Kinetics of Proteoheparan Sulfate Synthesis, Secretion, Endocytosis, and Catabolism by a Hepatocyte Cell Line*

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The metabolism of heparan sulfate proteoglycan was studied in monolayer cultures of a rat hepatocyte cell line. Late log cells were labeled with $^{35}$SO$_4^{2-}$ or [H]$^{3}$H]glucosamine, and labeled heparan sulfate, measured as nitrous acid-susceptible product, was assayed in the culture medium, the pericellular matrix, and the intracellular pools. Heparan sulfate in the culture medium and the intracellular pools increased linearly with time, while that in the matrix reached a steady-state level after a 10-h labeling period. When pulse-labeled cells were incubated in unlabeled medium, a small fraction of the intracellular pool was released rapidly into the culture medium while the matrix heparan sulfate was taken up by the cells, and the resulting intracellular pool was rapidly catabolized. The structures of the heparan sulfate chains in the three pools were very similar. Both the culture medium pool and the cell-associated fraction of heparan sulfate contained proteoheparan sulfate plus a polydisperse mixture of heparan sulfate chains which were attached to little, if any, protein. Pulse-chase data suggested that the free heparan sulfate chains were formed as a result of catabolism of the proteoglycan. When NH$_4$Cl, added to inhibit lysosomal function, was present during either a labeling period or a chase period, the total catabolism of the heparan sulfate chains to monosaccharides plus free SO$_4^{2-}$ was blocked, but the conversion of the proteoglycan to free heparan sulfate chains continued at a reduced rate.

Heparan sulfate has been reported in a variety of animal species and appears to be ubiquitous in the animal kingdom (1, 2). Its biosynthesis seems to parallel the early steps in heparin biosynthesis (3), leading to a proteoglycan product with multiple heparan sulfate chains on a single core protein (4-6). The heparan sulfate chains are made up of blocks of N,O-disulfated uronic acid-GlcN disaccharides which contain both GlcA and IdoA residues with intervening blocks of unsulfated GlcA-GlcNAc disaccharides (7-9). The extent of polymer maturation, as indicated by the degree of sulfation and the GlcA:IdoA ratio, varies and is reflected in the relative proportions of the sulfated and unsulfated blocks. The amounts and structures of heparan sulfate vary among species and tissues and are altered in embryonic (10) and transformed cells (5, 6, 11-13). Labeled heparan sulfate proteoglycan formed in cultured cells is secreted into the culture medium or into the pericellular matrix (5, 14) and is lost from both the intracellular and pericellular pools when the labeled cells are incubated in cold medium (6, 14). Heparan sulfate has been shown to self-associate (15), to bind to fibronectin (16-19), and to play roles in cell-substratum adhesion in cell cultures (16, 19, 20) and in glomerular filtration (21). However, no general physiological role for heparan sulfate reflective of its wide occurrence in animal cells, its potential for extensive structural variability, and its rapid turnover has been demonstrated.

The present study was undertaken to determine the distribution of different heparan sulfate species in the cellular pools of a rat hepatocyte cell line and to observe the kinetics of heparan sulfate synthesis, secretion, and turnover in these cells. The data show that there are multiple heparan sulfate species in these cells and that these species exist in a dynamic steady state involving both the pericellular matrix and the intracellular pools of heparan sulfate.

EXPERIMENTAL PROCEDURES

Cell Culture—A permanent rat hepatocyte cell line, originally isolated by Gerschenson et al. (22) and adapted to grow in serum-free medium (23), was obtained from Dr. David J. Shapiro of this Department. The cells were cultured in Higuchi's medium (23, 24) containing 1% fetal calf serum. Cells were maintained as monolayer cultures in Falcon T-75 flasks and were passaged weekly. For labeling experiments, cells were plated at 100 cells/cm$^2$ in 12.5 ml (T-25 dishes) or 5 ml (T-29 dishes) of medium. Medium was changed at day 4, and the cultures reached confluence after 6-7 days. Cell densities were determined by removing the culture medium, washing the cells twice with 2 ml of phosphate-buffered saline, releasing the cells from the dish with isotonic trypsin, and counting the released cells in a Coulter counter.

