A Unique Heparan Sulfate in the Nuclei of Hepatocytes: Structural Changes with the Growth State of the Cells

Neal S. Fedarko and H. Edward Conrad

Department of Biochemistry, University of Illinois, Urbana, Illinois 61801

Abstract. Growing and confluent cultures of a rat hepatocyte cell line were labeled with $^{35}SO_4^{2-}$ and the heparan sulfate in the culture medium, the pericellular matrix, the nucleus, the nuclear outer membrane, and the remaining cytoplasmic pool was purified by DEAE-cellulose chromatography. The heparan sulfate in all pools from the confluent cells was bound more strongly on the DEAE-cellulose column than the corresponding pools from the growing cells. Gel filtration of each pool before and after β-elimination showed that the heparan sulfate from the nuclear and nuclear membrane pools was composed of primarily free chains, whereas the heparan sulfate in all of the other pools was a mixture of proteoglycans and free chains. The heparan sulfate in each pool was cleaved with nitrous acid to obtain mixtures of di- and tetrasaccharides. Analysis of these mixtures showed that the structural features of the heparan sulfates in each pool were different and were altered significantly when the growing cells became confluent. The nuclear-plus-nuclear membrane pools represented 6.5% and 5.4% of the total cell-associated heparan sulfate in the growing cells and the confluent cells, respectively. The structural features of the heparan sulfate in the two nuclear pools were very similar to each other, but were markedly different from those of the heparan sulfate from the other pools or from any previously described heparan sulfate or heparin. The most unusual aspect of these structures was the high content of β-D-glucuronosyl(2-SO_4)→D-glucosamine-N,O-$(SO_4)_2$ disaccharide units in these sequences. The mode of biosynthesis and delivery of these unusual sequences to the nucleus and the potential significance of these observations are discussed.

HEPARAN sulfate proteoglycan (HSPG)[1] is a structurally variable polymer which is turned over rapidly in animal cells (2, 64, 68, 77). Heparan sulfate (HS) is structurally related to heparin, even though the core proteins on which the two polymers are synthesized are different (61, 67). The biosynthesis of heparin and HS appear to follow similar pathways (66, 69). Heparin is synthesized as a polymer of repeating D-glucuronic acid (GlcUA)→N-acetyl-D-glucosamine (GlcNAc) disaccharide units which then undergoes a series of maturation reactions initiated by N-deacetylation, which occurs randomly at some of the GlcNAc residues along the polymer chain, followed by N-sulfation of the resulting D-glucosamine (GlcN) residues. Further maturation, which occurs in the regions around the N-sulfo-D-glucosamine (GlcNSO_3) residues, involves additional N-deacetylation/N-sulfation at GlcNAc residues adjacent to the GlcNSO_3 residues, C5 epimerization of some of the GlcUA residues to form L-iduronic acid (IdoUA) residues, and O-sulfation of some of the GlcNAc and GlcNSO_3 residues at C6 and some of the uronic acid residues at C2 (69). The resulting polymer contains blocks of unsulfated disaccharides interspersed with blocks of disaccharides which are sulfated to varying degrees (13, 48, 73). HS chains are less highly matured than heparin chains. Because the disaccharide units of the heparin and HS chains undergo different extents of maturation, these polymers occur as mixtures of polysaccharide chains which differ in their sulfated disaccharide sequences and in their contents of GlcUA, IdoUA, and N- and O-sulfates (16). A large portion of the newly synthesized HSPG in hepatocytes is secreted into the extracellular matrix, then endocytosed, and catabolized with a $t_{1/2}$ of ~4 h (2). Thus, the HS chains of HSPG, which may exhibit a relatively broad range of structures, have the potential to undergo several significant structural changes during a single cell cycle in response to the metabolic state of the cells. The present study shows that HS is distributed in several cellular compartments, including the nucleus; that the structural features of the HS in the different compartments are different; and that, when cells pass from logarithmic growth to confluency, the rate of HSPG synthesis is markedly increased and the structures of the HS chains in the different cellular pools are altered. Thus, HS undergoes changes in its rate of synthesis and in its structure and exhibits the potential to play a role in the regulation of cellular metabolism.

[1] *Abbreviations used in this paper:* AMan, 2,5-anhydro-D-mannose; $AMan_R$, 2,5-anhydro-D-mannitol; CP pool, cytoplasmic pool; GlcN, D-glucosamine; GlcNAc, *N*-acetyl-D-glucosamine; GlcUA, D-glucuronic acid; HPLC, high performance liquid chromatography; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; IdoUA, L-iduronic acid; Ma pool, matrix pool; NM pool, nuclear membrane pool; NUC pool, nuclear pool; TKM buffer, 0.05 M Tris, 0.025 M KCl, 0.005 M $MgCl_2$.

Materials and Methods

Cell Culture

The permanent rat hepatocyte cell line (29) used previously (2) was maintained and cultured in Higuchi's medium (34) containing 1% fetal calf serum as before. For labeling experiments, cells were plated at 100 cells/cm^2 in 12.5 ml (T-75 dishes) or 5 ml (T-25 dishes) of medium. Medium was changed at day 4, and the cultures reached confluence after 6–7 d. Primary rat liver hepatocytes were cultured as described by Clancy et al. (14). Chick embryo tibial chondrocytes from zones 1–3 of the tibiotarsi of 12-d embryos were cultured in Dulbecco's modified Eagle's medium (40, 41). 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.

Labeling and Cell Fractionation

After 5 d in culture (growing cultures) or 9 d (confluent cultures), cells were labeled for 18 h with 25–100 μCi/ml of carrier-free $H_2{}^{35}SO_4$ (New England Nuclear, Boston, MA) in fresh medium. 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 (CM pool). The culture dish was then shaken at 100 rpm with 2 ml of 0.05% trypsin (Sigma Type III) and 0.02% EDTA in Puck's 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 a clinical centrifuge for 5 min to pellet the cells. The supernatant was termed the matrix pool (Ma pool). All subsequent steps were performed at 4°C. The cells were lysed and nuclei were isolated by the method of Blobel and Potter (5). Briefly, the cells were resuspended in 1.0 ml of 0.05 M Tris, 0.025 M KCl, 0.005 M $MgCl_2$ (TKM buffer) with 0.25 M sucrose and homogenized in a Potter-Elvehjem-type homogenizer (20 strokes). The homogenate and a 1.0 ml rinse of TKM buffer with 0.25 M sucrose were then added to a pre-chilled 15-ml polyallomar centrifuge tube. TKM buffer (4 ml) containing 2.3 M sucrose was then mixed with the cell homogenate. TKM buffer (2 ml) containing 2.3 M sucrose was then layered through the solution to the bottom of the tube using an 11-gauge needle and a crude nuclear fraction was isolated by centrifugation at 124,000 *g* for 30 min. The supernatant was termed the cytoplasmic pool (CP pool). The pellet was resuspended in 1 ml of TKM buffer with 0.5% Triton X-100, vortexed, and re-pelleted by centrifugation at 800 *g* for 5 min. The supernatant was taken as the nuclear membrane pool (NM pool) and the pellet was resuspended in 1 ml of buffer X (2) and extracted overnight at 4°C with shaking at 1,000 rpm. The supernatant was then taken as the nuclear pool (NUC pool).

For non-aqeuous isolation of nuclei, the CM and Ma pools were generated as above. The cell pellet was frozen immediately in liquid nitrogen and lyophilized. Nuclei were isolated by the method of Kirsch et al. (42), with all steps carried out at 2°C. Cells (1×10^6) were resuspended in 0.5 ml glycerol and disrupted by homogenization at 20,000 rpm for 4.5 min in a motor-driven Potter-Elvehjem homogenizer. The homogenate (2 ml) was layered over 0.5 ml of 85% glycerol, 15% 3-chloro-1,2-propanediol in a cellulose nitrate tube, and crude nuclei were pelleted by centrifugation at 120,000 *g* for 30 min. The supernatant was taken as the CP pool. The gelatinous pellet was resuspended in 0.1 ml glycerol with 0.5% Triton X-100 and pelleted at 500 *g* for 5 min. The supernatant was taken as the NM pool, and the pellet as the NUC pool.

