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Disulfide-Bonded Aggregates of Heparan Sulfate Proteoglycans†

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ABSTRACT: Heparan sulfate proteoglycans have been isolated from Swiss mouse 3T3 cells by using two nondegradative techniques: extraction with 4 M guanidine or 2.5% 1-butanol. These proteoglycans were separated from copurifying chondroitin sulfate proteoglycans by using ion-exchange chromatography on DEAE-cellulose in the presence of 2 M urea. The purified heparan sulfate proteoglycans are substantially smaller, ca. M_r 20 000, than those isolated from these same cells with trypsin, ca. M_r 720 000 [Johnston, L. S., Keller, K. L., & Keller, J. M. (1979) *Biochim. Biophys. Acta 583*, 81–94]. However, all of the heparan sulfate proteoglycans extracted by these three methods contain similar glycosaminoglycan chains (M_r 7500) and are derived from the same pool of cell surface associated molecules. The trypsin-released heparan sulfate proteoglycan (ca. M_r 720 000) can be significantly reduced in size (ca. M_r 33 000) under strong denaturing conditions in the presence of the disulfide reducing agent dithiothreitol, which suggests that this form of the molecule is a disulfide-bonded aggregate. The heparan sulfate proteoglycan isolated from the medium also undergoes a significant size reduction in the presence of dithiothreitol, indicating that a similar aggregate is formed as part of the normal release of heparan sulfate proteoglycans into the medium. These results suggest that well-shielded disulfide bonds between individual heparan sulfate proteoglycan monomers may account for the large variation in sizes which has been reported for heparan sulfate proteoglycans isolated from a variety of cells and tissues with a variety of extraction procedures.

The presence of heparan sulfate proteoglycans (HSPGs)[1] on the surface of many cell types, in the culture medium, and in basement membranes and other extracellular matrices has been amply documented (e.g., Kraemer, 1971; Dietrich et al., 1976; Mutoh et al., 1976; Keller et al., 1978; Kanwar & Farquhar, 1979; Hassel et al., 1980; Prinz et all., 1980; Kjellén et al., 1980, 1981). Despite considerable interest in the physiological function of these molecules, detailed structural models needed for the interpretation of structure/function data such as exist for cartilage proteoglycans (Hascall & Hascall, 1981) are generally lacking [however, see Oldberg et al. (1979)]. A major reason for this deficit is the fact that the HSPGs in these various cell culture/tissue compartments have been difficult to rigorously purify and no consensus as to their size currently exists. For example, the HSPG from cell surfaces has been reported to range from ca. M_r 20 000 to ca. M_r 1 000 000 (Johnston et al., 1979; Radhakrishnamurthy et al., 1980; Vogel & Peterson, 1981; Rapraeger & Bernfield, 1983) while that from basement membranes varies from M_r 130 000 to M_r 750 000 (Hassell et al., 1980; Kanwar et al., 1981; Oohira et al., 1983). While species and tissue-specific differences may account for such observations, differences in extraction techniques alone have been reported to yield cell surface HSPGs of differing molecular weights (Oldberg et al., 1977, 1979).

The protein moiety from nonproteolytically extracted cell surface HSPG has been shown to vary from ca. M_r 6000 to ca. M_r 40 000 (Oldberg et al., 1979; Mutoh et al., 1980; Lowe-Krentz et al., 1981). Although some of this variation may be due to polymeric forms (Lowe-Krentz et al., 1981), species and tissue variations may also account for some of these differences. However, since the most commonly used test for purity of HSPG is the susceptibility of the heparan sulfate glycosaminoglycan chain to degradation with nitrous acid, the presence of tightly bound nonglycosylated proteins has generally not been excluded. Additionally, all of the present methods commonly used to remove the carbohydrate chains from the protein moiety contain steps which could result in the alteration of the HSPG core protein by either enzymatic or chemical degradation [e.g., protease contamination of chondroitin ABC lyase (Oike et al., 1980) used to remove contaminating chondroitin sulfate proteoglycans (CSPGs) or acidic conditions during nitrous acid treatment (Cifonelli, 1968; Shively & Conrad, 1976) used to remove susceptible heparan sulfate glycosaminoglycans].

During our studies on the HSPGs from Swiss mouse 3T3 cells and the development of methods to purify the HSPG core proteins, we have employed several extraction methods. These

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[1] Abbreviations: HSPG, heparan sulfate proteoglycan; CSPG, chondroitin sulfate proteoglycan; Tris, tris(hydroxymethyl)aminomethane; Gdn·HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