Isolation of Heparan Sulfate from Labeled Cultures—After 5 days in culture (late log phase of growth), cells were labeled with 25-100 $\mu$Ci/ml of carrier-free $^{35}$H$_2$SO$_4$ (New England Nuclear) in fresh medium or with 25 $\mu$Ci/ml of [H]$^{3}$H[GlcN (26.8 Ci/mmol, Amersham Radiochemicals) in the Higuchi's medium containing only 1 g of glucose/l. At the end of the labeling period, the cultures were chilled on ice and the culture medium was removed. The cells were rinsed twice with 2 ml of cold phosphate-buffered saline, and the washes were combined with the original culture medium to obtain the culture medium pool. The culture dish was then shaken at 100 rpm with 2 ml of 0.1% trypsin (Sigma Type III) in phosphate-buffered saline at 37 °C for 10 min to remove the cell layer, and the cells were transferred to a chilled 12-ml conical centrifuge tube. The dish was rinsed with an additional 2 ml of cold phosphate-buffered saline, and the cells plus wash were centrifuged in the clinical centrifuge for 5 min to pellet the cells. The supernatant was termed the matrix pool, while the intracellular pool was obtained by extraction of the cell pellet with 1 ml of cold water, which gave complete solubilization of the

*This work was supported by Public Health Service Grant HL21763. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: GlcA, D-glucuronic acid; IdoA, L-iduronic acid; GlcNAc, N-acetyl-D-galactosamine; 2,5-AnMan, 2,5-anhydro D-mannose; ECTA, ethylene glycol bis(diaminoethyl ether)-N,N,N',N'-tetraacetic acid; CM pool, culture medium pool; Ma pool, pericellular matrix pool; IC pool, intracellular pool.
intracellular heparan sulfate.

In experiments for examination of the intact heparan sulfate proteoglycan, the cell-associated pool, which included both the Ma and the IC pools, was prepared by extracting the washed cell layer with 0.5% Triton X-100 in a buffer containing 0.1 M 6-amaminocaproic acid, 5 mM benzamidine, and 10 mM EDTA in 0.05 M Tris, pH 8.0. Blood was added to this detergent solution by shaking the culture dish at 175 rpm overnight at 4 °C. The dish was washed with an additional 2 ml of the detergent solution, and the combined extract and wash were centrifuged at 25,000 rpm for 30 min at 4 °C. The supernatant was saved for analysis.

Determination of Glicosaminoglycan Content of Pools—Heparan sulfate content of the labeled pools was determined by analysis of the products of nitrous acid degradation at pH 1.5 (25). Labeled pools were dialyzed versus water in 88% formic acid/glacial acetic acid/water at 25 °C. For analysis of the structures of the heparan sulfate chains, the heparan sulfate to a level which remained constant while both the IC and the Ma heparan sulfate pools continued to fall suggested that the CM pool was derived as a product secreted directly from the IC pool and not as a product released from the IC and the Ma heparan sulfate pools decreased rapidly while the CM pool of heparan sulfate increased, reaching a level during the first 2 h of the chase which did not change during the further incubation. During the 24-h chase period, 64% of the labeled heparan sulfate in the IC pool and 87% of that in the Ma pool disappeared with only 12% of the lost label appearing in the CM heparan sulfate pool and the remainder being released as free 35SO42− (not shown). The early rise of CM heparan sulfate to a level which remained constant while both the IC and the Ma heparan sulfate pools continued to fall suggested that the CM pool was derived as a product secreted directly from the IC pool and not as a product released from the Ma pool.

From these results, it was not clear whether the Ma heparan sulfate was being taken up and degraded in the lysosomes or being degraded extracellularly. To resolve this question, the chase was carried out in the presence of NH4Cl added to raise the pH of the lysosomes and thus block lysosomal function. In a preliminary experiment, it was demonstrated that NH4Cl had no effect on the rate of heparan sulfate synthesis in these cells in the late log phase of growth (25,000 cells/cm2) were labeled with 25 μCi 35SO42−/ml, and separate dishes, taken at the intervals indicated, were analyzed for cell number and for the amount of 35SO4 in the CM (C), Ma (Δ), and IC (Ο) pools.