Table I. Cellular Distribution of $^{35}SO_4$-labeled Heparan Sulfate

Cell type	Growth stage*	Cellular pool‡ CM	Ma	CP	NM	NUC	Total
		nmol $^{35}SO_4$/10^6 cells					
Hepatocytes							
Cell line	L	1.2	0.37	0.22	0.019	0.022	1.83
		(46)	(45)	(58)	(48)	(38)	
	C	1.7	1.0	2.3	0.11	0.08	5.19
		(74)	(51)	(55)	(55)	(52)	
Primary	—	1.11	0.69	0.78	0.045	0.129	2.75
Chondrocytes	L	0.95	0.12	0.055	0.005	0.004	1.36

* Cells in the logarithmic (*L*) or confluent (*C*) growth stage were labeled with $^{35}SO_4{}^{2-}$ for 18 h and cellular pools were isolated and analyzed for $^{35}SO_4{}^{2-}$-labeled heparan sulfate. For the hepatocyte cell line, the cell doubling times were 12 and 130 h, respectively, in the L and C growth stages. The primary hepatocytes do not divide in culture.

‡ Numbers in parentheses represent the percent of the total $^{35}SO_4$ in the heparan sulfate that was released as free $^{35}SO_4{}^{2-}$ by treatment with nitrous acid.

Analysis of Chondroitin Sulfate and Heparan Sulfate

The HS content of the labeled pools was determined by treatment of an aliquot of each pool with nitrous acid at pH 1.5 (72) and analysis of the products by paper chromatography for 24 h in *n*-butanol, glacial acetic acid, 1 N NH_4OH (2:3:2) as described previously (2). The nitrous acid treatment converted all of the HS to oligosaccharides and free $^{35}SO_4{}^{2-}$ which migrated away from the origin of the strip, leaving sulfated polymers at the origin. The nmol of $^{35}SO_4$ in the HS fraction spotted on the strip was calculated as the value obtained by dividing the total cpm of labeled $^{35}SO_4$ in the migrating oligosaccharides plus free $^{35}SO_4{}^{2-}$ by the cpm/nmol of the $^{35}SO_4{}^{2-}$ supplied in the culture medium. The nmol of N-linked SO_4 in the HS was calculated by dividing the cpm in the free $^{35}SO_4{}^{2-}$ by the cpm/nmol of the $^{35}SO_4{}^{2-}$ supplied in the culture medium.

A second aliquot of the sample was analyzed for chondroitin sulfate by treatment with a mixture of chondroitinases ABC and AC (39) to convert the chondroitin sulfate to disaccharides which were analyzed by paper chromatography for 18 h in *n*-butanol, glacial acetic acid, 1 M NH_4OH (2:3:1). The nmol of $^{35}SO_4$ in chondroitin sulfate on the strip were calculated by dividing the cpm

Table II. Effect of Cell Fractionation Procedures on Distribution of $^{35}SO_4{}^{2-}$-labeled Heparan Sulfate

Isolation procedure	Cellular pool* CM	Ma	CP	NM	NUC	Total
	nmol $^{35}SO_4$/10^6 cells					
Aqueous‡	1.1	1.5	3.2	0.3	0.08	6.18
Non-aqueous§	1.1	1.7	3.0	0.08	0.25	6.13

* Hepatocytes in the late log stage of growth were labeled for 18 h with $^{35}SO_4{}^{2-}$ and the cellular pools were isolated and analyzed for the nanomoles of $^{35}SO_4$-labeled heparan sulfate.

‡ Blobel and Potter (5).

§ Kirsch et al. (42).

*Table III. Adventitious Binding of HS to Nuclei**

Labeled pool added Pool	(cpm/10^6 cells) × 10^{-6}	cpm in isolated nuclei (NUC + NM Pools) Percent of added cpm‡
Experiment 1		
CP	1.5	0.23
CP	1.6	0.40
Experiment 2		
CM	4.0	0.15
CM	8.0	0.02
CM	12.0	0.02
Ma	1.0	0.42
Ma	2.0	0.13
CP	1.0	0.18
CP	2.0	0.13
Experiment 3		
[^{3}H]heparin	0.7	0.21
[^{3}H]heparin	1.25	0.42

* In Experiment 1 nuclei from unlabeled cells were mixed with $^{35}SO_4$-labeled CP pools. In Experiments 2 and 3 unlabeled cells (2×10^6 cells) were mixed with purified cellular pools (*Experiment 2*) or ^{3}H-labeled heparin (*Experiment 3*) and the cells were lysed and the NUC and NM pools were isolated and counted. The sum of the cpm in the two NUC pools was used to calculate the percent of the total added cpm recovered in the nuclear fraction. See text for details.

‡ All experiments were run in duplicate. Values represent averages of the two results obtained in each experiment.

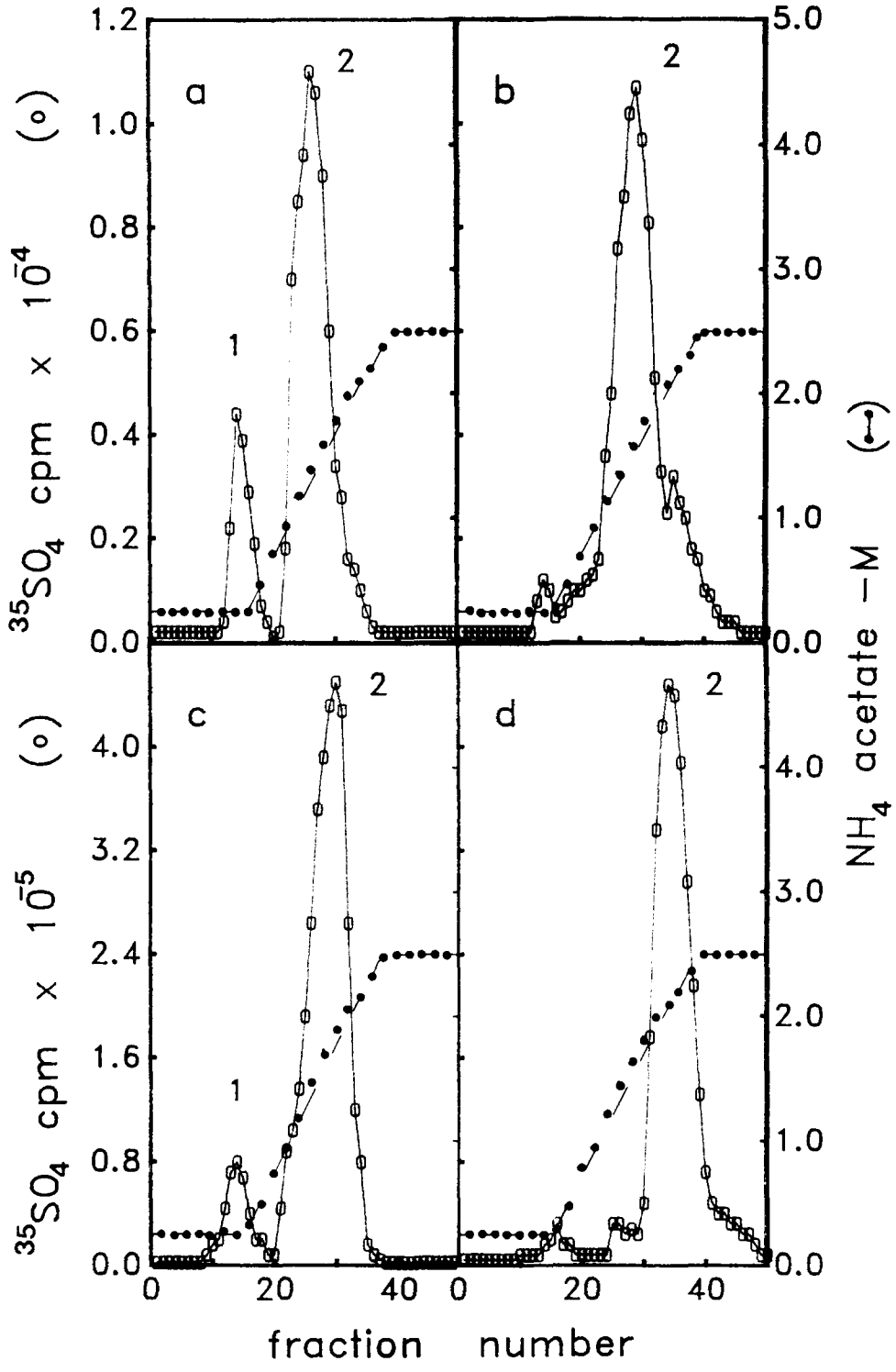

Figure 1. DEAE-cellulose chromatography of $^{35}SO_4$-labeled heparan sulfate from cultured hepatocytes. Cells were harvested during logarithmic growth (*a* and *b*) or after reaching confluency (*c* and *d*) after labeling with $^{35}SO_4^{2-}$ for 18 h. The CP pool (*a* and *c*) and the NUC pool (*b* and *d*) were isolated and fractionated on a DE52 column. In each panel, peak 1 is primarily free $^{35}SO_4^{2-}$, and peak 2 is heparan sulfate.

in the labeled disaccharides by the cpm/nmol of the $^{35}SO_4^{2-}$ supplied in the culture medium.