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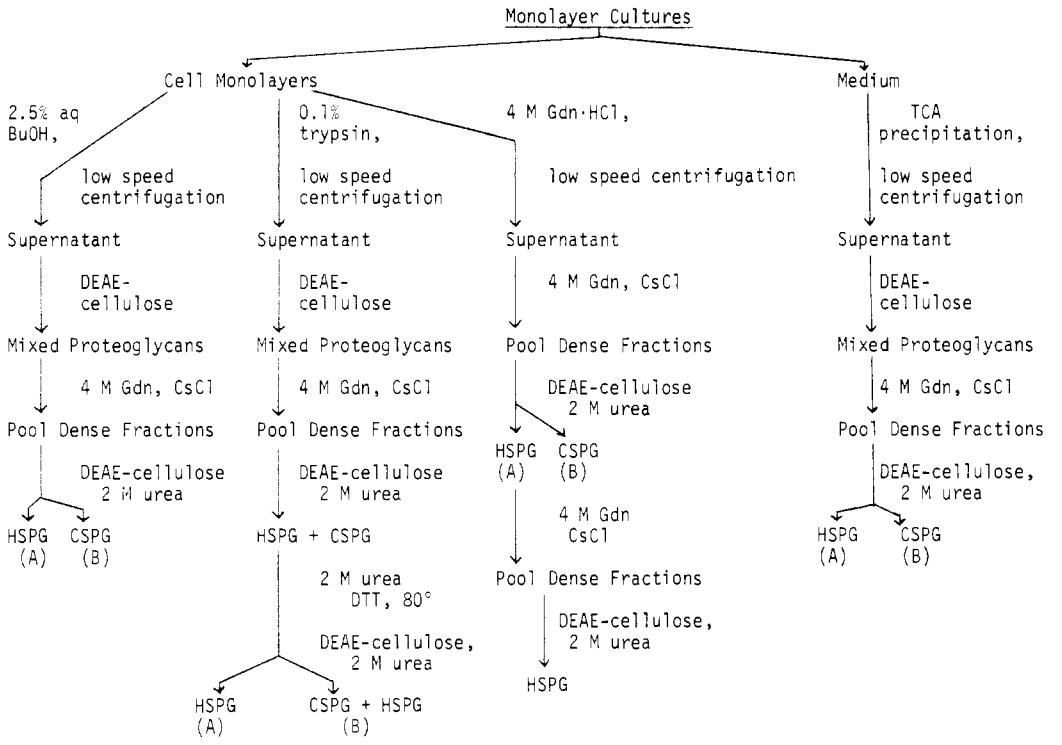

FIGURE 1: Preparation of heparan sulfate proteoglycans.

protocols have yielded HSPGs of differing sizes which are derived from the same cellular pool and contain identical glycosaminoglycan chains. In this paper we report that disulfide bonds, formed or destroyed under certain extraction conditions, may account, in large part, for significant variations in the size of HSPGs from some cell systems.

Materials and Methods

Materials. Trypsin (1× crystallized) was obtained from Worthington Biochemical Corp. The radioisotope $H_2{}^{35}SO_4$ (carrier free) was purchased from New England Nuclear. Unless otherwise indicated, all reagents and chemicals were of the highest grade and purity available. The source and maintenance of the Swiss mouse 3T3 cells have been described previously (Underhill & Keller, 1975).

Isolation of Radioisotopically Labeled Cell Surface Proteoglycan. Subconfluent cell cultures [about 60 dishes (150 mm) or 8 roller bottles (49 cm) in each preparation] were grown for 2 days with $H_2{}^{35}SO_4$ (5 μCi/mL) in modified media as described previously (Underhill & Keller, 1977). The confluent cell sheets were washed 2 times with cold phosphate-buffered saline (0.137 M NaCl, 10 mM KH_2PO_4, 20 mM KCl, and 80 mM Na_2HPO_4, pH 7.3) prior to harvest by one of the following methods (Figure 1).

Extractions: Butanol Extraction. The washed cell sheet was scraped off the plates in the presence of cold phosphate-buffered saline containing protease inhibitors (1 mM *o*-phenanthroline, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM iodoacetic acid, and 1 μg/mL soybean trypsin inhibitor), and the cell clumps were disrupted by aspiration through a Pasteur pipet. The cold cell suspension was then made up to 2.5% (v/v) 1-butanol, rapidly mixed, and immediately centrifuged at 12000*g* for 10 min at 4 °C (Noll et al., 1979). Subsequent treatment of butanol-extracted cells with trypsin removes less than 5% additional sulfated proteoglycan which indicated that the cell surface HSPGs isolated by butanol and trypsin extractions represent the same pool (see below).

Guanidine Extraction. Washed cells, as described above, were scraped off the plates and placed into a cold solution of phosphate-buffered saline containing 4 M guanidine hydrochloride (Gdn·HCl), which lysed the cells and released most internal components, or alternatively the cold Gdn·HCl solution was added directly to washed cells on plates (the same results were obtained with both methods). With very large preparations, this procedure produced a viscous solution. Such samples were sonicated until a homogeneous solution was obtained. This treatment had no effect on the quantity or quality of the final HSPG isolated. All samples were subjected to CsCl gradient centrifugation (described below) before further purification.

Trypsin Extraction. Washed cell sheets were incubated at 37 °C for 20–30 min with 1 mg/mL trypsin in phosphate-buffered saline (Underhill & Keller, 1975). The digestion was terminated with an equal volume of the cold phosphate-buffered saline solution containing protease inhibitors (above), and the cells released from the plate were removed by centrifugation as described above.

