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Heparan Sulfates of Cultured Cells. I. Membrane-Associated and Cell-Sap Species in Chinese Hamster Cells*

Paul M. Kraemer

ABSTRACT: Heparan sulfate has been isolated and identified from three cellular fractions of Chinese hamster cells (line CHO) grown in suspension culture: as a cell surface component removable with trypsin under conditions preventing irreversible cell damage; as a free, directly acid-soluble component of the "cell-sap"; and as a part of the residual acid precipitate that became acid soluble following papain digestion. Heparan sulfate from the latter cell fraction could be divided into two portions: a minor portion that labeled rapidly and a major portion that labeled slowly. These two forms also differed slightly in apparent molecular size and charge.

All four forms of cellular heparan sulfate were identified by cellulose acetate electrophoresis, by depolymerization by direct nitrous acid treatment, and by comparisons with authentic bovine heparan sulfate. The isolated forms differed in both *N*- and *O*-sulfate composition and may differ also in residual peptide content. It is proposed that the various forms represent different maturation and/or storage forms of a single metabolic system.

We have previously reported (Kraemer, 1968) the isolation of a glycosaminoglycan with the characteristics of heparan sulfate from Chinese hamster cells grown in suspension culture. The same glycosaminoglycan material was isolated quantitatively and qualitatively from either the direct trichloroacetic acid soluble fraction of whole cells or the trichloroacetic acid soluble fraction of the "cell-sap" (*i.e.*, supernatant fluid of freeze–thaw broken cells after 105,000*g*, 60 min). The glycosaminoglycan was identified as heparan sulfate on the basis of sugar composition, presence of *N*-sulfate groups, resistance to degradation by testicular hyaluroni-

* From the Biomedical Research Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87544. *Received May 21, 1970.* This work was performed under the auspices of the U. S. Atomic Energy Commission.

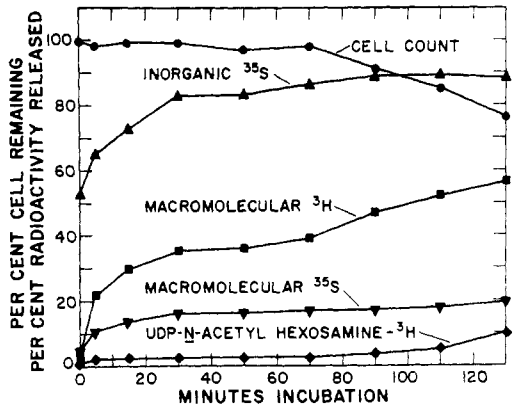

FIGURE 1: Release of radioactivity from cells during cell stripping with 0.1 mg/ml of crystalline trypsin in BSS, pH 7.2, 37°. Cells (4.8×10^8) labeled for one generation with glucosamine-6-*t* and $Na_2{}^{35}SO_4$ were harvested, washed, and resuspended in 200 ml of BSS plus 0.01 mg/ml of DNase at *t* = minus 5 min. At time 0 trypsin was added, and at the indicated times aliquots were removed and mixed with 0.2 mg/ml of soybean trypsin inhibitor. Suspension supernatant fluids were subjected to Bio-Gel P-2 chromatography and the data expressed in terms of the initial values for papain-digested, washed, prelabeled cells: 100% macromolecular-*t* = 45,000 cpm/aliquot; macromolecular-^{35}S = 2400 cpm/aliquot; UDP-*N*-acetylhexosamine = 70,000 cpm/aliquot; and inorganic ^{35}S = 350 cpm/aliquot.

dase, and enhanced degradation with heparin-grown *Flavobacterium heparinum*.

The present report concerns further studies of the properties of the directly trichloroacetic acid soluble heparan sulfate and also structurally distinct varieties of heparan sulfate associated with the cell surface membrane and with internal cellular particulate material. Further evidence of the identification of these heparan sulfates is provided, particularly as a result of studies of direct nitrous acid cleavage products and cellulose acetate electrophoresis. In addition, general procedures for the isolation from cultured cells of small amounts of heparan sulfate in the presence of much larger quantities of other complex carbohydrates are reported.