Isolation of Labeled Heparan Sulfate Pools

The HS in the CM, Ma, CP, NM, and NUC pools was purified by chromatography on columns of DEAE-cellulose (2). The proteoglycan nature of the HS was determined by comparison of the gel permeation profiles before and after β-elimination of the polysaccharide from the proteoglycan. For gel filtration, a stainless steel column (0.94 × 100 cm) packed with Fractogel TSK-HW-55(F) (Pierce Chemical Co., Rockford, IL) and equilibrated in 4 M guanidine-HCl was used for proteoglycan resolution. Chromatographic elution was performed at a flow rate of 0.5 ml/min using a Perkin-Elmer Series 10 pump. For analytical runs, 0.5-ml fractions were collected and taken directly for analysis by liquid scintillation counting. For preparation of larger quantities, 1.0-ml fractions were collected and 0.05-ml aliquots were taken for scintillation counting. The fractions in peaks 1, 2, and 3 from the TSK columns were combined separately, desalted on a BioGel P-2 column run in water, and analyzed for their content of HS and CS. For β-elimination, the desalted pools were treated with alkaline borohydride as before (2).

HS for analysis of the oligosaccharide composition of each pool was isolated by subjecting the pool to β-elimination and treating the products with a mixture of chondroitinases ABC and AC (39). The freed HS chains were recovered as the void volume material obtained from gel filtration on a TSK HW 40(F) column (1.27 × 120 cm) developed in 0.5 M NH_4 acetate and lyophilized twice to remove the NH_4 acetate (2).

Isolation of Heparan Sulfate Oligosaccharides Formed by Nitrous Acid Cleavage

The HS from the different pools was treated with nitrous acid at pH 1.5 (72) and an aliquot of the resulting mixture of oligosaccharides was removed and analyzed for free $^{35}SO_4^{2-}$ by paper electrophoresis in 88% formic acid/glacial acetic acid/water (100:348:3552) (pH 1.7) for 80 min at 25 V/cm. The remainder of the reaction mixture was aldehyde-reduced with $NaBH_4$ and separated according to size by gel filtration on Bio-Gel P2 (3). The disaccharide and tetrasaccharide mixtures were then analyzed by paper electrophoresis and by high performance liquid chromatography (HPLC). Paper electrophoretograms were run for 2 h at 25 V/cm on strips (1 × 22 in) of Whatman No. 3 paper in 88% formic acid/glacial acetic acid/water (100:348:3552) (pH 1.7). HPLC was run on a 0.4 × 25 cm SAX column (Whatman Inc., Clifton, NJ) developed with step gradients of KH_2PO_4 buffers (2, 3, 17). The peaks were identified by comparison of their retention times with those of standards (3, 17).

Preparation of ^{3}H-labeled Heparin

^{3}H-Labeled heparin was prepared by the procedure of Nakajima et al. (59). Briefly, heparin (13 mg) was reacted with 2.6 mg NaB^3H_4 (450 mCi/mmol) in 200 μl of 0.1 M Na borate buffer, pH 8.0, for 5 h at room temperature. The reaction mixture was then acidified with 200 μl of 1 N H_2SO_4 in the fume hood and separated from extraneous ^{3}H by gel filtration on a Fractogel TSK-HW-40(S) column (50 × 1.1 cm) equilibrated in 4 M guanidinium Cl. The ^{3}H-labeled material in the void volume was desalted by passage over a Fractogel TSK-HW-40(S) column (17 × 4.6 cm) run in 0.05 M NH_4 acetate. The void volume peak was lyophilized twice to remove the NH_4 acetate and further purified by chromatography on DE52 cellulose as described for the purification of the cellular HS pools.

Results

Distribution of $^{35}SO_4$-labeled Heparan Sulfate in Cellular Pools

The extracellular and intracellular pools of a hepatocyte cell line, primary rat hepatocytes, and chick embryo chondrocytes, isolated from cells cultured in the presence of $^{35}SO_4^{2-}$, were analyzed for $^{35}SO_4$-labeled HS. The results in Table I show that all of the cell types contained HS in all of the intracellular pools as well as in the extracellular pools. The largest amounts of HS were found in the extracellular pools and in the CP pool. Of particular note were the NUC and NM pools which together contained from 0.3% (chondro-

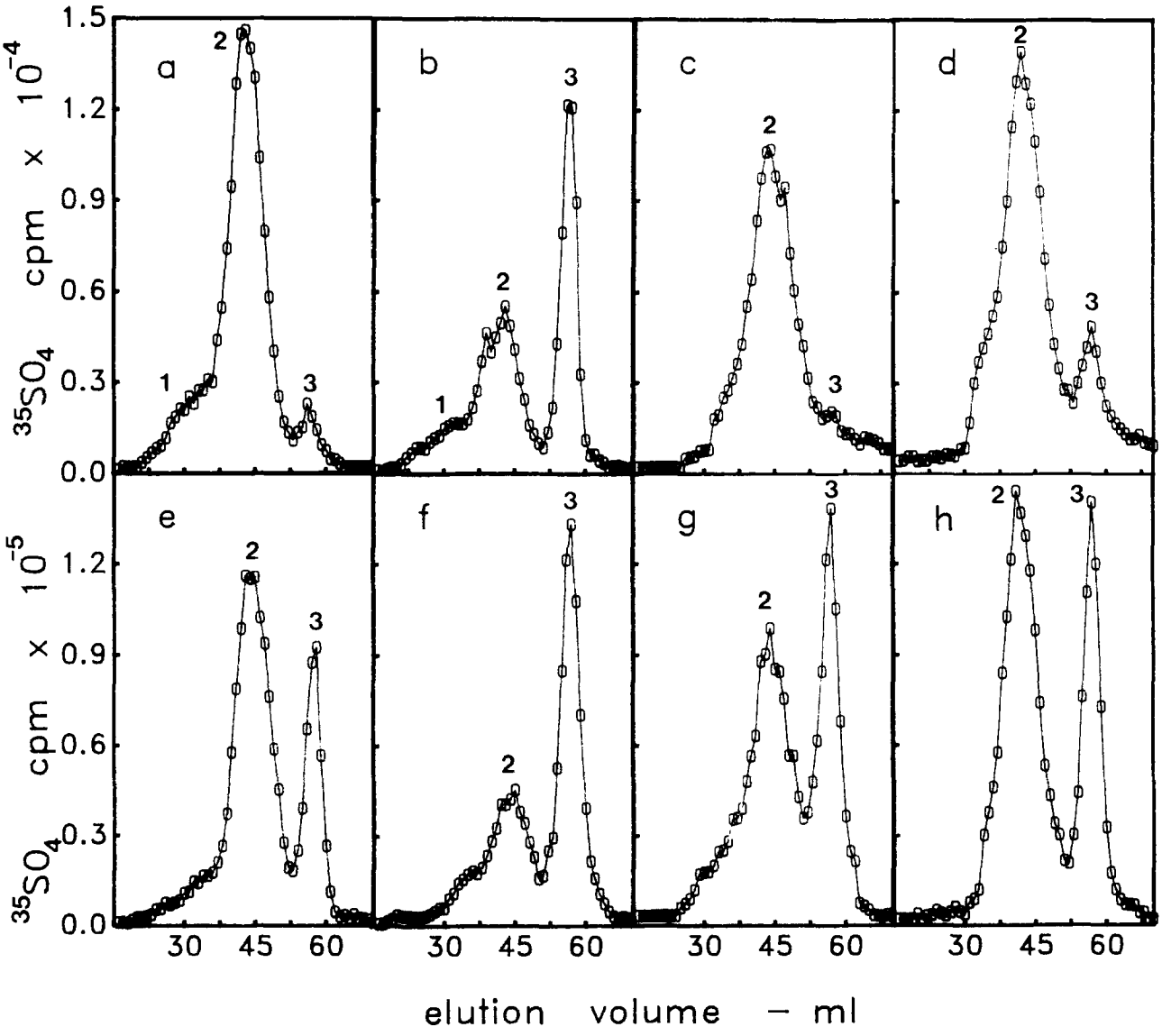

Figure 2. Gel filtration analysis of CP (*a*, *b*, *e*, and *f*) and NUC (*c*, *d*, *g*, and *h*) pools from log phase (*a*, *c*, *e*, and *g*) and confluent (*b*, *d*, *f*, and *h*) cells. The top panels show the TSK 55 profiles before β-elimination. The bottom panels show the corresponding fractions after β-elimination.

cytes) to 2.7% (primary hepatocytes) of the total cellular HS and from 8% (confluent hepatocyte cell line) to 18% (primary hepatocytes) of the total intracellular pool. For the rat hepatocyte cell line, which was chosen for further study, the results in Table I show that the amounts of $^{35}SO_4^{2-}$ incorporated into the HS in all of the cellular pools were markedly increased when the cells became confluent. In all of the pools except the CM pool, ~50% of the $^{35}SO_4$ in the HS was released as free $^{35}SO_4^{2-}$ when the sample was treated with nitrous acid, indicating that about half of the sulfate substituents in these polymers were present as N-sulfate groups on the GlcN residues (72).

