areas was not improved by

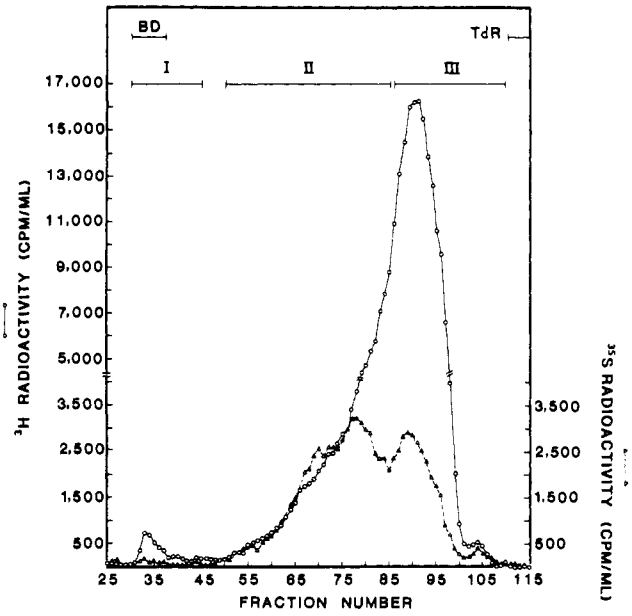

FIGURE 1: Column chromatography of substrate-attached material from long-term [^{3}H]glucosamine–[^{35}S]O_4 radiolabeled 3T3 cells. 3T3 cells were grown for 72 h in the presence of [^{3}H]glucosamine and $Na_2{}^{35}SO_4$ and then detached with EGTA containing PMSF. The substrate-attached material was extracted with $NaDodSO_4$ (also containing PMSF), and, after vacuum dialysis, it was chromatographed on a column of Sepharose CL-2B [1 × 120 cm, eluted with 120 mM Tris-HCl (pH 7.4) and 0.2% $NaDodSO_4$]. ^{3}H (○) and ^{35}S (△) radioactivities were determined for each fraction by using scintillation counter settings allowing less than 15% cross-over. Fractions were combined as indicated by the bars (BD, blue dextran; TdR, [^{14}C]-thymidine).

chromatography on Sepharose CL-4B, Sepharose CL-6B, or Sephadex G-100. This profile was not altered by omitting PMSF during cell detachment or extraction of substrate-attached material, nor was it altered by reduction and alkylation of substrate-attached material. Nearly identical profiles were obtained during analysis of substrate-attached material from SVT2 cells.

Proteoglycans in Long-Term Radiolabeled Substrate-Attached Material. The areas of radioactivity shown in Figure 1 were analyzed for their carbohydrates as described under Materials and Methods. The fractions designated as area I in Figure 1 were combined, and an aliquot was rechromatographed on Sepharose CL-2B (Figure 2A, solid line). This material was separated into two areas, one completely excluded (2BI) and one partially included (2BII). Both areas consisted entirely of hyaluronic acid (Table I). A second aliquot of the starting material was digested extensively with Pronase and chromatographed on the same column (Figure 2A, dotted line). The resulting profile was essentially unchanged from the untreated material (which had been incubated without enzyme as a control). Again, areas 2BIP and 2BIIP contained only hyaluronic acid (Table II). This apparent lack of structurally important, Pronase-digestible protein associated with hyaluronic acid is consistent with the absence of any leucine-radiolabeled material coelectrophoresing with a hyaluronic acid band on $NaDodSO_4$–polyacrylamide gel electrophoresis (data not shown).

The fractions corresponding to area II in Figure 1 were then combined and rechromatographed on Sepharose CL-4B (Figure 2B, solid line). Carbohydrate analysis of this material (designated 4BI) is shown in Table I. An aliquot of the area II material was digested with Pronase and chromatographed on the same Sepharose CL-4B column as the sham-digested material. This profile (Figure 2B, dotted line) differs sub-

Table I: Distribution of Long-Term Radiolabeled Polysaccharides in Sepharose Fractions of Substrate-Attached Material from 3T3 Cells[a]

poly-saccharide[b]	% radioact. in Sepharose fractions[c]				
	2BI[d]	2BII	4BI	6BI	6BII
HS			43.8	2.7	1.7
6S			2.3	0.0	
4S			16.8	0.5	
DS			4.8	0.3	
OS			17.3	0.5	
HA	100.0	100.0	13.3	0.1	
gp			1.7	95.9	98.3

[a] 3T3 cells were grown in the presence of [^{3}H]glucosamine for 72 h, after which substrate-attached material was collected and chromatographed on Sepharose CL-2B. The three fractions (Figure 1) were rechromatographed on Sepharose CL-2B, CL-4B, and CL-6B, and the various fractions (Figure 2A–C, solid lines) were analyzed for carbohydrate content as described under Materials and Methods. [b] HS, heparan sulfate; 6S, chondroitin 6-sulfate; 4S, chondroitin 4-sulfate; DS, dermatan sulfate; OS, unsulfated chondroitin; HA, hyaluronic acid; gp, glycopeptide. [c] Percentage of radioactivity associated with a particular polysaccharide within a given fraction. [d] Fractions are described in Figure 2.

stantially from the control. Area 4BIP contained heparan sulfate along with some hyaluronic acid and glycopeptide (Table II). Area 4BIIP, which eluted at a position where no material from the undigested preparation chromatographs, contained all the chondroitin species along with some heparan sulfate and no hyaluronic acid. This suggests that all of the chondroitins and at least 53% of the heparan sulfate in 4BI are structurally connected to protein and can thus be considered as proteoglycans. Whether the heparan sulfate remaining in 4BIP may have undergone a slight shift in position due to Pronase digestion cannot be determined, and this analysis may have underestimated the proportion of protein-linked heparan sulfate.