Isolation of Radioisotopically Labeled Medium Proteoglycan. Proteoglycans were isolated from protein-depleted medium as described previously (Johnston et al., 1979) except that the proteolytic enzyme digestions were omitted. The recovery of macromolecular [^{35}S]sulfate label in the trichloroacetic acid soluble fraction was always quantatative within experimental error.

Purification. The supernatants from the butanol and trypsin extractions were dialyzed in the cold against a low ionic strength buffer (20 mM Tris-HCl, pH 7.4, and 0.01 M NaCl) or deionized water before ion-exchange chromatography. The dialyzed supernatants were adsorbed to DEAE-cellulose columns in 0.01 M NaCl and then eluted with a linear salt gradient between 0.01 and 1.0 M NaCl (in 20 mM Tris-HCl, pH 7.4) as described previously (Underhill & Keller, 1975). Alternatively, samples were dialyzed against the low ionic strength buffer containing 2 M urea, and the DEAE-cellulose ion-exchange chromatography was performed in the presence of 2 M urea.

Removal of Carbohydrate. The glycosaminoglycan chains of the heparan sulfate proteoglycans were degraded by nitrous

acid by using the conditions described (Underhill & Keller, 1975; Johnston et al., 1979). Alkaline release of the glycosaminoglycan chains was performed in 1.0 M NaOH containing 1 M $NaBH_4$ for 24–72 h at 45 °C.

Analytical Methods. Analysis for radioactivity was performed as described (Underhill & Keller, 1975; Keller et al., 1978). Gel filtration on 1 × 120 cm of Sepharose CL-4B or CL-6B (Pharmacia) was executed in 1.0 M NaCl (in 2 mM Tris-HCl, pH 7.0). Alternatively, columns were equilibrated in phosphate-buffered saline containing 4 M Gdn·HCl. The recovery of radiolabel from the gel filtration was quantatative within experimental error estimated to be less than 5%. Blue dextran and phenol red were used for high and low molecular weight markers, respectively. Columns were calibrated with defined dextran fractions as described previously (Johnston et al., 1979). The molecular weight values obtained are only estimates since the physical parameters of the molecules which determine their behavior on these columns (e.g., frictional coefficients and protein–carbohydrate contents) are unknown; thus, a definitive set of standards cannot be selected. The values obtained, however, do give relative size estimates and are consistent with values obtained by others. To avoid emphasizing the molecular weight values themselves, most of our results are reported in terms of K_{av}. Density gradient centrifugation was performed in CsCl gradients containing 4 M Gdn·HCl in phosphate-buffered saline. The sample and 1.2 g of CsCl were dissolved in 3.6 mL of buffer, and the centrifugation was carried out at 20 °C for 72 h at 100 000*g* in a Beckman SW 50.1 rotor (Lowe-Krentz & Keller, 1983b).

Results

The isolation of proteoglycans from the various extracts of cells (see Materials and Methods and Figure 1) was performed as described previously (Underhill & Keller, 1975; Keller et al., 1978; Johnston et al., 1979). However, the presence of large amounts of cellular material in the 4 M Gdn·HCl extract necessitated a modification in the standard protocol. Much of the extraneous material was removed from this crude extract by density gradient centrifugation in CsCl–4 M Gdn·HCl. The [^{35}S]sulfate-labeled proteoglycans, present in the most dense gradient fractions, were pooled as indicated (Figure 2, inset) and dialyzed against 2 M urea in order to prevent precipitation of the proteoglycans, which occurred upon dialysis against water or low ionic strength buffers. When the cells were grown close to each other in time and labeled with one batch of [^{35}S]sulfate, which had decayed to the same extent, the macromolecular radiolabel in the Gdn·HCl extract was about 1.5 times that present in trypsin or butanol extracts. The source of this increased amount of material in the Gdn·HCl extract is probably from intracellular pools of newly synthesized or endocytosed proteoglycans which are not released by the trypsin or butanol extraction.[2]

All three [^{35}S]sulfate-labeled extracts (butanol, Gdn·HCl, and trypsin) were subjected to ion-exchange chromatography on DEAE-cellulose as described previously (Keller et al., 1978; Materials and Methods), except that the buffers used for the Gdn·HCl-extracted proteoglycans recovered from the CsCl gradient contained 2 M urea to avoid precipitation (see above). The elution profiles for the butanol- and trypsin-extracted and medium proteoglycans were identical with those published previously (Underhill & Keller, 1975; Keller et al., 1978), consisting of a single almost symmetrical proteoglycan peak eluting at a salt concentration of about 0.50 M. As expected