Table II shows that essentially the same distribution of HS is found in the intracellular fractions when nuclei were isolated using a non-aqueous fractionation procedure. The only significant difference in distribution is seen in the NM and NUC pools where the total HS in the two pools remains the same while the partitioning of the HS into the NUC pool was higher when the non-aqueous method was used for isolation of the nuclei. The properties of the NM and NUC pools isolated using the aqueous method were virtually identical in their structural properties (below).

Table IV. Chromatographic Properties of Heparan Sulfate

Pool	Growth stage*	DEAE-Cellulose position	TSK 55 Chromatography: Peak‡	% of $^{35}SO_4$ in peak: HS	CS	Elution position: Original	β-Eliminated
		M NH_4Cl					K_{av}
CM	L	1.2	1 (49)	75	14	0.03	0.38
			2 (11)	87	10	0.46	0.94
			3 (40)	87	9	0.97	0.97
	C	1.4	1 (69)	72	14	0.12	0.32
			2 (18)	90	6	0.41	0.94
			3 (12)	88	5	0.97	0.97
Ma	L	1.2	1 (32)	70	20	0.03	0.26
			2 (58)	85	10	0.53	0.56
			3 (10)	90	8	0.97	0.97
	C	1.4	1 (73)	85	17	0.12	0.38
			2 (23)	78	14	0.53	0.56
			3 (4)	81	13	0.97	0.97
CP	L	1.2	1 (16)	68	18	0.21	0.53
			2 (78)	95	4	0.56	0.56
			3 (7)	95	5	0.94	0.94
	C	1.6	1 (13)	63	19	0.21	0.53
			2 (40	94	4	0.49	0.50
			3 (47)	92	5	0.94	0.94
NM	L	1.4	1 (0)	—	—	—	—
			2 (89)	95	3	0.56	0.56
			3 (11)	97	4	0.96	0.96
	C	1.9	1 (0)	—	—	—	—
			2 (97)	94	3	0.54	0.54
			3 (3)	97	4	0.96	0.96
NUC	L	1.5	1 (0)	—	—	—	—
			2 (91)	99	2	0.56	0.56
			3 (9)	99	1	0.94	0.94
	C	2.1	1 (0)	—	—	—	—
			2 (94)	100	0	0.47	0.47
			3 (6)	99	0	0.91	0.91

* See Table I, footnote 1.

‡ Values in parentheses are percentages of the total $^{35}SO_4$ in each pool found in peaks 1, 2, and 3.

Adventitious Adsorption of Heparan Sulfate and Heparin to Cell Nuclei

Three experiments were performed to determine whether the labeled HS recovered in the nuclear pools was HS that was adsorbed in a nonspecific manner to the original nuclear pellets. In these experiments, unlabeled nuclei were isolated by the aqueous procedure from cultures to which different labeled HS or heparin fractions had been added. The results of these experiments are shown in Table III. In the first experiment, parallel cultures were grown to the late log stage and one of the cultures was labeled for 18 h with $^{35}SO_4^{2-}$ while the other was not. Both cultures were harvested at a cell density of 2×10^6 and crude nuclei (NUC + NM pools) were separated from the CP pools. The nuclear fraction from the unlabeled culture was mixed with the TKM-1.6 M sucrose solution containing the $^{35}SO_4$-CP pool from the labeled culture. The NUC and the NM pools were re-isolated from the mixture and the total $^{35}SO_4$ cpm in the nuclear pools was measured. In the second experiment, the CM, Ma, and CP pools were isolated from a $^{35}SO_4$-labeled culture by DE52 chromatography and desalted by gel filtration. Each of the purified pools was added to unlabeled cells from late log cultures. The cells were lysed in the presence of the labeled pools and the NUC and NM pools were isolated and counted. Finally, ^3H-labeled heparin, which had a net charge similar to that of the HS found in the nuclear HS pools, was added to unlabeled cells. The cells were lysed and the nuclear pools were isolated and analyzed for ^3H cpm. In all of these experiments, the amount of labeled HS or heparin recovered in the NUC-plus-NM pools was <0.5% of the total cpm added to the cells or cell fraction containing the unlabeled nuclei—well below the percentage of the total labeled HS which was recovered in the nuclear fractions when the cells were labeled with $^{35}SO_4^{2-}$.

Table V. Oligosaccharides from Growing and Confluent Hepatocytes

Oligosaccharide	Sulfates per mol*	Growth State‡	Cellular pool: CM	Ma	CP	NM	NUC
			pmol/10^6 cells§				
Disaccharides	1	L	9.0	18.0	5.6	0.5	0.7
		C	20.0	30.0	66.0	3.0	4.8
	2	L	10.0	14.0	5.0	1.8	2.4
		C	10.0	28.0	90.0	5.5	8.0
Tetrasaccharides	1	L	16.0	6.6	6.3	1.0	0.8
		C	8.0	4.8	90.0	21.0	4.2
	2	L	1.1	0.5	1.0	1.2	0.9
		C	2.7	0.8	12.0	5.0	1.5
	3	L	1.8	0.3	0.3	0	0
		C	1.1	0.3	3.7	0	0
	4	L	0	0.1	0.2	0	0
		C	0	0.1	0.8	0	0
Hexasaccharides	1	L	0	5.7	2.1	0	0.2
		C	0	16.0	31.0	2.7	7.2
	2	L	0	2.1	2.7	0	0.3
		C	0	2.2	20.0	7.6	0.9

* Determined by comparison of rates of migration on paper electrophoretograms with the rates observed for standards (3); see Figs. 3 and 4.

‡ See Table I, footnote 1.

§ The number of pmols of each oligosaccharide was calculated by dividing the $^{35}SO_4$ cpm in the oligosaccharide by the cpm/nmol of $^{35}SO_4^{2-}$ in the culture and the number of SO_4 substituents on the oligosaccharide.

Properties of Heparan Sulfate from Different Pools

The HS from the different pools was purified first by DEAE-cellulose chromatography and then by gel filtration on TSK 55 columns. The chromatographic behaviors of the HS from the CM and Ma pools of these cells were reported previously (2). The behaviors of the CP and NUC pools on DE52 columns and TSK 55 columns are illustrated here. The DE52 and gel filtration elution profiles of the HS obtained from the CP and NUC pools of growing and confluent cells are shown in Figs. 1 and 2, respectively. The chromatographic behaviors of the HS from all pools are summarized in Table IV. Several features of the chromatographic behaviors of the HS in the different pools are of note. First, in the DE52 profiles, the HS in each pool is recovered exclusively in peak 2 as before (2). Although the HS from the CM, Ma, and CP pools of the log-phase cells all elute at the same position, the HS in the NM and NUC pools is bound more tightly and requires a higher concentration of NH_4 acetate for elution (Table IV). Second, the HS from each pool of the confluent cells was more strongly bound than the corresponding HS pool from the growing cells (Table IV and Fig. 1), indicating a change in the HS structure in the confluent cells. Third, on the TSK columns the HS in the CM, Ma, and CP pools of both log and confluent cells separated into three peaks, whereas only peaks 2 and 3 were obtained for the HS in the NM and NUC pools (Table IV and Fig. 2). It was shown previously that most of the HS in proteoglycan form was present in peak 1 and this is confirmed in the present results by the finding that β-elimination causes an increase in K_{av} of the peak 1 material (Table IV). Peak 2