Finally, the fractions corresponding to area III in Figure 1 were combined and rechromatographed on Sepharose CL-6B (Figure 2C, solid line). This gave rise to a main peak (6BI) with a shoulder of lower molecular weight (6BII). Carbohydrate analysis of 6BI is shown in Table I. 6BII is essentially all glycopeptide with a small amount of heparan sulfate.

When the area III material from Figure 1 was digested with Pronase and chromatographed on the same column as the control, four areas of radioactivity could be separated (Figure 2C, dotted line). Their carbohydrate analyses are shown in Table II. Again, the presence of chondroitin 4-sulfate, unsulfated chondroitin, and dermatan sulfate in 6BIIIP (which coeluted with 6BII, an area containing none of these species in the undigested sample) indicated their proteoglycan nature.

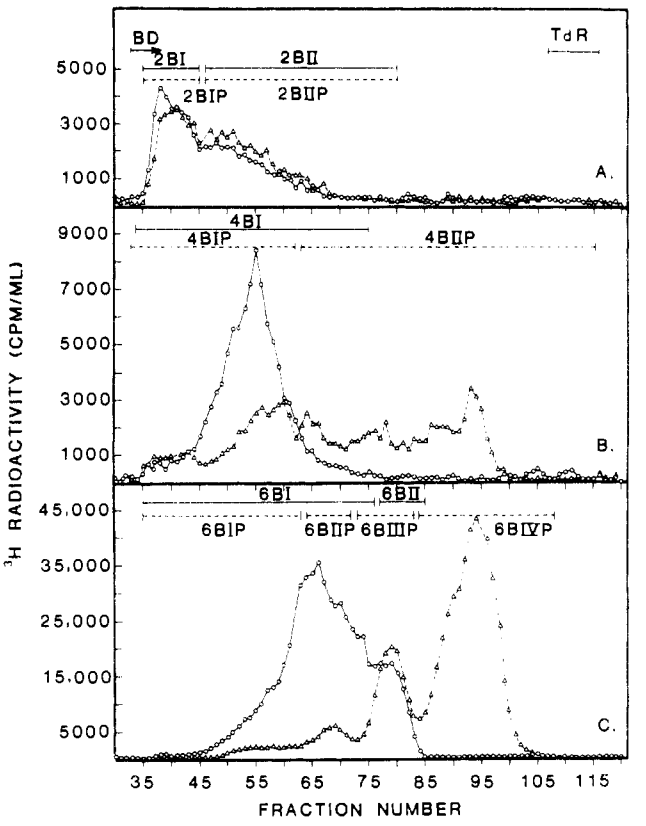

FIGURE 2: Column chromatography of substrate-attached material from long-term [^{3}H]glucosamine-radiolabeled 3T3 cells before and after Pronase digestion. Fractions in areas marked I, II, and III in Figure 1 (substrate-attached material from long-term [^{3}H]glucosamine-radiolabeled 3T3 cells) were combined and divided into two aliquots. One aliquot of each area was digested with Pronase as described under Materials and Methods, while the other aliquot was incubated under the same conditions in the absence of Pronase. The sham-digested (○) and Pronase-digested (△) materials were then chromatographed on Sepharose columns [all 1 × 120 cm, eluted with 150 mM Tris-HCl (pH 7.4) and 0.2% NaDodSO$_4$]. (A) Area I material from Figure 1 chromatographed on Sepharose CL-2B. (B) Area II material from Figure 1 chromatographed on Sepharose CL-4B. (C) Area III material from Figure 1 chromatographed on Sepharose CL-6B. Fractions were combined as indicated by the bars. The position of blue dextran (BD) and [^{14}C]thymidine (TdR) elution was very similar for all three columns.

Proteoglycans in the Substrate-Attached Material of Reattaching Cells. Sepharose CL-2B chromatography of doubly radiolabeled substrate-attached material from reattaching 3T3 cells was also performed, and the results are shown in Figure 3. Again, results with substrate-attached material from SVT2 cells were essentially identical. There is little

Table II: Distribution of Long-Term Radiolabeled Polysaccharides in Sepharose Fractions of Pronase-Digested Substrate-Attached Material from 3T3 Cells[a]

polysaccharide[b]	% radioact. in Sepharose fractions[c]							
	2BIP[d]	2BIIP	4BIP	4BIIP	6BIP	6BIIP	6BIIIP	6BIVP
HS			54.0	47.5	72.9	67.3		
6S				3.7	1.6	1.4		
4S				26.9	4.7	11.1	11.6	
DS				3.2	8.8	8.9	5.5	
OS				18.7	4.4	10.3	13.5	
HA	100.0	100.0	38.5		7.6	1.0		
gp			7.5				69.4	100.0

[a] Long-term [^{3}H]glucosamine-radiolabeled substrate-attached material from 3T3 cells was collected and chromatographed on Sepharose CL-2B. The three fractions (Figure 1) were digested with Pronase, the digests were chromatographed on Sepharose CL-2B, CL-4B, and CL-6B, and the resulting fractions (Figure 2A–C, dotted lines) were analyzed for carbohydrate content as described under Materials and Methods. [b] HS, heparan sulfate; 6S, chondroitin 6-sulfate; 4S, chondroitin 4-sulfate; DS, dermatan sulfate; OS, unsulfated chondroitin; HA, hyaluronic acid; gp, glycopeptide. [c] As in footnote *c* in Table I. [d] Fractions are described in Figure 2.