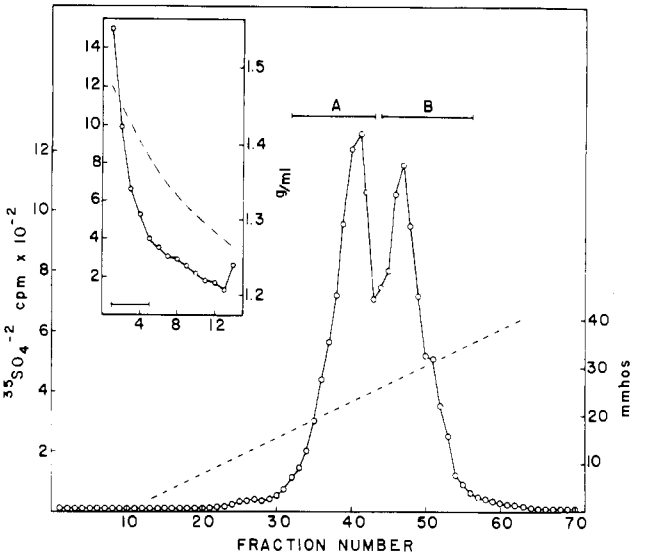

FIGURE 2: CsCl gradient and ion-exchange chromatography of Gdn·HCl extracts. [^{35}S]Sulfate-labeled Gdn·HCl cell extracts were subjected to dissociative CsCl gradient centrifugation as described under Materials and Methods (inset). The most dense fractions were pooled as shown by the bar, dialyzed against 2 M urea, and further fractionated by using ion-exchange chromatography on DEAE-cellulose in 2 M urea as described under Materials and Methods. The [^{35}S]sulfate-labeled (O) proteoglycans were pooled as shown by the bars: (---) g/mL, inset; (---) $m\Omega^{-1}$, main panel.

(Underhill & Keller, 1975), when aliquots of the butanol-, trypsin-, and medium-derived proteoglycans purified by ion-exchange chromatography were subjected to nitrous acid degradation, 50–75% of the [^{35}S]sulfate label was reduced to small products. The remaining [^{35}S]sulfate was present in CSPG as described earlier (Underhill & Keller, 1975; Keller et al., 1978). The [^{35}S]sulfate-labeled material in the Gdn·HCl extract, however, was separated into two major peaks, which were pooled as indicated by the bars (Figure 2) and which contained about 53% (peak A) and 47% (peak B) of the total applied [^{35}S]sulfate. Greater than 95% of peak A, but only 17% of peak B, was susceptible to degradation with nitrous acid. These data indicate that ion-exchange chromatography in 2 M urea effected separation of sulfated proteoglycans, with peak A consisting of essentially only HSPG and peak B primarily CSPG. The homogeneity of peak A (HSPG) from the Gdn·HCl-extracted proteoglycans was demonstrated by the single well-defined peak obtained upon rechromatography on DEAE-cellulose (see Figure 4A).

The Gdn·HCl-extracted HSPG and the butanol, trypsin, and medium sulfated proteoglycan mixtures were further purified by CsCl density gradient centrifugation (Figure 3). In each sample greater than 90% of the [^{35}S]sulfate-labeled proteoglycans sedimented in the densest fractions which were pooled as indicated by the bars. Because ion-exchange chromatography on DEAE-cellulose in the presence of 2 M urea had effected separation of Gdn·HCl-extracted HSPGs from CSPGs (Figure 2), the trypsinate, butanol-released, and medium [^{35}S]sulfate-labeled proteoglycans were dialyzed against 2 M urea and now also subjected to ion-exchange chromatography in the presence of 2 M urea. A double peak pattern similar to that obtained for the guanidine proteoglycans was obtained with the proteoglycans present in both the butanol extract and the medium (compare Figure 2 with Figure 4B,C). The first peak in each case (peak A) eluted at the same conductivity as the rechromatographed Gdn·HCl HSPG (Figure 4A–C). The trypsin-released proteoglycans (Figure 4D) eluted as a single peak coincident with the second peak obtained in the other separations. The [^{35}S]sulfate-labeled

[2] K. M. Keller and J. M. Keller, unpublished observations.

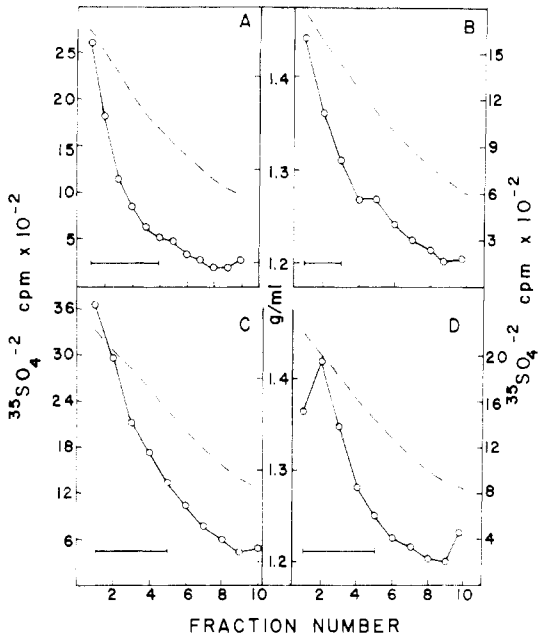

FIGURE 3: CsCl gradient centrifugation of proteoglycans. [^{35}S]-Sulfate-labeled (O) proteoglycans isolated after ion-exchange chromatography (see Materials and Methods) were subjected to CsCl density gradient centrifugation in the presence of 4 M Gdn·HCl (see Materials and Methods). The proteoglycan fractions were pooled as shown by the bars. (A) Butanol proteoglycans, (B) medium proteoglycans, (C) Gdn·HCl HSPGs, and (D) trypsin proteoglycans: (---) g/mL.