FIG. 1. Synthesis and secretion of heparan sulfate. Cells in the late log phase of growth (25,000 cells/cm2) were labeled with 25 μCi 35SO42−/ml, and separate dishes, taken at the intervals indicated, were analyzed for cell number and for the amount of 35SO4 in the CM (C), Ma (Δ), and IC (Ο) pools.

Kinetics of Precursor Incorporation into Heparan Sulfate—Fig. 1 shows the rates of incorporation of 35SO42− into the intracellular, matrix, and culture medium heparan sulfate pools by monolayer cultures of hepatocytes. The rates of appearance of heparan sulfate in the IC and CM pools were linear for the duration of the labeling period while the accumulation of heparan sulfate in the Ma pool appeared to approach a steady-state level. When cells were prelabeled for 20 h and then incubated in unlabeled medium (Fig. 2), the IC and the Ma heparan sulfate pools decreased rapidly while the CM pool of heparan sulfate increased, reaching a level during the first 2 h of the chase which did not change during the further incubation. During the 24-h chase period, 64% of the labeled heparan sulfate in the IC pool and 87% of that in the Ma pool disappeared with only 12% of the lost label appearing in the CM heparan sulfate pool and the remainder being released as free 35SO42− (not shown). The early rise of CM heparan sulfate to a level which remained constant while both the IC and the Ma heparan sulfate pools continued to fall suggested that the CM pool was derived as a product secreted directly from the IC pool and not as a product released from the Ma pool.

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Effect of NH4Cl on distribution of heparan sulfate in a chase of pulse-labeled cells

Cells in the late log phase of growth (25,000 cells/cm²) were labeled with 85SO₄⁻ (93 pCi/ml) for 4 h. The labeling medium was then replaced with fresh medium containing 1.0 mM Na₂SO₄ + 10.0 mM NH₄Cl. Separate cultures were analyzed at 0, 4, and 21 days after addition of the chase medium.

| Chase period | IC | Ma | CM | Total |
|--------------|----|----|----|-------|
| h            |    |    |    | pmol 85SO₄ |
| 0            | 165| 165| 120| 120   | 0    | 0    | 285 | 285 |
| 4            | 130| 225| 58 | 67    | 45   | 43   | 253 | 335 |
| 21           | 80 | 248| 31 | 55    | 46   | 45   | 162 | 320 |

cultures. The results of the chase experiments in the absence and presence of NH₄Cl are presented in Table I. In the absence of NH₄Cl, the pools changed in the same manner as described above (Fig. 2). In contrast, when NH₄Cl was present, there was a slight increase in total heparan sulfate, presumably due to incorporation of the remaining pool of intracellular 85SO₄⁻ into heparan sulfate. However, the Ma heparan sulfate pool dropped and the IC pool rose by a corresponding amount. The CM pool rose by an amount too small to account for the drop in the Ma pool and did not change after the first 4 h of the chase. Thus, NH₄Cl prevented heparan sulfate catabolism, but did not prevent its endocytosis. The constant level of heparan sulfate in the CM pool after the first 4 h of the chase period suggested that the heparan sulfate in this pool was metabolically inert. This was confirmed by the finding that the amount of 85SO₄⁻ heparan sulfate in the culture medium from a 85SO₄⁻-labeled culture did not change when the medium was incubated for 20 h with a washed monolayer of unlabeled cells, whether NH₄Cl was present or not (25). Thus, the heparan sulfate in the CM pool was not taken up either into the Ma or into the IC pool. Therefore, the increased heparan sulfate in the IC pool during the chase in the presence of NH₄Cl must have been due to endocytosis of heparan sulfate from the Ma pool.