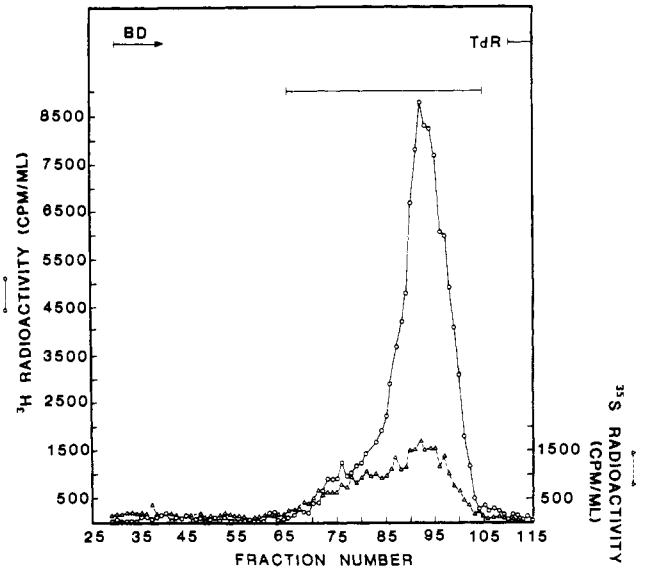

FIGURE 3: Column chromatography of substrate-attached material from reattaching [^{3}H]glucosamine–[^{35}S]O_4 radiolabeled 3T3 cells. 3T3 cells were grown for 72 h in the presence of [^{3}H]glucosamine and $Na_2{}^{35}SO_4$. They were detached with EGTA, washed, and allowed to readhere for 1 h to new dishes in the presence of $Na_2{}^{35}SO_4$, after which time the cells were removed and the substrate-attached material was extracted as described in the legend to Figure 1. Substrate-attached material was chromatographed on a column of Sepharose CL-2B [1 × 120 cm, eluted with 150 mM Tris-HCl (pH 7.4) and 0.2% NaDodSO$_4$]. ^{3}H (○) and ^{35}S (△) radioactivities were determined as described in the legend to Figure 1. Fractions were combined as indicated by the bar (BD, blue dextran; TdR, [^{14}C]thymidine).

Table III: Distribution of Polysaccharides in Sepharose Fractions of Substrate-Attached Material from Reattaching 3T3 Cells[a]

polysaccharide[b]	% radioact. in Sepharose fractions[c] 6BI[d]	6BII
HS	17.4	3.9
6S	0.1	
4S	0.5	0.1
DS	0.4	0.7
OS	0.5	
HA	1.7	
gp	79.4	95.3

[a] Long-term[^{3}H]glucosamine-radiolabeled 3T3 cells were detached with EGTA and allowed to attach to fresh dishes for 1 h, after which substrate-attached material was collected and chromatographed on Sepharose CL-6B (Figure 4, solid line). The resulting fractions were analyzed for carbohydrate content as described under Materials and Methods. [b] HS, heparan sulfate; 6S, chondroitin 6-sulfate; 4S, chondroitin 4-sulfate; DS, dermatan sulfate; OS, unsulfated chondroitin; HA, hyaluronic acid; GP, glycopeptide. [c] As in footnote *c* in Table I. [d] Fractions are described in Figure 4.

radioactivity in the area corresponding to area I in Figure 1, and unlike the long-term radiolabeled preparation, there is no easily discernible separation in the material eluting in fractions 60–105. The single tritium peak does, however, elute at the same position as the material in area III of Figure 1 and the bulk of ^{35}S-radiolabeled material is shifted to a smaller apparent size as compared to long-term radiolabeled substrate-attached material.

An analysis similar to that described for long-term radiolabeled substrate-attached material was carried out for the substrate-attached material from reattaching 3T3 cells. The carbohydrate composition of the two areas indicated in Figure 4 (solid line) was determined and is shown in Table III. Although only two size classes were assessed, hyaluronic acid,