proteoglycans were pooled as peaks A and B for medium- (peak A is 58% and peak B is 42% of the applied [^{35}S]sulfate) and butanol- (peak A is 49% and peak B is 51% of the applied [^{35}S]sulfate) extracted proteoglycans (see bars in Figure 4B,C) and as indicated for the Gdn·HCl and trypsin samples (Figure 4A,D). Aliquots of the ^{35}S-radiolabeled A pools from the butanol and medium fractions were completely degraded to small products by nitrous acid treatment when analyzed by chromatography on Sepharose CL-4B or CL-6B (data not shown), indicating that these pools are composed of HSPGs free of CSPGs. However, the single peak of trypsin proteoglycans was still composed of a mixture of about 60% HSPG and 40% CSPG.

Characterization of Proteoglycans. The relative sizes of the three chondroitin sulfate free HSPGs and the trypsin-released proteoglycans were obtained by filtration on Sepharose CL-4B in 1 M NaCl (Figure 5). As is evident, the butanol- and Gdn·HCl-extracted HSPGs elute at K_{av} = 0.68 (ca. M_r 20 000) (Figure 5A,B), while the trypsin-extracted proteoglycans and medium HSPGs appear to be polydisperse with the major portion eluting at K_{av} = 0.27 M_r >500 000 [Johnston et al. (1979) obtained similar sized HSPGs after removing CSPGs from the trypsinate and medium using chondroitin ABC lyase] and a small amount eluting in the K_{av} = 0.68 region (Figure 5C,D). The extreme difference in size between the HSPGs present in the trypsinate and butanol extract was particularly surprising because both techniques remove the same population of [^{35}S]sulfate-labeled molecules from the cell (see Materials and Methods). In addition, a similarity of the HSPGs extracted with Gdn·HCl, butanol, and trypsin is established by the fact that the heparan sulfate glycosaminoglycan chains released by alkali from the butanol-, trypsin-, and Gdn·HCl-extracted proteoglycans elute at the same K_{av} (0.69) from a Sepharose CL-6B column in 4 M guanidine. As expected, the intact butanol and guanidine HSPGs appear larger when analyzed by these same column

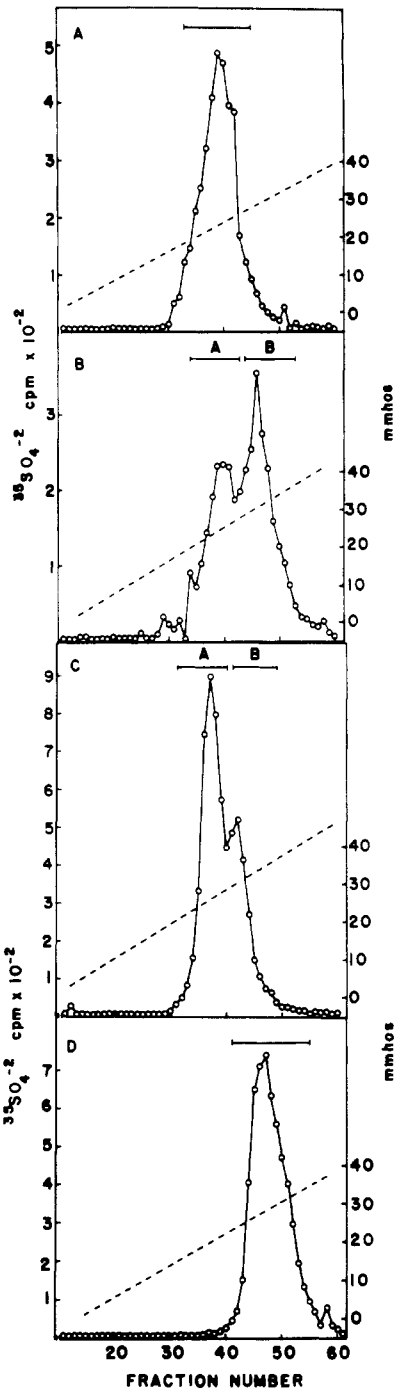

FIGURE 4: Ion-exchange chromatography of proteoglycans in 2 M urea. [^{35}S]Sulfate-labeled (O) proteoglycans obtained after CsCl gradient centrifugation (as shown in Figure 2) were further purified by ion-exchange chromatography on DEAE-cellulose in 2 M urea, and pools were made as indicated by the bars. (A) Gdn·HCl HSPG, (B) butanol proteoglycans, (C) medium proteoglycans, and (D) trypsin proteoglycans.

conditions (K_{av} = 0.62) (data not shown). The heparan sulfate chains released from the medium proteoglycan, analyzed on a Sepharose CL-6B column in 4 M Gdn·HCl, elute somewhat earlier (K_{av} = 0.58 on CL 6B) than those released from the butanol, trypsin, and Gdn·HCl HSPGs by the same treatment (above); however, some preliminary unpublished data suggest that the chain release from the medium HSPG may not be complete (see below and discussion).