Binding of the Ma Pool of Heparan Sulfate to the Cell Surface—Attempts were made to displace the heparan sulfate pool from the cell surface with compounds observed previously to be involved in heparan sulfate binding (29) or receptor-mediated endocytosis (30, 31). Table II shows the effects of mannose-6-P0₄, GalNAc, heparin, and EGTA on the release of the matrix heparan sulfate. The data show that quite high concentrations of mannose-6-P0₄, GalNAc, heparin, and EGTA were required for the release of significant amounts of the Ma pool, while GalNAc at the same high concentration and heparin and EGTA were relatively ineffective.

Comparison of the Structures of the Heparan Sulfate Chains in the Different Cellular Pools—To compare the structural features of the heparan sulfate, a culture was labeled with [3H]GlcN so that both the unsulfated and the sulfated disaccharide units of the heparan sulfate chains would be labeled. The cellular pools were recovered and the [3H]heparan sulfate was isolated by chromatography as shown in Fig. 3. Three

![Figure 3](image-url)

**Figure 3.** DEAE-cellulose chromatography of [3H]glucosamine-labeled IC (panel a), Ma (panel b), and CM (panel c) pools. Five dishes of near-confluent cells (2.5 × 10⁵ cells/dish) were labeled for 18 h with 25 pCi [3H]glucosamine/ml. The cells were harvested, and the pools were isolated and chromatographed on DE52 in a linear NH₄Cl gradient as described under "Experimental Procedures."
peaks of $^3$H-labeled material were obtained for each of the pools. Analyses showed that the labeled products in the peaks which eluted before the beginning of the gradient contained free GlcN, hexosamine phosphates UDP-hexosamines, and glycoproteins, while the only nitrous acid-susceptible material was found in the peak that eluted in the gradient. The fractions in those peaks that are bracketed in Fig. 3 were combined and dialyzed, and the heparan sulfate chains were released from the core proteins by $\beta$-elimination. The free heparan sulfate chains were isolated on a Fractogel TSK HW40 column and cleaved by treatment with nitrous acid. The resulting oligosaccharides were rechromatographed on the Fractogel column. Fig. 4 shows the elution profile for the heparan sulfate obtained from the Ma pool before and after the nitrous acid treatment. Similar separations were obtained for the oligosaccharides from the other heparan sulfate pools. The fractions in each peak were combined and desalted, and the di-, tetra-, and hexasaccharide mixtures were analyzed by high-performance liquid chromatography, as shown in Fig. 5 for the Ma oligosaccharides. Similar profiles were obtained for the oligosaccharide fractions from all three cellular pools. The di- and tetrasaccharide peaks were identified by comparison of their elution positions with those for previously established structures (27, 28). The structures of the hexasaccharide peaks are not known. However, the paper-electrophoretic migrations of the three major hexasaccharides obtained from the high-performance liquid chromatograms suggest that they are all unsulfated. The high-performance liquid chromatographic retention times for the octa- and higher oligosaccharides were too long to obtain good yields from the column. However, when the higher oligosaccharide mixture was analyzed by paper electrophoresis, 75–80% of the labeled material migrated at the same rate as the di- and tetrasaccharides, while the remainder remained at the origin of the electrophotogram. It was concluded that the migrating material contained alternating uronic acid and GlcNAc residues and represented higher heparan sulfate oligosaccharides, while the origin material represented glycoproteins or glycopeptides.