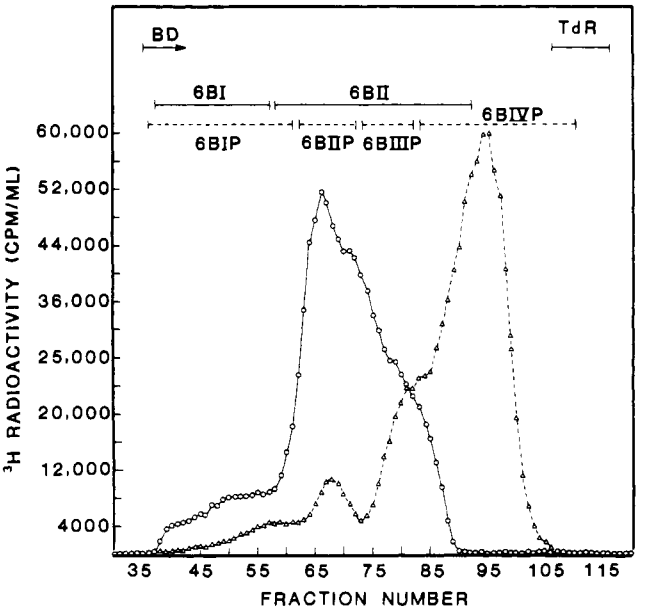

FIGURE 4: Column chromatography of substrate-attached material from reattaching [^{3}H]glucosamine-radiolabeled 3T3 cells before and after Pronase digestion. Fractions in the area marked in Figure 3 (substrate-attached material from reattaching 3T3 cells) were combined and divided into two aliquots. One aliquot was digested with Pronase as described under Materials and Methods, while the other aliquot was incubated under the same conditions in the absence of Pronase. The aliquots were then chromatographed on the same column of Sepharose CL-6B [1 × 120 cm, eluted with 150 mM Tris-HCl (pH 7.4) and 0.2% NaDodSO$_4$]. Fractions from the sham-digested (○) and Pronase-digested (△) materials were combined as indicated by the bars (BD, blue dextran; TdR, [^{14}C]thymidine).

Table IV: Distribution of Polysaccharides in Sepharose Fractions of Pronase-Digested Substrate-Attached Material from Reattaching 3T3 Cells[a]

poly-saccharide[b]	% radioact. in Sepharose fractions[c] 6BIP[d]	6BIIP	6BIIIP	6BIVP
HS	78.8	81.9	18.2	
6S	1.0	0.3		
4S	2.0	2.0	1.3	
DS	11.4	13.6	3.4	
OS	1.9	1.0		
HA	4.9	1.2		
gp			77.1	100.0

[a] [^{3}H]Glucosamine-radiolabeled substrate-attached material was collected from reattaching 3T3 cells as described in Table III and digested with Pronase. The digest was chromatographed on Sepharose CL-6B (Figure 4, dotted line), and the resulting fractions were analyzed for carbohydrate content as described under Materials and Methods. [b] HS, heparan sulfate; 6S, chondroitin 6-sulfate; 4S, chondroitin 4-sulfate; DS, dermatan sulfate; OS, unsulfated chondroitin; HA, hyaluronic acid; gp, glycopeptide. [c] As in footnote *c* in Table I. [d] Fractions are described in Figure 4.

unsulfated chondroitin, and chondroitin 6-sulfate are entirely in 6BI, while heparan sulfate and chondroitin 4-sulfate are distributed about evenly in 6BI and 6BII (in terms of total radioactivity). Dermatan sulfate and glycopeptide are found almost entirely in 6BII. After Pronase digestion (Figure 4, dotted line), the shift into 6BIIP of a significant amount of the heparan sulfate and all of the chondroitin and chondroitin 6-sulfate, along with some of the chondroitin 4-sulfate (all of these had not been in 6BII), again argues for the proteoglycan nature of these species (Table IV).

Guanidine Hydrochloride Extraction of Substrate-Attached Material. For examination of proteoglycan interactions in substrate-attached material, techniques used in studies on

Table V: Distribution of Guanidine Hydrochloride Extractable and Resistant Polysaccharides in Substrate-Attached Material from SVT2 Cells[a]

radiolabeling conditions[b]	polysaccharide[c]	% radioact.[d] extractable	resistant
long term	glycopeptide	23.4	64.6
	GAG	76.6	35.4
	total	100.0	100.0
	HS	52.5	83.3
	6S	1.8	0.8
	4S	12.8	5.4
	DS	7.7	4.8
	OS	11.6	4.0
	HA	13.6	1.7
	total	100.0	100.0
reattaching	glycopeptide	33.3	68.6
	GAG	66.7	31.4
	total	100.0	100.0
	HS	66.3	90.8
	6S	3.0	0.6
	4S	8.5	2.9
	DS	9.7	3.5
	OS	8.0	1.3
	HA	4.5	0.9
	total	100.0	100.0

[a] Substrate-attached material from SVT2 cells was radiolabeled with [^{3}H]glucosamine as indicated and then extracted with 4.0 M guanidine hydrochloride. Material resistant to guanidine hydrochloride extraction was then recovered by NaDodSO$_4$ extraction, and the carbohydrates of both pools of material were analyzed as described under Materials and Methods. [b] Cells were radiolabeled by growth for 72 h in the presence of [^{3}H]glucosamine, and substrate-attached material was either collected directly (long term) or the EGTA-detached cells were washed and allowed to adhere to fresh dishes for 1 h, after which time the substrate-attached material was collected (reattaching). [c] HS, heparan sulfate; 6S, chondroitin 6-sulfate; 4S chondroitin 4-sulfate; DS, dermatan sulfate; OS unsulfated chondroitin; HA, hyaluronic acid. [d] Glycopeptide and GAG are shown as the percentage of total radioactivity, while the individual GAG's are shown as the percentage of total GAG radioactivity.

hyaline cartilage were applied, beginning with guanidine hydrochloride extraction (Sajdera & Hascall, 1969; Hascall & Sajdera, 1969). This extraction is not quantitative, removing only 73% of the long-term [^{3}H]glucosamine-radiolabeled material extractable by NaDodSO$_4$ and only 50% of the reattaching cell substrate-attached material. Similarly, only 24% of the [^{14}C]leucine and 40% of the $^{35}SO_4$ long-term radiolabeled material is extractable with guanidine hydrochloride. These amounts were minimally increased by raising the temperature of incubation (to 25 or 37 °C) and were not affected at all by increasing the molarity of guanidine hydrochloride (up to 8.0 M) or by adding dithiothreitol or EDTA to the extractant.