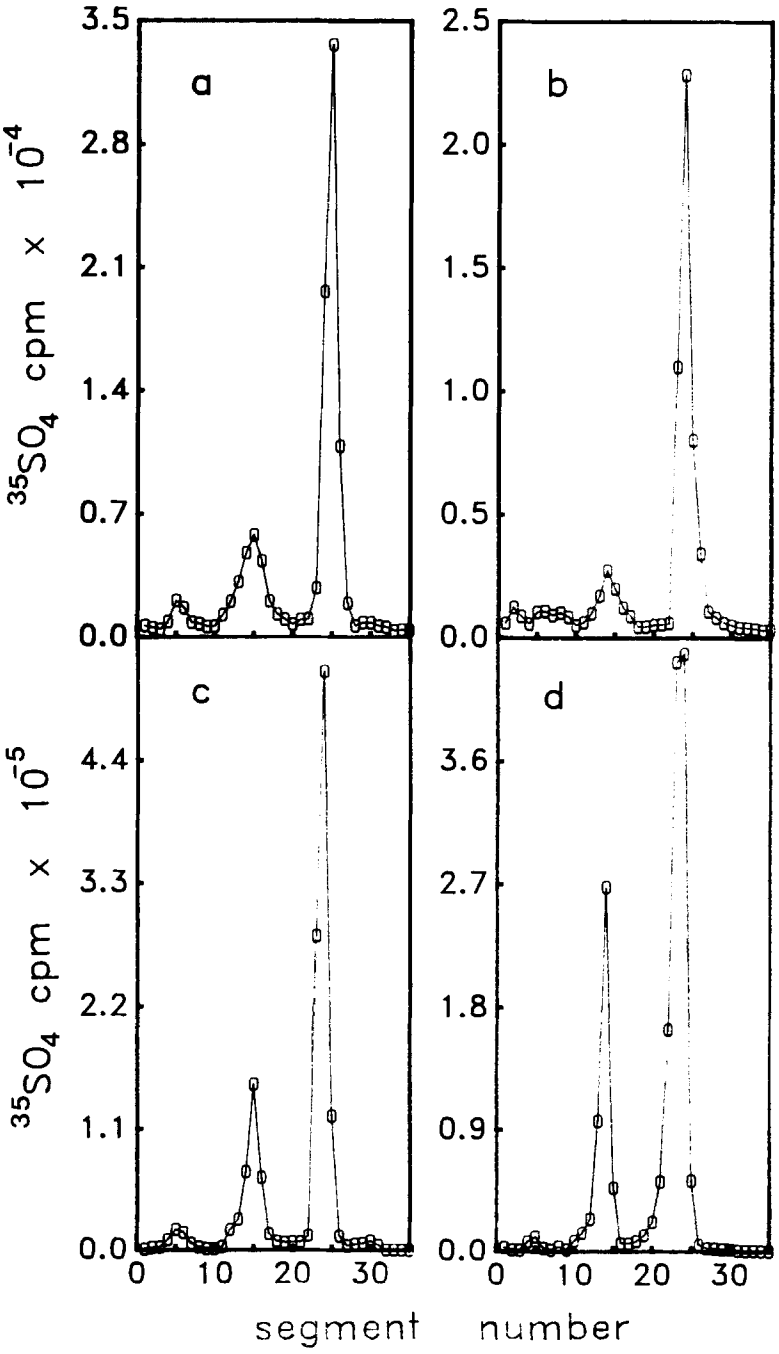

Figure 3. Paper electrophoretic analysis of disaccharides obtained by cleavage of heparan sulfate from the CP (*a* and *c*) and NUC (*b* and *d*) with nitrous acid. Heparan sulfate was isolated from log-phase (*a* and *b*) and confluent (*c* and *d*) hepatocytes. The peaks at segment 24 of the electrophoretograms migrate in the position of disulfated disaccharides, whereas the peaks at segment 15 migrate in the position of monosulfated disaccharides. The total cpm in each peak was used to calculate the nanomoles of disaccharide in each fraction (Table IV).

of the CM pools also shows an increase in K_{av} on β-elimination, but in all of the other pools the K_{av} of peak 2 is not altered by β-elimination, indicating that these pools contain HS associated with little if any core protein. Finally, the analyses of the peaks for their HS and CS content show that the CS is concentrated primarily in the peak 1 material with smaller amounts of CS in peaks 2 and 3. The material in the NM and NUC pools is almost entirely HS.

Isolation of Oligosaccharides Formed by Nitrous Cleavage of Heparan Sulfate

Each purified pool of HS was treated with nitrous acid and the resulting $^{35}SO_4$-labeled oligosaccharides were separated into di-, tetra-, and hexasaccharides by gel filtration (2). The distribution of each size class according to number of SO_4 substituents per mol was determined by paper electrophoresis. This is illustrated for the di- and tetrasaccharides in Figs. 3 and 4, respectively. The recoveries of each oligosaccharide size and charge class are given in Table V. The results show that, for all pools, the sulfated oligosaccharides were recovered primarily in the disaccharide fraction. Approximately equal amounts of mono- and disulfated disaccharides were found in all pools except for the NM and NUC pools in which the amounts of disulfated disaccharides were from two- to four-fold greater than the amounts of monosulfated disaccharides. This is consistent with the stronger retention of the HS from these pools on the DEAE-cellulose columns (Fig. 1). Much greater differences were observed for the ratios of tetra- and

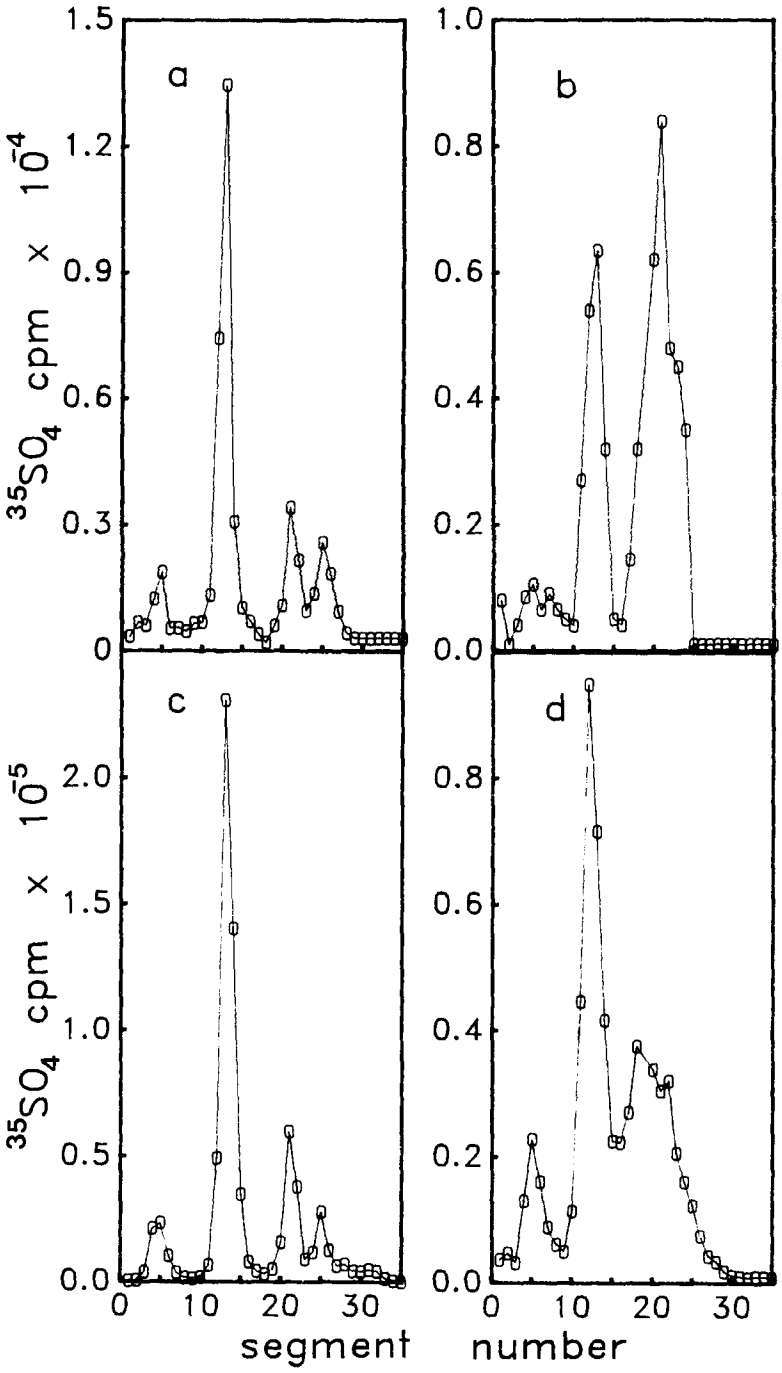

Figure 4. Paper electrophoretic analysis of tetrasaccharides obtained by cleavage of heparan sulfate from the CP (*a* and *c*) and NUC (*b* and *d*) pools with nitrous acid. Heparan sulfate was isolated from log phase (*a* and *b*) and confluent (*c* and *d*) hepatocytes. Mono-, di-, and tri-, and tetrasulfated tetrasaccharides migrate at segments 14, 21, 25, and 28, respectively. The total cpm in each peak was used to estimate the nanomoles of disaccharide in each fraction (Table IV).