The CSPGs separated from either the Gdn·HCl, butanol, or medium extract elute at a position midway between the T 40 and T 110 markers (c and d, respectively, in Figure 5). The CSPG from the trypsin sample that remained undegraded by

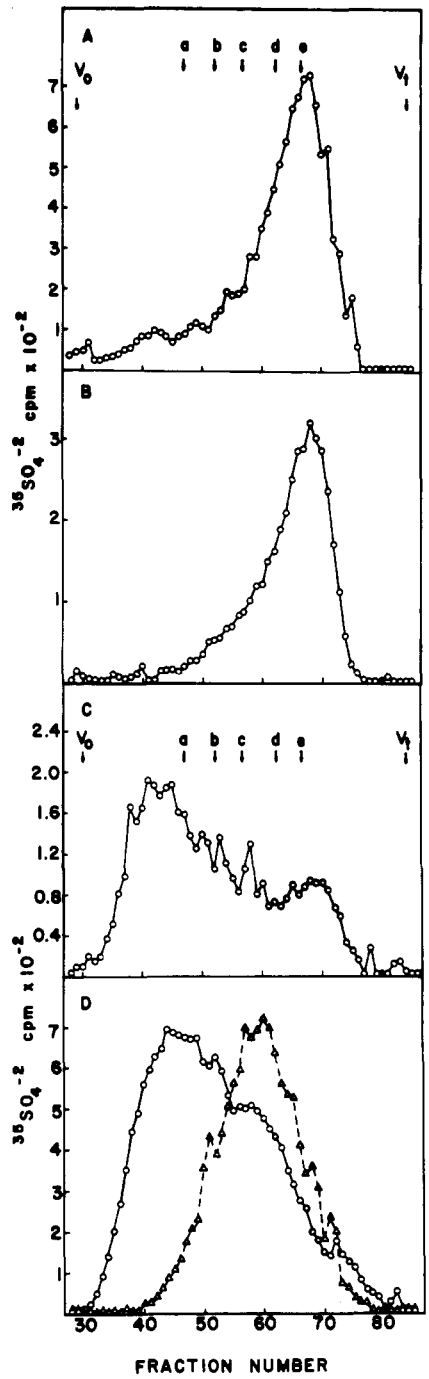

FIGURE 5: Gel filtration of HSPGs and proteoglycans. Aliquots of [^{35}S]sulfate-labeled (○) (A) Gdn·HCl HSPG, (B) butanol HSPG, (C) medium HSPG, and (D) trypsin HSPG and CSPG mixture [before (○) or after (△) incubation at 80 °C in 2 M urea and 2 mM DTT; see Materials and Methods. Arrows at the top of the panels show the elution of polydextran fractions (a) M_r 500000, (b) M_r 250000, (c) M_r 110000, (d) M_r 40000, and (e) M_r 20000. The CSPG from Gdn·HCl or butanol extracts as well as that from trypsin extracts obtained after HNO_2 digestion of the HSPG migrates midway between markers c and d.

nitrous acid elutes at this same position. These data (not shown) suggest that the CSPGs present in these different extracts are very similar in molecular size.

Proteoglycan Aggregates. Since the large trypsin proteoglycan mixture could be aggregated with adventitious protein, the preparation was examined for possible protein contaminants by SDS–polyacrylamide gel electrophoresis. The proteoglycans were prepared in sample buffer containing DTT as a reducing agent and subjected to electrophoresis; 80% or more of the [^{35}S]sulfate label was present at the interface of

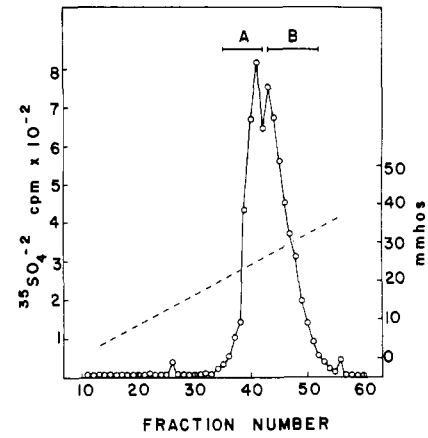

FIGURE 6: Reduced trypsin proteoglycans separated by ion-exchange chromatography. [^{35}S]Sulfate-labeled (○) trypsin-extracted proteoglycans were incubated in 2 M urea containing 2 mM DTT at 80 °C for 2 h. The reduced proteoglycans were then subjected to ion-exchange chromatography on DEAE-cellulose as described under Materials and Methods, and fractions were pooled as shown by the bars.