Materials and Methods

CHO cells were grown in F-10 medium in suspension culture as previously described (Kraemer, 1968). Periodic checks for mycoplasmal contamination using the House and Waddell (1967) modification of the method of Chanock *et al.* (1962) were negative. Radioactive glucosamine (-6-*t*, sp act. (Ci/mole) 1150; -1-^{14}C, sp act. 51, New England Nuclear) and $Na_2{}^{35}SO_4$ (about 300, New England Nuclear) were added when the cell count was about 300,000/ml, and the cells were harvested and washed with balanced salt solution (BSS) 21 hr later when the cell count had reached about 600,000/ml.

Gel filtration with Bio-Gel P-2 (100–200 mesh) or Bio-Gel P-10 (200–400 mesh) was done with 1.2 × 140 cm columns equilibrated and eluted with 0.1 M ammonium acetate, pH ~ 7.0. Bio-Gel P-2 runs were made at a flow rate of ~24 ml/hr and Bio-Gel P-10 at 16 ml/hr; in both cases 2-ml fractions were collected. DEAE-cellulose (Whatman DE52 microgranular, preswollen) was precycled and preequilibrated with 0.01 M ammonium acetate, then loaded into 0.8 × 40 cm columns. Samples were eluted with a linear salt gradient, 0.01–2.0 M ammonium acetate, in a total of 400 ml. Three-milliliter fractions were collected at a flow rate of 24 ml/hr. Aliquots from chromatography fractions were counted in Instagel (Packard) by liquid scintillation methods; both ^{3}H and ^{35}S counting efficiencies were usually near 26% with ^{35}S to ^{3}H channel crossover between 0.30 and 0.40.

Enzyme sources and incubation conditions were as previously described (Kraemer, 1968). Bovine heparan sulfate was a gift from Dr. J. A. Cifonelli and had the following composition: uronic acid, 36.9%; glucosamine, 21.6%; sulfate to hexosamine ratio 1.0 with equal concentrations of *N*-sulfate and *O*-sulfate (personal communication, J. A. Cifonelli). Heparin, hyaluronic acid, and chondroitin sulfate (mixed) were purchased from Calbiochem.

Uronic acid assays were done by the orcinol method of Mejbaum (1939); nitrous acid degradations were done as follows: the lyophilized sample was dissolved in 0.8 ml of water, then 0.1 ml of glacial acetic acid and 0.1 ml of 18% ammonium nitrite were added, mixed, stoppered, and allowed to remain at room temperature for 80 min. The sample was then frozen and relyophilized.

Cellulose acetate electrophoresis, using Sepraphore III strips (Gelman), was done in 0.15 M pyridine–formic acid, pH 3.0 (Matalon and Dorfman, 1968), sodium barbital, pH 8.6, μ 0.1, and 0.1 M barium acetate (Wessler, 1968). Current was 1–2 mA per strip, and the time was 40 min. Strips were stained with toluidine blue (0.05% in 65% ethanol) or Alcian blue (0.05% in 95% ethanol).

Preparation of Surface, Cell-Sap, and Residual Acid-Precipitable Fractions from Cells Grown with Glucosamine-6-t and $Na_2{}^{35}SO_4$. Cells were harvested by centrifugation following 21 hr of growth in medium containing 0.1 mCi/l. of glucosamine-6-*t* and 1 mCi/l. of $Na_2{}^{35}SO_4$. The cells were washed three times with cold Earle's balanced salt solution (BSS), then resuspended in BSS containing 0.1 mg/ml of crystalline trypsin and 0.01 mg/ml of DNase, and incubated at 37° for 35 min with occasional agitation. Both trypan blue impermeability (a crude measure of cell viability) and cell number remained essentially constant during the trypsin–DNase treatment. Following this treatment, the cells were pelleted, the trypsin supernatant was harvested, and the cell pellet was resuspended in cold water to which 40% trichloroacetic acid was added to a final concentration of 7%. This preparation was kept in an ice bath for 30 min, then centrifuged to pellet the precipitate, and the trichloroacetic acid soluble and precipitable fractions were separated. In the meantime, incubation at 37° of the trypsin supernatant was continued, and in some cases activated papain was added. After 2 hr, this supernatant was chilled, trichloroacetic acid was added to 7%, and after 30 min in ice, the trichloroacetic acid soluble portion of this fraction was harvested.