| Oligosaccharide distribution on heparan sulfate chains | % of total $^3$H in heparan sulfate |
|------------------------------------------------------|------------------------------------|
| Disaccharides                                         |                                   |
| GlcA-2,5-AnMan                                        | 0.2 0.3 1.3                       |
| IdoA-2,5-AnMan                                        | 0.9 0.2 0.7                       |
| GlcA-2,5-AnMan(6SO$_4$)                               | 0.2 0.2 0.1                      |
| IdoA-2,5-AnMan(6SO$_4$)                               | 9.5 7.2 5.3                      |
| IdoA(2SO$_4$)-2,5-AnMan                               | 3.3 5.3 4.2                      |
| Tetrasaccharides                                      |                                   |
| GlcA-GlcNAc-GlcA-2,5-AnMan                             | 8.1 9.5 11.4                     |
| IdoA-GlcNAc-GlcA-2,5-AnMan                             | 12.1 12.5 15.9                   |
| GlcA-GlcNAc(6SO$_4$)-GlcA-2,5-AnMan                   | 0.7 1.1 2.1                      |
| IdoA-GlcNAc(6SO$_4$)-GlcA-5,5-AnMan                   | 1.5 3.6 2.0                      |
| IdoA(2SO$_4$)-GlcNAc-GlcA-2,5-AnMan                   | 1.5 2.3 1.4                      |
| Hexasaccharides                                       |                                   |
| h1 (unsulfated)                                       | 3.2 4.7 4.1                      |
| h2 (unsulfated)                                       | 6.8 6.6 5.2                      |
| h3 (unsulfated)                                       | 1.9 0.9 0.6                      |
| monosulfated                                          | 1.0 1.0 1.0                      |
| Higher oligosaccharides$^a$                           | 4.0 33.3 34.0                    |
| Remainder$^a$                                         | 10.1 10.0 10.5                   |

$^a$The combined material from Fig. 4, peak 1, was analyzed by paper electrophoresis. The fraction of this material which electrophoresed at the same rate as the unsulfated di- and tetrasaccharides is designated as "higher oligosaccharides," while the fraction that remained at the origin is designated as "remainder." The remainder represents glycoproteins and glycopeptides (see text).
Proteoglycan Nature of the Heparan Sulfate—The procedure used above for the separation of the Ma and the IC pools involved treatment of the washed cells with isotonic trypsin to release the matrix before extraction of the IC pool from the remaining cells, a procedure that would cleave the core proteins in the Ma and perhaps the IC pools. To investigate the proteoglycan nature of the heparan sulfate, the CA and only about 10% of the total polymer-bound 35S04 found in chondroitin sulfate are also shown. Calculation of the distribution used above for the separation of the Ma and the IC pools

| Sulfate type  | Heparan sulfate pool |
|--------------|---------------------|
|              | IC  | Ma  | CM  |
| O-Sulfates/GlcN | 0.21 | 0.24 | 0.19 |
| Total pool    | 1.23 | 1.38 | 1.27 |
| Disaccharides | 0.16 | 0.24 | 0.17 |
| Tetrasaccharides | 0.08 | 0.12 | 0.09 |
| Hexasaccharides | 0.00 | 0.00 | 0.00 |
| Higher oligosaccharides | 0.42 | 0.43 | 0.42 |

Effects of NH4Cl on the Composition of the Heparan Sulfate Pools—When hepatocytes were labeled in the presence of NH4Cl, proteoglycan synthesis, secretion, and endocytosis continued, but catabolism was inhibited (Table I). The amount of heparan sulfate accumulated in the CA pool was elevated in the presence of NH4Cl, but the amount in the CM pool was not affected. The DE52 elution profiles (not shown) of the CM and CA pools from cultures that had been labeled in the presence of NH4Cl showed that both pools gave a single peak for the 35S04-labeled glycosaminoglycans, just as in Fig. 3 for the cultures labeled in the absence of NH4Cl. When the DE52 peaks from the NH4Cl-treated cultures were chromatographed on Sepharose CL-6B, the elution profile for the CA pool isolated from cells labeled in the presence of 10 mM NH4Cl.

![Fig. 6. DE52 chromatography of the 35SO4-labeled polymers from the CA (panel a) and the CM (panel b) pools. Five dishes of cells (2.5 x 10^6 cells/dish) were labeled with 25 μCi/ml 35SO4 for 20 h. The CM and CA pools were isolated and chromatographed on a DE52 column at 4 °C in a linear NaCl gradient.](image)