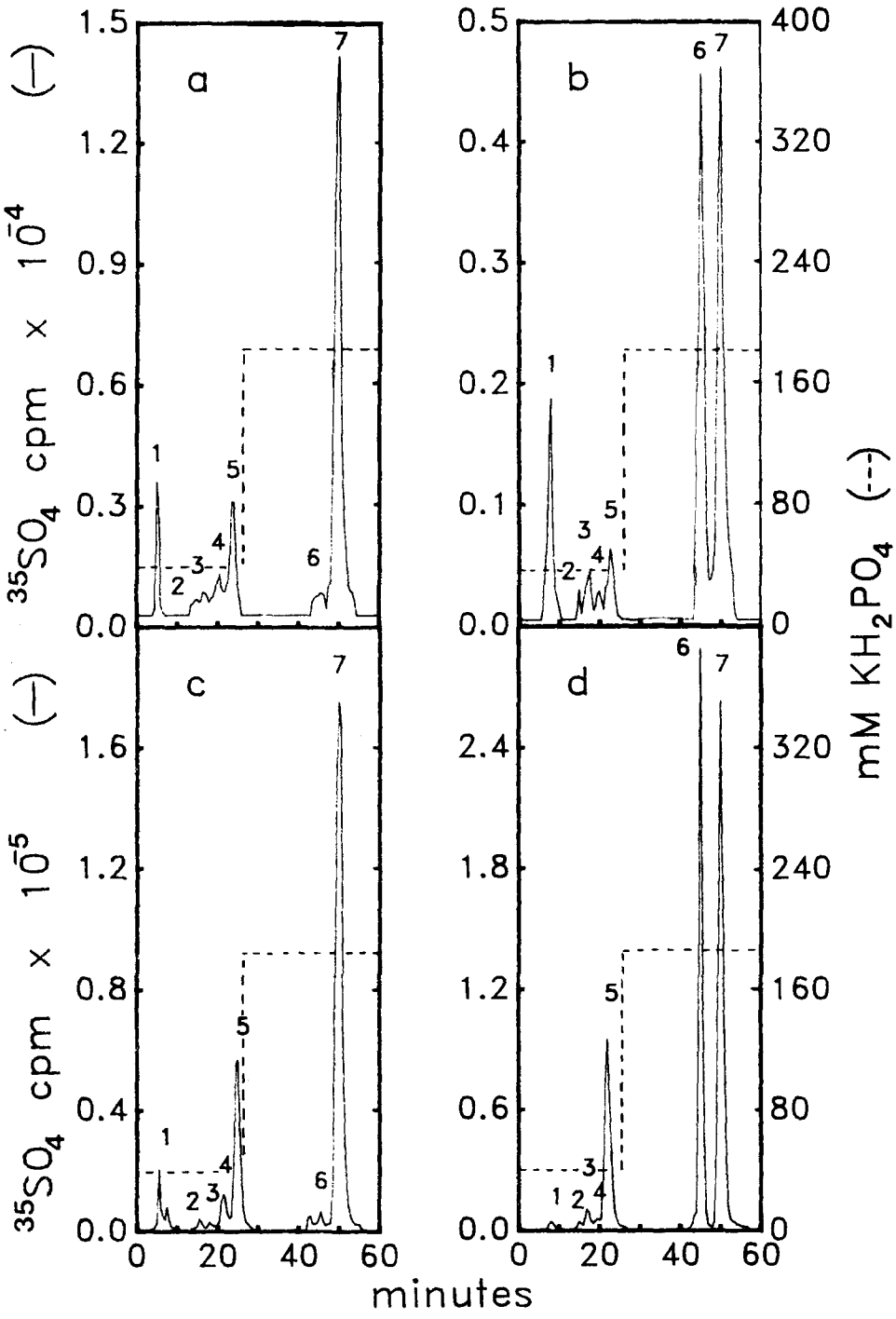

Figure 5. HPLC of disaccharide fractions obtained by cleavage of heparan sulfate from the CP (*a* and *c*) and NUC (*b* and *d*) pools with nitrous acid. Heparan sulfate was isolated from log phase (*a* and *b*) and confluent (*c* and *d*) hepatocytes. Peaks 1–7 were identified by comparison of their retention times with those of standards (3) as $AMan_R(6\text{-}SO_4)$, $GlcUA(2\text{-}SO_4)\rightarrow AMan_R$, $GlcUA\rightarrow AMan_R(6\text{-}SO4)$, $IdoUA\rightarrow AMan_R(6\text{-}SO4)$, $IdoUA(2\text{-}SO_4)\rightarrow AMan_R$, $GlcUA(2\text{-}SO_4)\rightarrow AMan_R(6\text{-}SO_4)$, and $IdoUA(2\text{-}SO_4)\rightarrow AMan_R(6\text{-}SO_4)$, respectively. The total cpm in each peak was used to calculate the nanomoles of disaccharide in each fraction (Table VI).

hexasaccharide groups in the different pools. Consistent with the observed increase in total HS in confluent cultures (Table I), the amounts of each oligosaccharide type were much higher in the pools obtained from confluent cells. The largest changes in oligosaccharide content with cell density were observed for the CP pool.

HPLC Analysis of the Oligosaccharide Composition of the Heparan Sulfate Pools

The disaccharides and tetrasaccharides were analyzed by HPLC. Figs. 5 and 6 show the elution profiles of the disaccharides and tetrasaccharides, respectively, of the HS from the CP and NUC pools from growing and confluent cells. From these profiles it is seen (*a*) that there are striking differences in the oligosaccharide compositions of the HS from these pools and (*b*) that the oligosaccharide compositions of the HS from these pools change with cell density. The results of the HPLC analyses of the oligosaccharides from all of the HS pools, summarized in Table VI, show that each HS pool is structurally different from the other HS pools, and that the structure of the HS in each pool changes with cell density. These differences are summarized in Fig. 7, where the disaccharide content of each HS pool is expressed as the mol percent of total disaccharides in the HS pool.