the separating and stacking gels with the remaining label being distributed through the center of the gels. No protein bands were detected in the trypsin proteoglycan fraction [quantities used (greater than 100 μg) were identical with those for butanol and Gdn·HCl HSPGs], although some minor protein bands were visualized in the butanol HSPG (M_r 15000) and Gdn·HCl HSPG preparations (eight or more bands, M_r 15000–70000). None of the [^{35}S]sulfate-labeled HSPGs located by autoradiography were visualized with the protein stain (data not shown). Most of the contaminating proteins in the Gdn·HCl and butanol preparations were subsequently removed by heating (37 °C) the proteoglycans in 2 M urea with 2 mM DTT for 30 min and separating the released proteins by ion-exchange chromatography on DEAE-cellulose in 2 M urea. After this treatment, there was no change in elution profiles from gel filtration or ion-exchange chromatography for any of the proteoglycans. Although small quantities of M_r 15000 protein remained with the Gdn·HCl and butanol HSPGs, it seemed unlikely that this low level of protein contaminants was responsible for the great differences in HSPG elution on gel filtration. However, it was still possible that due to the lack of protein and/or carbohydrate chain unfolding, the mild reducing conditions employed had not completely reduced all or prevented reformation of disulfide bonds between proteoglycan monomers. Accordingly, the proteoglycans were subjected to the more stringent conditions of heating to 80 °C for 2 h in 2 M urea with 2 mM DTT. This treatment did not result in changes in the elution behavior of the butanol or Gdn·HCl HSPGs on Sepharose CL-4B gel. However, the size of the trypsin proteoglycan mixture now was significantly reduced and eluted in a position identical with that of the chondroitin sulfate proteoglycans from the other fractions (see Figure 5D). On subsequent ion-exchange chromatography on DEAE-cellulose in the presence of 2 M urea (Figure 6), the treated trypsin proteoglycan mixture resolved into two peaks (A and B) similar to those in the other proteoglycan preparations (Figure 6). Fractions were pooled as indicated by the two bars and analyzed for HSPG content: 95% of peak A and 60% of peak B were degraded by nitrous acid, which indicated that separation of heparan sulfate was still incomplete. The K_{av} of the trypsin HSPG (pool A) was 0.49 as determined on Sepharose CL-6B in 4 M Gdn·HCl.

The requirement for DTT in order to effect the disaggregation of the trypsin proteoglycans was demonstrated by the

lack of change in elution behavior on Sepharose CL-4B when the incubation in 2 M urea was performed in the absence of DTT. Possible effects by any cyanate present in the urea were eliminated by the fact that the same changes in size were observed if 4 M Gdn·HCl was used with DTT. The size of the medium HSPG on Sepharose CL-4B after treatment with 2 M urea and 2 mM DTT at 80 °C for 2 h was also reduced. The K_{av} changed from 0.27 to 0.50, although the elution profile indicated that the size of the molecules was still polydisperse. Even more stringent conditions such as higher concentrations of urea or inclusion of a detergent further increased the K_{av} of the medium HSPG. However, our efforts have not yet produced proteoglycans or glycosaminoglycan chains from medium HSPGs which are identical in size with those from the butanol- or Gdn·HCl-extracted cell surface HSPGs.

As is stated under Materials and Methods, the trypsin release of proteoglycans is accomplished at 37 °C, while butanol and Gdn·HCl extractions were performed at 4 °C. If the butanol extraction is carried out at room temperature, a large proteoglycan aggregate is recovered which behaves on Sepharose CL-4B as the trypsin proteoglycan. However, extraction with 4 M Gdn·HCl at room temperature leads to the recovery of only the small proteoglycans described above (data not shown). Apparently, the release of HSPGs from these cells at elevated temperatures, by trypsin or 1-butanol extraction, or natural release into the growth medium results in the formation of disulfide-bonded aggregates by an as yet undefined mechanism which is blocked by Gdn·HCl.

Discussion

Two nondegradative extraction techniques (cold 2.5% 1-butanol or 4 M Gdn·HCl) have been used to isolate sulfated proteoglyans from Swiss mouse 3T3 fibroblasts. In order to recover HSPGs with intact protein cores, the HSPGs in these fractions have been purified and separated from contaminating CSPGs by a method obviating the use of chondroitin ABC lyase, which has been reported to contain protease activity (Oike et al., 1980). The HSPGs extracted and isolated by these methods are significantly smaller than those previously studied in this laboratory which were isolated from the same cell type by trypsin treatment (Johnston et al., 1979). Despite the size disparity, a variety of evidence, including glycosaminoglycan chain size and mutually exclusive removal of HSPGs by butanol or trypsin, indicates that these HSPGs represent the same pool.

Previous work in this laboratory has shown that 1-butanol extraction at room temperature produced large (M_r >500 000) HSPGs (Lowe-Krentz et al., 1981) which contain protein cores consisting of a family of disulfide-bonded multimers.[3] This observation is supported by our current data which show that treatment of the large trypsin HSPG with DTT in either 2 M urea or 4 M Gdn·HCl resulted in a significant size reduction and by our unpublished results which show that [^{35}S]cysteine is incorporated into the purified HSPGs. Similar treatement of either the cold Gdn·HCl-extracted or butanol-extracted HSPGs with DTT did not cause any detectable size changes in these molecules. The larger size of the reduced trypsin HSPG relative to the butanol or Gdn·HCl HSPG indicates that the conditions we have used are insufficient to disrupt all of the bonds between monomer HSPGs. Experiments to clarify this are currently under way. However, the possible involvement of glycosaminoglycan chain interactions [self-association (Fransson et al., 1981)] were ruled out by two pieces of data. First, butanol and Gdn·HCl HSPGs, which have heparan sulfate chains identical with those from trypsin HSPG, would also be expected to aggregate if self-association were the cause of the trypsin aggregates; this does not occur. Second, no disassembly of the trypsin HSPG aggregates occurs in the presence of urea or Gdn·HCl.