The composition of the carbohydrates extracted from the substrate-attached material of long-term radiolabeled and reattaching SVT2 cells is shown in Table V. The material resistant to guanidine hydrochloride extraction was subsequently extracted with NaDodSO$_4$, and its carbohydrate composition is shown as well. In both long-term radiolabeled and reattaching cell substrate-attached material, the guanidine-soluble material is relatively deficient in glycopeptide and highly enriched for GAG's. Guanidine-resistant material is enriched for glycopeptide and heparan sulfate. The striking aspect of the resistant material from long-term radiolabeled substrate-attached material is its close similarity to whole substrate-attached material from reattaching cells (Rollins & Culp, 1979). In guanidine-resistant material from reattaching cells, heparan sulfate accounts for 91% of the GAG.

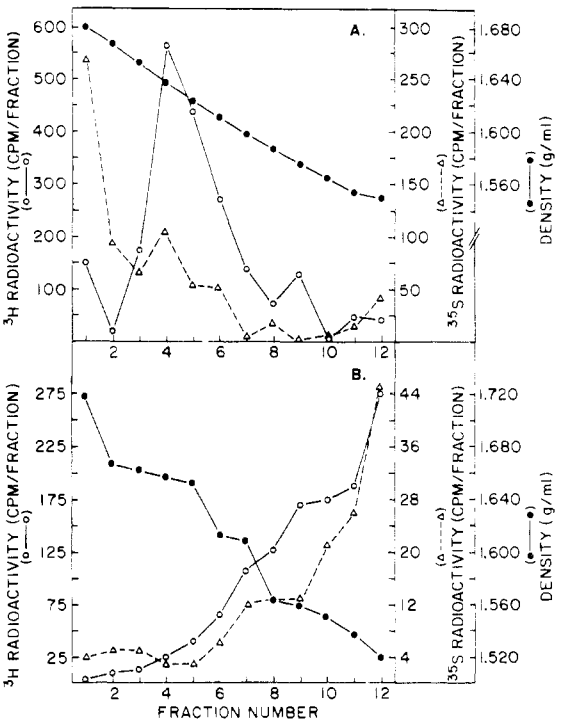

FIGURE 5: Associative and dissociative isopycnic centrifugation of substrate-attached material from long-term [^{3}H]glucosamine–$^{35}SO_4$ radiolabeled 3T3 cells. Substrate-attached material from long-term [^{3}H]glucosamine–[^{35}S]O$_4$ radiolabeled 3T3 cells was extracted with 4.0 M guanidine hydrochloride, concentrated, and dialyzed to 0.4 M guanidine hydrochloride as described under Materials and Methods. Cesium chloride was added to this extract to a density of 1.63 g/mL, and the mixture was centrifuged to equilibrium (A). Fractions 1–8 from (A) were combined and made 4.0 M in guanidine hydrochloride. Cesium chloride was added to a density of 1.54 g/mL, and the mixture was centrifuged to equilibrium (B). Approximately 1-mL fractions were collected from the bottoms of the centrifuge tubes, and the density (●) and ^{3}H (○) and ^{35}S (△) radioactivities of each fraction were determined as described under Materials and Methods.

Isopycnic Centrifugation of Associative and Dissociative Extracts of Substrate-Attached Material. When a 4.0 M guanidine hydrochloride extract of long-term [^{3}H]glucosamine–[^{35}S]O$_4$ radiolabeled substrate-attached material is dialyzed to associative conditions (0.4 M guanidine hydrochloride) (Hascall & Sajdera, 1969) and centrifuged to equilibrium in a cesium chloride gradient, the profile of Figure 5A results. There are two major peaks of radioactivity at densities greater than 1.580 g/mL. The denser peak, at the bottom of the gradient, has a much higher ratio of ^{35}S/^{3}H radioactivity than does the peak of intermediate density. There is also a small area of ^{35}S and ^{3}H radioactivity at the top of the gradient.