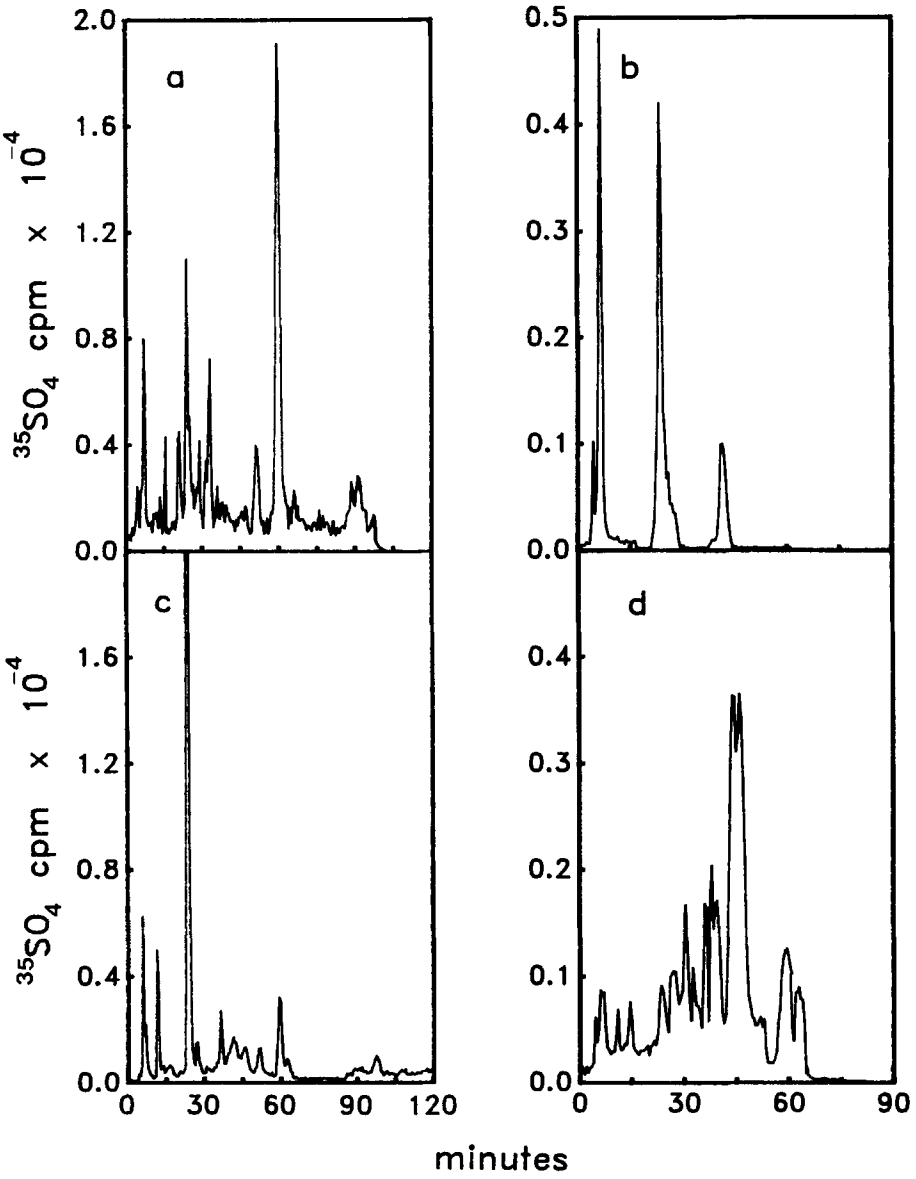

Figure 6. HPLC of tetrasaccharide fractions obtained by cleavage of heparan sulfate from the CP (*a* and *c*) and NUC (*b* and *d*) pools with nitrous acid. Heparan sulfate was isolated from log-phase (*a* and *b*) and confluent (*c* and *d*) hepatocytes. Peaks were identified by comparison of their retention times with those of standards (3) (see Table VI). The total cpm in each peak was used to calculate the nanomoles of disaccharide in each fraction (Table VI).

Discussion

The core protein of HSPG is synthesized in the rough endoplasmic reticulum and transported to the Golgi region where the sulfated polysaccharide chains are attached prior to secretion of the proteoglycan from the cell (69). In cultured hepatocytes, the Ma pool, but not the CM pool, is endocytosed and catabolized in the lysosomes (2). Catabolism of the HS is initiated by the action of an endo-β-glucuronidase. Each cleavage of the HS chain by this enzyme yields one fragment having a GlcUA residue on its reducing terminal and a second fragment which must have a nonreducing terminal GlcN residue (35, 43, 60, 62, 78). The specificity of the enzyme has not been established in terms of the N- and O-substituents on the newly formed nonreducing terminal GlcN residue. The intracellular pool of HS consists of a mixture of the intermediates in the biosynthesis of HSPG and the intermediates in the catabolism of HS. Consistent with these conclusions, it has been demonstrated that a polyclonal antibody directed against the core protein of HSPG binds to the sinusoidal plasmalemmal domain on the surface of hepatocytes, to the cisternae of the rough endoplasmic reticulum, to the Golgi stack, and to endosomes and lysosomes (75). The occurrence of HS (4, 56) and of chondroitin sulfate (4, 22, 56, 74), hyaluronic acid (22, 23, 55, 73), and other uncharacterized glycosaminoglycans (21, 57) in the nucleus has been reported previously. The antibody against the HSPG core protein did not detect nuclear HSPG (75). However, the nuclear HS observed in the present study contained little if any core protein and therefore could not have been detected by antibody binding. In none of the previous cases where HS has been found in isolated nuclei has the HS structure been characterized.

Because the $^{35}SO_4$ substituents of HS are concentrated in blocks of sulfated disaccharides which are interspersed in the polymer with blocks of unsulfated disaccharides (13, 48, 73), the structural analysis of the $^{35}SO_4$-labeled oligosaccharides formed by nitrous acid cleavage of HS gives no indication of lengths of the nonsulfated blocks of the HS chains or the relative proportions of sulfated and nonsulfated blocks in the HS. However, the present study has shown significant structural differences in the sulfated blocks of the HS in the

Table VI. Oligosaccharide Composition of Pools

Oligosaccharide	Growth stage*	Cellular pool				
		CM	Ma	CP	NM	NUC
		pmol/10⁶ cells‡				
Disaccharides						
GlcUA(2-SO₄)→AMan$_R$	L	0.64	1.70	0.50	0.03	0.04
	C	0.40	0.80	3.20	0.11	0.17
GlcUA→AMan$_R$(6-SO₄)	L	1.10	1.40	0.70	0.07	0.12
	C	2.30	3.80	2.50	0.23	0.58
IdoUA→AMan$_R$(6-SO₄)	L	3.20	2.50	1.30	0.07	0.08
	C	5.60	2.80	12.00	0.37	0.20
IdoUA(2-SO₄)→AMan$_R$	L	3.40	12.00	2.60	0.13	0.23
	C	12.00	23.00	49.00	1.80	3.70
GlcUA(2-SO₄)→AMan$_R$(6-SO₄)	L	0.00	1.70	0.40	0.90	1.10
	C	0.80	1.80	2.80	3.60	3.60
IdoUA(2-SO₄)→AMan$_R$(6-SO₄)	L	11.00	13.00	5.90	1.20	1.50
	C	8.90	29.0	92.00	2.50	2.40
Tetrasaccharides§						
Unknown	L	0.7	0.4	1.1	0.4	0.1
	C	1.2	1.9	33.0	1.0	0.3
t3	L	0.7		0.9		
	C	0.3	0.1	0.8	0.5	0.4
t4	L	0.7		0.2	0.1	0.1
	C	0.1	0.8	3.0	0.2	0.3
t5	L	0.8	0.2	0.3	0.0	0.0
	C	0.3	1.2	4.8	0.3	1.1
t6	L	0.6		0.2		
	C	0.5	0.3	1.4	0.1	0.6
t7	L	0.25		0.15	0.1	
	C	0.1	0.15	1.0	0.1	0.6
t8	L	0.5	0.15	4.3		
	C	0.2	0.2	1.6		0.1
t9	L			0.17		0.03
	C	0.07	0.03	0.17		0.2
t10	L			0.4		
	C	0.07	0.1	0.5		
t14	L			0.4		
	C	0.1	0.03	0.6		

* See Table I, footnote 1.

‡ See Table V, footnote 3.

§ The structures of the tetrasaccharides are given in reference 3. The structure of the unknown tetrasaccharide, which is the major tetrasaccharide peak in the CP pool from the confluent cells, has not been established.

different pools as indicated by the marked differences in the profiles of both the sulfated disaccharides and the sulfated tetrasaccharides. The NUC and NM pools, which gave very similar profiles, contain the most unique HS structures. In fact, the unusual structural features of the nuclear HS offer strong support for the conclusion from the data in Table III that the HS found in the nuclear fractions does not result from contamination of the nuclei with HS from any of the other cellular pools. When the nuclei were isolated using a non-aqueous method, very little HS was found in the NM pool but there was a proportionate increase in the amount of HS in the NUC pool. Thus, the NM pool obtained when nuclei are isolated using aqueous procedures may be formed by leakage of the HS from the nuclear pool into the nuclear membrane. However, leakage into other cellular pools does not seem to occur since HS with the structural features of that in the NM and NUC pool is not found in the other pools. The loss of proteins from the nucleus when nuclei are isolated by aqueous methods is well documented (63).

The formation of large amounts of GlcUA(2-SO₄)→AMan$_R$(6-SO₄) from the nuclear HS is of special note. Studies on the structure and the biosynthesis of heparin have indicated that IdoUA is the only uronic acid in heparin that is sulfated. Recently, however, both GlcUA(2-SO₄)→AMan$_R$(6-SO₄) and GlcUA(2-SO₄)→AMan$_R$ have been found among the deamination products of heparin, albeit at levels which are <1% of the total disaccharides formed (3). Thus, the high proportion of GlcUA(2-SO₄)→AMan$_R$(6-SO₄) recovered from the nuclear HS reflects a very unusual species of HS. Recently, a sulfatase which specifically cleaves the sulfate group from GlcUA(2-SO₄) residues in glycosaminoglycans has been observed in both human skin fibroblasts and chick embryo chondrocytes (71). Curiously, the GlcUA(2-SO₄) sulfatase activities in these cell extracts were much higher than the IdoUA(2-SO₄) sulfatase activities in the same extracts. This suggested that 2-o-sulfated GlcUA residues in glycosaminoglycans might be of greater significance than previously believed. The novel presence of such a high proportion of GlcUA(2-SO₄) in nuclear HS may be the primary reason for the presence of high levels of this sulfatase.