![Fig. 7. Sepharose CL-6B chromatography of 35SO4-labeled polymers from the CA (panel a) and CM (panel b) pools. The major peaks from the DE52 columns (Fig. 6), which contained the heparan sulfate (see text), were combined, dialyzed, concentrated, and chromatographed on Sepharose CL-6B as described under "Experimental Procedures." The curves with the open symbols show the profile for the pools isolated from cells labeled in medium without NH4Cl, that with the closed symbols shows the profile for the CA pool isolated from cells labeled in the presence of 10 mM NH4Cl.](image)
a relative increase in the size of Peak 1 in the CA pool from the NH$_4$Cl-treated culture. The effect of labeling in the presence of NH$_4$Cl on the distribution of label in the Sepharose CL-GB peaks is further illustrated by the results shown in Table VI. These data show that NH$_4$Cl has no effect on the composition of the CM pool, but results in an increase in all of the peaks in the CA pool. A comparison of the relative increase in the size of the three peaks from the NH$_4$Cl-treated culture shows that Peak 1 is increased 7-fold while Peaks 2 and 3 are increased to a much lesser extent.

Changes in Distribution of Heparan Sulfate Proteoglycan and Free Chains in a Pulse-Chase Experiment—The demonstrations that 1) the fraction of heparan sulfate which emerges in the early part of the Sepharose CL-6B column is a proteoglycan while those which emerge later appear to be free chains of heparan sulfate, and 2) inhibition of lysosomal function with NH$_4$Cl results in an increase in the heparan sulfate proteoglycan relative to the free chains of heparan sulfate suggest a precursor-product relationship between the heparan sulfate proteoglycan in the CA pool and the free chains. This possibility was investigated by examining the Sepharose CL-6B profiles at timed intervals after addition of the unlabeled chase medium to $^{35}$S0$_4$-labeled cells. The results, see Fig. 8, show that the proteoglycan peak, which emerges between fractions 30 and 60, decreases as the chase progresses and, after 8 h, the major component remaining in the CA pool is the most included material, shown above to be free chains of heparan sulfate.

**DISCUSSION**

Previous studies have shown that the heparan sulfate synthesized by cultured cells is distributed among CM, Ma, and IC pools (6, 14) and that both the CM and the cell-associated pools consist of a mixture of heparan sulfate proteoglycan and free heparan sulfate chains (14, 31-33). Hook and his collaborators (29) have shown that two-thirds of the cell surface heparan sulfate of primary cultures of hepatocytes can be displaced by heparin while the remaining one-third is an integral membrane proteoglycan tightly associated with the plasma membrane via interactions of its hydrophobic core protein with the lipid bilayer. The latter fraction can be released from the cells with trypsin (34). Alternatively, it can be solubilized from the isolated plasma membrane with detergent to obtain a proteoglycan that can be re-embedded in liposomes (34). Other studies have confirmed the hydrophobic nature of the core protein of the heparan sulfate proteoglycan (35, 36).

The hepatocyte cell line described here exhibits similar behavior regarding heparan sulfate distribution and properties.

**TABLE V**

Properties of heparan sulfate peaks from Sepharose CL-6B

| Pool                  | Peak | $K_r$ Actual | % of $^{35}$SO$_4$-polymer in culture |
|----------------------|------|-------------|---------------------------------------|
|                      |      |             | Original $\beta$-Eliminated$^b$        | Heparan SO$_4$ | Chondroitin SO$_4$ |
| Cell-Associated      | 1    | 0.23        | 0.70 (84) | 1.00 (16) | 4.7 (73) | 1.8 (29) |
|                      | 2    | 0.75        | 0.72 (80) | 1.00 (20) | 22.7 (96) | 1.9 (8)  |
|                      | 3    | 0.84        | 0.85 (85) | 1.00 (15) | 33.6 (99) | 1.0 (3)  |
| Total                | 29.5 | (80)        | 7.2 (20)  |

*From the Sepharose column chromatography (Fig. 7).
Numbers in parentheses show the percentages of the total $^{35}$SO$_4$-polymer in each fraction (before $\beta$-elimination) which was found in heparan sulfate and chondroitin sulfate.