When the material banding at densities greater than 1.580 g/mL (fractions 1–8 in Figure 5A) is brought to dissociative conditions (4.0 M guanidine hydrochloride) and again centrifuged to equilibrium in cesium chloride, the radioactivity is distributed as shown in Figure 5B. A significant amount of ^{35}S and ^{3}H radioactivity remains at the bottom of the gradient while over 80% of the ^{3}H radioactivity and 75% of the ^{35}S radioactivity band at densities less than 1.580 g/mL. This material can be dialyzed to reduce the guanidine hydrochloride concentration to 0.4 M and recentrifuged to give the same pattern as fractions 1–8 in Figure 5A (data not shown). Thus, there are carbohydrate-containing species in substrate-attached material which appear to undergo reversible dissociation in 4.0 M guanidine hydrochloride. The dissociated

Table VI: Polysaccharides in Guanidine Hydrochloride Extracted SVT2 Substrate-Attached Material Separated by Associative Isopycnic Centrifugation[a]

polysaccharide[b]	% radioact. in fraction[c] AI	AII	AIII	tube
HS	35.6	82.7	38.1	27.6
Ch-ase digestible	45.8	11.4	27.0	40.0
gp	18.6	5.9	34.9	32.4
total	100.0	100.0	100.0	100.0
% of total radioact.[d]	6.5	30.8	14.3	48.4

[a] Substrate-attached material from long-term [^{3}H]glucosamine-radiolabeled SVT2 cells was extracted with 4.0 M guanidine hydrochloride, concentrated, and dialyzed to 0.4 M guanidine hydrochloride. Cesium chloride was added to this associative extract, and the mixture was centrifuged to equilibrium. Fractions were collected, and the polysaccharides in each fraction were digested with Pronase and chondroitinase ABC as described under Materials and Methods. [b] HS, heparan sulfate; Ch-ase digestible, chondroitinase ABC digestible; gp, glycopeptide. [c] AI, fractions 1 and 2 from a profile similar to Figure 5A; AII, fractions 3-9; AIII, fractions 10–12; tube, an $NaDodSO_4$ extract of the polyallomer tube in which the centrifugation was performed. (This fraction contained 48% of the total radioactivity.) The chondroitinase ABC digest of each fraction was chromatographed on Sepharose CL-6B as described under Materials and Methods. This technique identifies heparan sulfate, glycopeptide, and chondroitinase digestible GAG's (Rollins & Culp, 1979). [d] Percentage of the total radioactivity recovered from the gradient and the centrifuge tube in each fraction.

moieties are then separable by isopycnic centrifugation. Substrate-attached material from [^{3}H]glucosamine-radiolabeled reattaching cells was shown to behave similarly.

Nearly all of the [^{14}C]leucine-radiolabeled material from these guanidine hydrochloride extracts bands at the top of associative gradients, although small amounts of radioactivity are detectable at densities corresponding to the major [^{3}H]-glucosamine peak (Figure 5A). Essentially the same gradient profiles were seen for substrate-attached material from 3T3 and SVT2 cells.

Identification of Polysaccharides in Associative Gradient Fractions. Three areas of radioactivity in an associative gradient of long-term [^{3}H]glucosamine-radiolabeled substrate-attached material from SVT2 cells were analyzed for carbohydrate as described under Materials and Methods. The areas were AI, corresponding to fractions 1 and 2 in Figure 5A, AII (fractions 3–9), and AIII (fractions 10–12). Also, it was routinely found that slightly less than 50% of the [^{3}H]glucosamine radioactivity was bound to the centrifuge tube in a uniform distribution (none was bound to any other surfaces used; for example, during dialysis steps). Therefore, the $NaDodSO_4$ extract of the centrifuge tube was also analyzed. These carbohydrate analyses are shown in Table VI. The most striking aspect of this analysis is the enrichment for heparan sulfate in AII. AI, however, is composed predominantly of chondroitinase-digestible species.

Effect of Pronase Digestion on Aggregation. A typical associative sedimentation equilibrium analysis of long-term radiolabeled SVT2 cell substrate-attached material is shown in Figure 6A. Fractions 1–9 were combined, and an aliquot was dialyzed against 150 mM Tris-HCl (pH 8.2) and 1.5 mM $CaCl_2$ and digested for 24 h with 2 mg/mL (8 mg total) Pronase at 56 °C. This material was extensively dialyzed against 0.05 M sodium acetate buffer (pH 5.8) and then made 0.4 M in guanidine hydrochloride and centrifuged under these associative conditions. This treatment had no effect on the behavior of the polysaccharides in the cesium chloride gradient (data not shown).