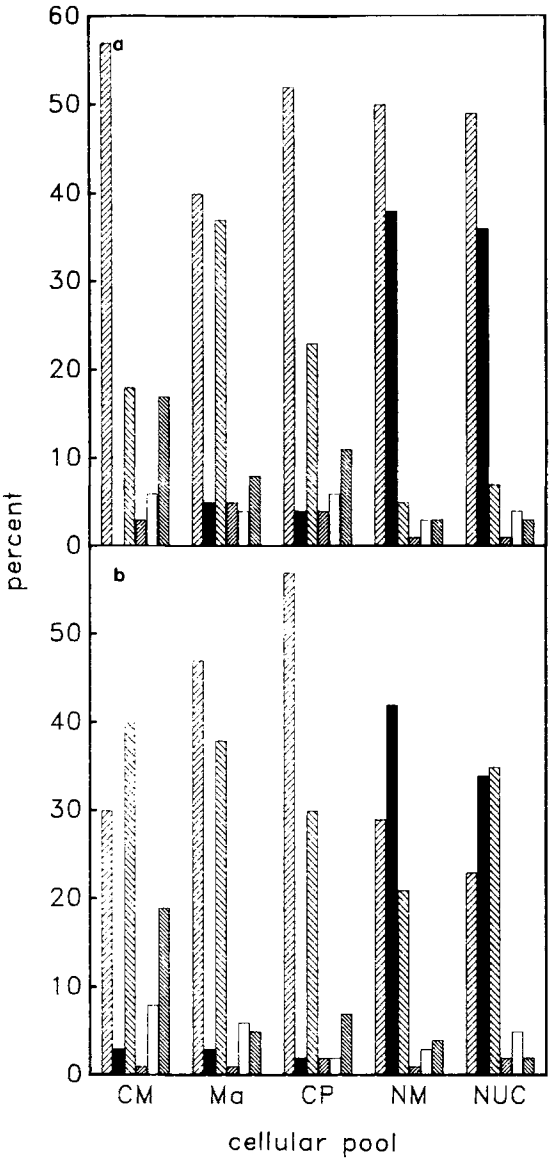

Figure 7. Distribution of disaccharides in cellular pools of log-phase (*a*) and confluent (*b*) hepatocytes. The six bars in each group of bar graphs are, from left to right, IdoUA(2-SO_4)→$AMan_R$(6-SO_4), GlcUA(2-SO_4)→$AMan_R$(6-SO_4), IdoUA(2-SO_4)→$AMan_R$, GlcUA(2-SO_4)→$AMan_R$, GlcUA→$AMan_R$(6-SO4), and IdoUA→$AMan_R$(6-SO4). Each bar is plotted as mol percent of the total disaccharides in the cellular pool.

Another important aspect of the present findings is the observation that both the amounts and the structural features of the HS in all of the pools change with cell density. Table I shows the marked elevation in the amount of $^{35}SO_4^{2-}$ incorporated into all of the pools of the confluent cells. The chromatographic properties of the HS chains (Fig. 1 and Table IV) reflect a higher degree of sulfation in the HS from confluent cultures than in that from the growing cells, presumably because these HS chains contain longer sequences of sulfated disaccharides and shorter sequences of unsulfated disaccharides than the HS from the dividing cells. An examination of the disaccharide and tetrasaccharide compositions of the HS from growing and confluent cells shows that there is also a qualitative difference in the structures of the HS chains with changes in cell density. For the NUC pool, this difference is reflected by an increased percentage of IdoUA(2-SO_4)→$AMan_R$ in the HS fragments in the confluent cultures and a decreased percentage of IdoUA(2-SO_4)→$AMan_R$(6-SO_4), with the percentage of GlcUA(2-SO_4)→$AMan_R$(6-SO_4) remaining the same (Fig. 7).

For the most part, the physiological functions that have been attributed to HS are those that are appropriate for structures that are primarily a part of the surfaces of eukaryotic cells. HS appears to play roles in (*a*) binding of cells to each other in vivo and to their growth substratum in cell culture (15, 47), (*b*) binding of lipoprotein lipase to endothelial cells (11), (*c*) preventing blood coagulation in capillary endothelial cells (10, 53–55), (*d*) filtration in glomerular basement membranes (38), and (*e*) controlling metastasis (58). However, a number of recent observations have suggested that HS may play other, equally important roles in controlling cell growth and activity. These roles include control of the growth (8–10, 20, 26, 31, 36, 52), migration (52), and activities (50, 51) of smooth muscle cells, stimulation of the growth of vascular endothelial cells (49, 76), control of angiogenesis (25, 27), neutralization of the effects of prostacyclin on platelet aggregation (70), and release of growth factors from endothelial cells (49). Most notable is the ability of heparin and HS to inhibit the proliferation of vascular smooth muscle cells (8–10, 20, 31, 36), an effect which may be mediated via interactions of cell surface structures either with heparin (9, 31, 36) or a unique species of HS derived from either endothelial cells (8, 10) or smooth muscle cells (20). Also, Majack and Bornstein have shown that heparin can modulate the secretory phenotype of vascular smooth muscle cells (50) and induce the synthesis of a unique low molecular weight collagen in these cells (51). The mechanisms of action of heparin and HS on smooth muscle cells are unknown. However, it is of note that only HS from confluent endothelial or smooth muscle cells will inhibit smooth muscle cell growth (10, 20). This implies that the HS secreted by these confluent cultures is a form that is structurally different from that synthesized by growing cells, just as observed for all of the cellular pools described here.

The origin of the free HS chains in the nucleus has not been established. The unique nuclear HS sequences may be synthesized as a product distinct from the bulk of the HS made by the cell, or these sequences may be formed as segments of larger HS chains. In either case, this HS presumably must be attached to the core protein during the biosynthesis in the Golgi region and then must be cleaved from the core protein before entering the nucleus. Several pathways from the Golgi region to the nucleus are possible. The HS may be cleaved during direct passage from the Golgi region to the nucleus. On the other hand, if it is formed as a segment of larger HS chains, it may first be secreted into the Ma pool (a pool that contains low but significant amounts of the GlcUA[2-SO_4] residues—Fig. 7) from which it is rapidly endocytosed, spliced out of the longer chain, and delivered to the nucleus. Since the initial step in HS catabolism involves cleavage of the chains by the endo-β-glucuronidase (35, 43, 60, 61, 78), it may be that 2-O-sulfation of the GlcUA residues prevents the action of this enzyme, thus, preserving sequences which contain GlcUA(2-SO_4) residues. It is also possible that a cell surface receptor for the HS could bind and protect the unique segments destined for the nucleus from catabolic

enzymes, and serve as the carrier to transport the HS segments into the nucleus. The nuclear HS offers obvious unique structural features that might be involved in binding to a specific carrier protein for transport into the nucleus, including both the nonreducing terminal GlcN residue formed by the action of the endo-β-glucuronidase and the GlcUA(2-SO_4) residues. Since the Ma pool is much larger than the NUC + NM pools, the differences in the overall structures of the HS in the Ma pool and the NUC pool do not preclude the possibility that the HS in the Ma pool contains short sequences like those found in the nucleus. Consistent with this possibility, Radoff and Danishefsky (65) have shown that GlcUA and IdoUA are not evenly distributed along the chains of anticoagulantly active heparin. We have observed recently that, under labeling conditions used here, HS does not appear in the nucleus until 2 h after hepatocytes are exposed to $^{35}SO_4^{2-}$, long after both newly synthesized HS and endocytosed HS appear in the CP pool (Ishihara, M., and H. E. Conrad, unpublished results). This observation is consistent with the possibility that the nuclear HS is formed as a unique catabolite of the HSPG endocytosed from the Ma pool. It also demonstrates further that unlabeled nuclei can be recovered from cells which have accumulated significant levels of labeled HS in the CP pool (2).

The nuclear localization of an HS with a structure totally different from those reported previously, the changes in rates of HSPG synthesis at confluence, the rapid turnover of HSPG in cells, and the structural variability of HS suggests that this HS species may play some unique role in regulation of cellular activities. Furthermore, HS appears to be ubiquitous in the animal kingdom (7, 30). The rapid secretion, endocytosis, and catabolism of HSPG has been observed for a number of different cell types and appears to represent the general metabolic behavior for HSPGs in all animal cells (2, 28, 64, 77). At present, no physiological mechanisms by which HS may regulate cellular functions are known. However, in nonphysiological experiments, it has been shown that heparin, when added to isolated nuclei, stimulates both DNA replication and transcription (1, 19, 24, 44, 45), apparently by interacting with the histones and causing a physical swelling of the chromosomes and exposure of the DNA to act as template (6, 18, 46). On the other hand, when these activities are assayed using a naked DNA template, heparin inhibits replication and transcription, apparently by interacting with the enzymes involved in these processes. Heparin also affects the activities of a number of other enzymes found in the nucleus, including cAMP-dependent protein kinase N II (32), topoisomerase I (37), and others, as well as some that are found in the cytoplasm of cells (12, 33). Thus, the potential for heparin-like structures to play a regulatory role in the activities of cells has been amply demonstrated. However, in none of these cases have the in vitro effects of heparin and HS been equated with a physiological role for these polymers.

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