The possibility that the small HSPGs isolated with Gdn·HCl or butanol result from reduction during isolation seems unlikely since no intermediate forms are seen nor are we aware of any mechanism for breaking large numbers of disulfide bonds in physiological saline at 4 °C. Thus, the identification of large disulfide-bonded aggregates in trypsin-released HSPGs from Swiss mouse 3T3 cells but not in their cold-extracted counterparts suggests that the large molecular weight forms may be (a) an artifact of the isolation techniques or (b) a mimicry of some naturally occurring biochemical event(s) leading to disulfide formation or intermolecular disulfide exchange at the cell surface itself or during release of HSPGs. Although our data indicate that the trypsin-released HSPGs are disulfide bonded, we have not totally eliminated the possible participation of another protein(s) in the formation of this disulfide-bonded aggregate. Likewise, we have not ruled out the possible presence of low molecular weight proteins which are disulfide bonded to the butanol or Gdn·HCl cell surface HSPG and thus prevent disulfide bond formation between individual HSPG monomers. Experiments to clarify this situation are under way.

Our data also suggest that medium HSPGs from the 3T3 cell lines are assembled into large disulfide-bonded aggregates during release. However, it remains to be shown whether the medium and cell surface HSPGs from the 3T3 cell line are of identical derivation [although the structures of the carbohydrate chains appear to be the same (Keller et al., 1980)]. Neither the HSPG nor glycosaminoglycan chains in the medium HSPG can be reduced to the size of the butanol-extracted cell surface HSPG or glycosaminoglycan chains under the conditions we have employed. Nevertheless, our results indicate that at least some disulfide bonds do contribute to the large size of the medium HSPG. Ongoing investigations with more stringent conditions and tests for other types of cross-linking should resolve this question.

In conclusion, our observations on disulfide bonds in HSPGs offer an explanation for much of the disparity in HSPG molecular weight values reported in the literature. A similar explanation may account for the identification of large HSPGs in the medium of basement membrane forming cells in contrast to the smaller HSPGs isolated directly from purified basement membranes (Kanwar et al., 1981; Oohira et al., 1982; Lowe-Krentz & Keller, 1983b). Investigations in these other systems similar to those in our current report will be needed to determine the relevancy of our observations to these and other data on the variation of HSPG sizes.

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Stopped-Flow Kinetic Studies on the Interaction between Echinomycin and DNA†

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ABSTRACT: The kinetics of association between the quinoxaline antitumor antibiotic echinomycin and DNA have been studied by stopped-flow methods. With natural DNAs, the reaction profile is completely described by a single exponential, the time constant for which varies linearly with the DNA concentration. This bimolecular rate constant is similar for both calf thymus and *Micrococcus lysodeikticus* DNA ($k = 6 \times 10^4$ M^{-1} s^{-1} at 25 °C, $I = 0.01$) and is probably dominated by interaction with relatively weak but abundant binding sites from which the antibiotic dissociates fairly quickly. The observed single exponential suggests a molecular mechanism of binding in which both chromophores of the antibiotic become intercalated simultaneously rather than sequentially; no transition from a mono-intercalated state to a bis-intercalated state could be detected. The reaction is slowed by a factor of about 3 on raising the salt concentration from $I = 0.01$ to $I = 0.5$. Binding to poly(dA-dT) is also described by a single exponential, the time constant for which is about 3 times faster than that seen with natural DNAs. By contrast, the interaction with poly(dG-dC) requires two exponentials for a proper description, the faster of which is similar to that seen with natural DNAs. This may reflect the initial interaction of the antibiotic with two types of sequences, tentatively identified as GpC and CpG, from which it dissociates at very different rates. The differences in kinetic behavior may be explicable on the basis of an alternating B structure for poly(dA-dT) and a more classical B form for poly(dG-dC).

The quinoxaline group of antibiotics, of which echinomycin (Figure 1) is the best known member, are highly active against Gram-positive bacteria, viruses, and a variety of experimental tumors (Katagiri et al., 1975). Their biological properties have been attributed to their ability to bind to DNA (Ward et al., 1965; Sato et al., 1967; Waring & Makoff, 1974). All of them seem to interact specifically with DNA (not RNA) via the process of bifunctional intercalation (Waring & Wakelin, 1974; Lee & Waring, 1978; Waring & Fox, 1983). Detailed studies with echinomycin and a variety of natural and synthetic DNA species have revealed differences in the apparent binding constants ranging over more than 1 order of magnitude (Wakelin & Waring, 1976). While there is no straight-forward correlation between the measured binding constants and

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