**TABLE VI**

Effect of NH$_4$Cl in the labeling medium on the distribution of heparan sulfate in the Sepharose CL-6B peaks

| Pool                  | Peak | Total $^{35}$SO$_4$-polymer in peak | Ratio of $^{35}$SO$_4$-polymer, $+\text{NH}_4\text{Cl} / -\text{NH}_4\text{Cl}$ |
|----------------------|------|-------------------------------------|--------------------------------------------------------------------------------|
|                      |      | $^{35}$SO$_4$-$\text{polymer}$, $-\text{NH}_4\text{Cl}$ | $+\text{NH}_4\text{Cl}$ / $-\text{NH}_4\text{Cl}$ |
| Cell-Associated      | 1    | 2.8                                  | 19.1                                                                                 |
|                      | 2    | 10.2                                 | 40.1                                                                                 |
|                      | 3    | 14.6                                 | 36.3                                                                                 |
| Total                | 27.6 | 95.5                                | 3.5                                                                                 |

$^{35}$SO$_4$-SO$_3$-$\text{polymer}$, $-\text{NH}_4\text{Cl}$

FIG. 8. Changes in the distribution of heparan sulfate proteoglycan and free chains in a pulse-chase experiment. Four dishes of cells in the late log phase of growth (25,000 cells/cm$^2$) were labeled for 4 h with 100 $\mu$Ci $^{35}$SO$_4}$/ml in 2 ml of medium. The cells were washed twice with phosphate-buffered saline, and the labeling medium was then replaced with cold medium containing 1 mM Na$_2$SO$_4$. The CA pool was isolated from one dish each at 0 (panel a), 2 (panel b), 4 (panel c), and 8 h (panel d) and chromatographed on Sepharose CL-6B as described under "Experimental Procedures."
released from the cells by isotonic trypsin under conditions that do not cause cell lysis, while the heparan sulfate that remains with the trypsinized cells is the IC pool. Very little of the Ma pool could be displaced from the cell surface by heparin or by the concentrations of reagents that normally displace ligands that are taken up by receptor-mediated endocytosis in hepatocytes (30, 31). Thus, it appears that the Ma pool in this hepatocyte cell line may be entirely in the form of a proteoglycan which has its hydrophobic core protein buried in the plasma membrane bilayer. A cell matrix made up entirely of the integral membrane proteoglycan has been reported in hepatoma cells (6). The CA pool in the present study includes both the IC and the Ma pools. It consists of a mixture containing a heparan sulfate proteoglycan species and a polydisperse mixture of free heparan sulfate chains.

Complete extraction of the CA pool from the washed cell monolayer requires dilute detergent solutions. In contrast, the IC pool is quantitatively recovered from the trypsinized cells by extraction with water. These observations suggest that the proteoglycan fraction of the CA pool is obtained primarily from the Ma pool while the free heparan sulfate chains are derived from the IC pool.

In contrast to a report describing a neuroblastoma cell line (37), the structural features of the heparan sulfate chains in the IC, Ma, and CM pools are very similar (Table III). The distributions and compositions of the di-, tetra-, and hexasaccharide fractions formed by nitrous acid cleavage were almost identical. The overall degree of sulfation of the heparan sulfate in these cells was somewhat lower than found in that from some other cell types for which structural data are available (37, 38). Less than half of the GlcN residues (42–43%) were N-sulfated, and 19–24% of the GlcN residues were O-sulfated. The O-sulfate was approximately evenly distributed between C-2 of the IdoA residues and C-6 of the GlcN residues. More than 97% of the O-sulfated residues were in the di- and tetrasaccharide fractions, consistent with the earlier conclusion that O-sulfation occurs in the regions of the chain where the N-sulfated GlcN residues are present in blocks (7–9). The octa- and higher oligosaccharides migrated on paper electrophoretograms at the same rate as the unsulfated di- and tetrasaccharides (not shown), indicating that these higher oligosaccharides contain few if any SO₃⁻ substituents. This is consistent with the observation that unsulfated disaccharides containing GlcNAc residues occurred in blocks (7–9). In each of the three pools, approximately 10% of the labeled product does not migrate on paper electrophoretograms. This material, which was present only in the higher oligosaccharide fractions, may contain O-linked or N-linked glycopeptides from the core protein. These oligosaccharide analyses were performed on the heparan sulfate chains from each of the pools without separation of the proteoglycan and free chain species. A preliminary experiment in which Sepharose CL-6B peaks 1, 2, and 3 of the CA pool were cleaved with nitrous acid showed that the di-, tetra-, and hexasaccharides were obtained from each peak in similar ratios. However, insufficient material was recovered for further analyses of the oligosaccharide fractions from each of the individual peaks.