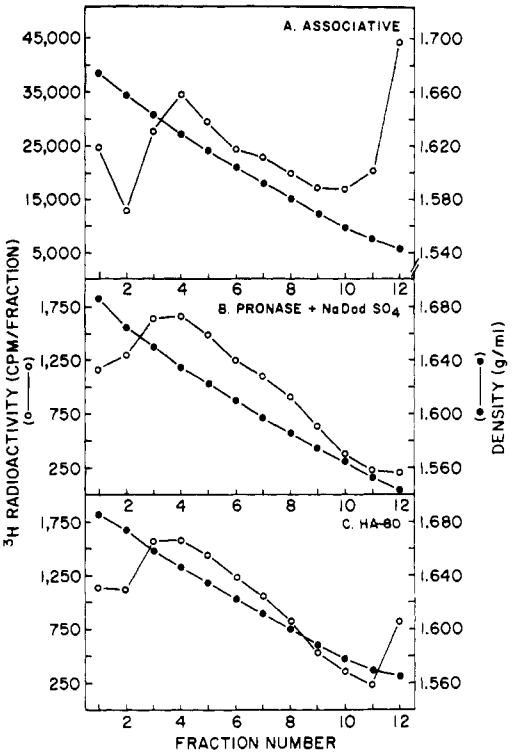

FIGURE 6: Effect of Pronase digestion and HA-80 on the behavior in isopycnic centrifugation of substrate-attached material. Substrate-attached material from long-term [^{3}H]glucosamine-radiolabeled SVT2 cells was extracted with 4.0 M guanidine hydrochloride, dialyzed to associative conditions, and centrifuged to equilibrium in cesium chloride (A). Fractions 1–9 of this associative gradient were digested with Pronase and recentrifuged under associative conditions to give a profile indistinguishable from that seen in fractions 1–9 in (A). This material was divided into two aliquots. One aliquot was made 0.2% in $NaDodSO_4$ and redigested with Pronase. The digest was dialyzed and recentrifuged (B). The other aliquot was made 4.0 M in guanidine hydrochloride, 100 μg of HA-80 was added, and the mixture was then dialyzed back to 0.4 M guanidine hydrochloride and recentrifuged (C). Fractions were collected, and their densities (●) and content of ^{3}H radioactivity (O) were measured as described in Figure 5.

For investigation of whether the protein in these preparations may be protected from proteolysis under associative conditions, the same material was digested with Pronase in the presence of 0.2% $NaDodSO_4$. The digest was then dialyzed extensively to remove proteolytic fragments and to lower the $NaDodSO_4$ concentration. Associative isopycnic centrifugation of this preparation is shown in Figure 6B. Again, little effect was seen although a shift of some material at the bottom of the gradient led to a loss of a distinct AI fraction.

Effect of HA-80 on Aggregation. For assessment of the possible multivalency of hyaluronic acid as having a role in proteoglycan complex formation (Hardingham & Muir, 1972; Hascall & Heinegard, 1974a,b), an aliquot of combined fractions 1–9 in Figure 6A was dissociated by adding an equal volume of 8.0 M guanidine hydrochloride in 0.05 M sodium acetate (pH 5.8). To this dissociated mixture was added 100 μg of HA-80, a hyaluronidase digestion product from hyaluronic acid consisting of approximately 80 repeating units. The size of this oligomer (a maximum of 0.003% of the size of most of the hyaluronic acid in substrate-attached material) corresponds to the distance between attached proteoglycan subunits in the cartilage aggregates (Hascall & Heinegard, 1974a,b; Hascall, 1977).

The dissociative mixture was kept at 4 °C for 24 h and then dialyzed against 9 volumes of 0.05 M sodium acetate (pH 5.8) to bring it back to associative conditions. This mixture was

centrifuged as usual, and the result is shown in Figure 6C. Approximately 10% of the total radioactivity was found in the least dense fraction of the gradient, indicating the ability of HA-80 to displace some glucosamine-radiolabeled material from the aggregated material. There is a concomitant loss of a distinct AI fraction, similar to the effect seen with Pronase digestion in the presence of $NaDodSO_4$.

Discussion

These experiments have demonstrated that a large proportion of the GAG's earlier identified in cellular adhesion sites (Terry & Culp, 1974; Roblin et al., 1975; Rollins & Culp, 1979) are present as proteoglycans. This was shown by the Pronase sensitivity of the apparent size of a percentage of all the GAG's in substrate-attached material, except hyaluronic acid (Mathews, 1971). The size of the undigested heparan sulfate proteoglycan (4BI of Figure 2B) is very similar to the size of the native heparin proteoglycan from mast cells (molecular weight estimated to be ~200000) (Yurt et al., 1977) and the macromolecular heparan sulfate of human skin fibroblasts (Kleinman et al., 1975). The smaller heparan sulfate pools, namely, the native 6BI and 6BII (Figure 2C) and the Pronase-digested 4BIIP, are comparable in size to the single polysaccharide chains of heparin (Yurt et al., 1977) and heparan sulfate (Kleinman et al., 1975) from these sources. Some of the high molecular weight heparan sulfate appears to be Pronase resistant (4BIP), which takes on significance in the light of the aggregation studies (see below).

The lack of any effect on the size of hyaluronic acid by Pronase digestion is consistent with the conventional view of this GAG as a protein-free polysaccharide (Lindahl & Hook, 1978). Most of the chondroitin 4-sulfate and unsulfated chondroitin, however, was shown to be proteoglycan by the elution of these GAG's in 4BIIP and 6BIIIP after Pronase digestion. No GAG had eluted in these positions before digestion. The chondroitin 6-sulfate and dermatan sulfate (Toole & Lowther, 1968) were also shown to be proteoglycan by the same reasoning.

As expected, over 90% of the glycopeptide elutes at a position where there are no GAG's (6BIVP) and nearly all of it elutes in areas where there is no heparan sulfate. These patterns occurred *before* chondroitinase digestion. The ease with which this material can be separated from all the other GAG's strongly supports the identification made in an earlier report (Rollins & Culp, 1979) of this material as being derived from glycoprotein.

Obviously, hyaluronic acid chromatographs with an apparent size larger than any of the other GAG's. Preliminary analysis of these data also suggests that chondroitin 6-sulfate and 4-sulfate and unsulfated chondroitin are somewhat higher in molecular weight than dermatan sulfate and heparan sulfate. That is, relatively more of the chondroitins are in the higher molecular weight fractions (4BI, 4BIP, and 4BIIP) than the lower molecular weight fractions (6BI, 6BII, and 6BIIVP), both before and after Pronase digestion. Whether this reflects the existence of different proteoglycan species, one with the chondroitins vs. another with predominantly dermatan sulfate, is currently under investigation. Data from reattaching cells bear out this trend. The size distributions of the GAG-containing species in the reattaching cell substrate-attached material differ somewhat from those seen in the long-term radiolabeled material, but whether this is an indication of the presence of different proteoglycan species has yet to be shown.