The present results give a picture of the overall metabolism and cellular transport of proteoglycan sulfate. From the data, it is clear that the heparan sulfate that is released into the culture medium is not derived from the general pericellular matrix pool since the amount of heparan sulfate in the medium does not increase after the intracellular heparan sulfate proteoglycan precursor is exhausted, even though relatively large amounts of heparan sulfate remain in the Ma pool. However, the CM heparan sulfate may be transiently associated with the matrix as it passes from the IC pool into the medium. The pericellular matrix of these cells contains a very dynamic pool of heparan sulfate. Labeled heparan sulfate of the Ma pool appears in the cell matrix rapidly when 35SO₄⁻ is added to the culture medium and disappears with a half-life of less than 4 h when the labeled precursor is removed. Since the Ma pool appears to contain heparan sulfate proteoglycan as an integral membrane proteoglycan, it may be that the Ma pool is endocytosed as a result of the normal internalization and recycling of the plasma membrane (39). Kraemer and Tobey (40) reported the loss of cell surface heparan sulfate from CHO cells just prior to mitosis and suggested that enough labeled heparan sulfate appeared in the culture medium to account for the loss from the cell matrix. However, the results from these studies were also consistent with the possibility that heparan sulfate was secreted directly from the IC pool into the medium while the matrix fraction was endocytosed and catabolized. The latter interpretation of the earlier results would be consistent with the observations for the hepatocytes studied here. This raises the possibility that the endocytosis of heparan sulfate from the Ma pool is a cell cycle-dependent process.

The IC pool obviously must contain a transient pool of newly synthesized heparan sulfate proteoglycan, but it also contains a large pool of intermediates formed in the degradation of heparan sulfate. The catabolism of heparan sulfate proteoglycan, reviewed recently by Roden (41), is a complex process. The proteoglycan is initially cleaved into large oligosaccharides by an endogluconidase, and the oligosaccharides are eventually converted to monosaccharides and 35SO₄⁻ by the action of N- and O-sulfatases, hexosaminidase, and β-glucuronidase. All of these enzymes are found in the lysosomes. In the present study, the overall catabolic events suggested by the changes in the Sepharose CL-6B elution profiles during a chase period (Fig. 8) appear to reflect the coordinate activities of these lysosomal enzymes. The proteoglycan peak disappears more rapidly than the heparan sulfate chains in Peaks 2 and 3, which apparently are linked to little, if any, protein. The changes observed are those that would be expected if the proteoglycan is converted to Peak 2 and on to Peak 3 by the endogluconidase. All three peaks show a progressive decrease during the chase consistent with the proposed precursor-product sequence. A similar sequence of changes has been reported for heparin synthesis and turnover in mouse mastocytoma cells (42).

When the cells were labeled in the presence of NH₄Cl, the relative amount of proteoglycan was markedly increased (Table I), but Peaks 2 and 3 were still formed and appeared in both the CA and the CM pools. Thus, if Peaks 2 and 3 are formed from Peak 1, the conversion of Peak 1 to the smaller products is slowed, but not completely blocked, by NH₄Cl. This may be due to the fact that the endogluconidase which initiates the breakdown of the heparan sulfate chains, although identified as a lysosomal enzyme (43), has a pH optimum between pH 5 and 7 (43–46). Thus, this enzyme may retain some of its activity in cells treated with NH₄Cl. The appearance of free heparan sulfate chains in the culture medium was unexpected since this suggests that such chains may be released as free chains from the lysosomes. Further studies on the metabolic flow of the heparan sulfate through the cellular pools are required to determine the source of the heparan sulfate in the medium.

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