The second part of this communication deals with interactions among these proteoglycans. Chondrocytes in tissue culture have been extracted with guanidine hydrochloride, and they have been shown to make matrix materials capable of aggregating in a manner similar to the elements of whole cartilage (Hascall et al., 1976; De Luca et al., 1978; Kimura et al., 1978). It was hoped that substrate-attached material could be treated in the same way and that similar interactions might be revealed. Figure 5 shows the striking effect of 4.0 M guanidine hydrochloride on the behavior of these proteoglycans during isopycnic centrifugation. Nearly all the material which bands at densities greater than 1.600 g/mL under associative conditions bands at densities less than 1.600 g/mL under dissociative conditions. A small amount of material with a high $^{35}S/^{3}H$ ratio remains at the bottom of the dissociative gradient. This suggests that complexes with a large number of negative charges can be dissociated into their subunits by 4.0 M guanidine hydrochloride and those subunits with a lower charge density can float to the top of the gradient. This is consistent with the fact that material from the bottom of the associative gradients (fractions AI and AII) is excluded from Sepharose CL-2B when chromatographed in 0.4 M guanidine hydrochloride (B. J. Rollins and L. A. Culp, unpublished experiments). Only hyaluronic acid showed this behavior in $NaDodSO_4$.

In cartilage, it has been elegantly shown that the formation of the protein–polysaccharide complex is due to the interaction of the core protein of the chondroitin–keratan sulfate proteoglycan subunit with hyaluronic acid (an interaction further stabilized by another glycoprotein) (Hascall & Sajdera, 1969; Hardingham & Muir, 1972; Gregory, 1973; Hascall & Heinegard, 1974a,b; Heinegard & Hascall, 1974). All of the elements of the cartilage aggregation system (except the glycoprotein link) have been shown to be present in the lower gradient fractions of guanidine-extracted substrate-attached material. Furthermore, digestion with Pronase in the presence of $NaDodSO_4$ and the addition of HA-80 to presumably aggregated material both lead to the displacement of *some* of the formerly aggregated material from the densest gradient fractions. All of this information is consistent with but in no way constitutes a demonstration of cartilage-like aggregates in substrate-attached material.

The existence of true aggregates in the higher density fractions of these associative gradients has not been rigorously demonstrated by this work. And the fact that 4.0 M guanidine hydrochloride chases these components to lower density while Pronase treatment of the midgradient fractions does not do so appears somewhat contradictory. Unlike the cartilage system, however, almost 75% of all the carbohydrate in the denser fractions is heparan sulfate and there are precedents for such protease-resistant macromolecules specifically involving heparin derivatives. Macromolecular heparin from rat skin (Horner, 1971) and native heparin from rat peritoneal mast cells (Yurt et al., 1977) have been shown to be large molecular weight proteoglycans (M_r 1.1×10^6 and 750000, respectively) which are resistant to mild protease digestion (including Pronase) but are degraded to free carbohydrate chains by alkali and can be shown to contain xylose and serine. Thus, the heparan sulfate in substrate-attached material could be engaging in protein-mediated interactions with itself or other components and yet this protein would remain insensitive to protease treatment.

Alternatively, the observed aggregation phenomenon could be due to carbohydrate–carbohydrate interaction. If these interactions are mediated by hydrogen bonds or if they depend in some way on specific conformations of the polysaccharides [some of which are known to be hydrogen bond directed (Rees, 1972; Atkins & Sheehan, 1973; Elloway & Atkins, 1977;

Atkins & Nieduszynski, 1977; Arnott & Winter, 1977; Winter et al., 1978)], then guanidine hydrochloride should be able to disrupt the interactions by its ability to disrupt hydrogen bonding.

These results are consistent with a model of adhesion presented earlier (Culp et al., 1978; Rollins & Culp, 1979) in which cell surface heparan sulfate could cross-link cell surface fibronectin and substrate-bound cold-insoluble globulin in the initial adhesive step. The presence of heparan sulfate proteoglycans in substrate-attached material suggests that this carbohydrate could be acting in such a multivalent capacity. Subsequent detachment of cells during the normal course of movement in tissue culture was suggested to occur as result of accumulating hyaluronate–chondroitin proteoglycan species competing against the heparan sulfate for fibronectin binding. Previous studies (Culp et al., 1978) have indicated a prospective function for hyaluronate–chondroitin complexes in labilization of fibronectin in the adhesion site with subsequent cytoskeletal disorganization and pinching off of footpads at the trailing edge of the cell.

That the aggregation phenomenon involving heparan sulfate is not identical with the cartilage paradigm is immensely interesting. Until now, no tissue system has been described in which "macromolecular" heparan sulfate or heparan sulfate proteoglycans are known to play a role. Whether this phenomenon is, indeed, functionally related to the enrichment for heparan sulfate in the early adhesion site is unknown, but further investigation of this question and of the nature of the protein species in the aggregates themselves is currently underway.

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