The trichloroacetic acid precipitate of the cell pellet was resuspended in 0.1 M ammonium acetate, digested with 1 mg/ml of activated papain for 16 hr at 56°, then chilled, and reprecipitated with 7% cold trichloroacetic acid. The trichloroacetic acid soluble portion of the papain-digested trichloroacetic acid precipitate was harvested. In each case, trichloroacetic acid soluble fractions were extracted with ether 3 times, residual ether was removed with a nitrogen stream, and the fraction was lyophilized.

Results

Removal of Cell Surface Materials with Trypsin. When cultured cells are treated with proteolytic enzymes under conditions where no irreversible cell damage occurs (Kraemer, 1967), materials are released into the supernatant which

appear to represent, in part, cell surface macromolecules. A kinetic study of this process of cell surface "stripping," using cells that had been doubly labeled with glucosamine-*6-t* and $Na_2{}^{35}SO_4$, is illustrated in Figure 1. The process can be divided into two phases: an early phase, during which the cell number remains stable, and a later phase, during which gradual and progressive destruction of cells occurs. As illustrated, analysis of the radioactivity released during the early phase showed that discrete portions of the total cellular macromolecular ^3H and ^{35}S were released, achieving plateau values of 36 and 17%, respectively, while only a minuscule portion of the total UDP-*N*-acetylhexosamine leached out of the cells. When such cells were returned to growth medium following this degree of treatment, exponential growth resumed after a lag of about 2 hr. Continued treatment beyond this early phase resulted in progressive loss of viability. For the purposes of this paper, preparations designated as surface materials refer to materials released during the early phase (35 min) of trypsin treatment.

Distribution of Radioactivity in Surface, Cell-Sap, and Residual Trichloroacetic Acid Precipitable Cell Fractions. In most experiments, three trichloroacetic acid soluble fractions were isolated from doubly labeled cells: trypsin supernatant from cells treated under conditions where no irreversible cell damage occurred (Kraemer, 1967), referred to as surface material; direct trichloroacetic acid soluble material from the cells previously "stripped" by trypsin, referred to as cell-sap material; and residual trichloroacetic acid precipitable material rendered trichloroacetic acid soluble by papain digestion. Together, the three fractions included between 95 and 100% of both ^3H and ^{35}S radioactivity of the washed cells. Designation of the direct trichloroacetic acid soluble material as cell-sap refers only to the free cytoplasmic heparan sulfate (obviously, many other cell-sap molecules are precipitated by trichloroacetic acid) and is based upon previous work (Kraemer, 1968) which showed that direct trichloroacetic acid treatment of whole cells yielded an identical amount of soluble heparan sulfate as trichloroacetic acid treatment of just the cell-sap fraction of whole cells.

The three cell fractions were submitted to Bio-Gel P-2 molecular sieve chromatography to permit an initial separation of macromolecular and low molecular weight acid-soluble materials. Such runs are illustrated in Figure 2 and show that radioactivity identifiable as both ^3H and ^{35}S eluted with the void volume in each cell fraction. Additional material containing ^3H and ^{35}S was retained on the columns in the case of surface and cell-sap fractions, but almost all of the radioactivity of the residual trichloroacetic acid precipitate appeared to be macromolecular.

If the trypsin stripping step was omitted and only two cell fractions were prepared (namely, direct trichloroacetic acid soluble and trichloroacetic acid soluble of papain-digested trichloroacetic acid precipitate), nevertheless, the radioactivity of the trichloroacetic acid precipitate was almost entirely macromolecular. In this case, the low molecular weight radioactive materials that had been found in the surface fraction added to those of the cell-sap, while the Bio-Gel P-2 excluded material of the surface fraction added to the trichloroacetic acid precipitate. The large middle ^3H peak, as illustrated in Figure 1B, has previously (Kraemer, 1968) been demonstrated to represent UDP-*N*-acetylhexosamine; the large retained ^{35}S peak in Figure 1A represents inorganic sulfate. Thus, the trypsin treatment, while not irreversibly damaging the cells, resulted in loss of the bulk of the intracellular inorganic ^{35}S, but only minor leaching out of the nucleotide amino sugars

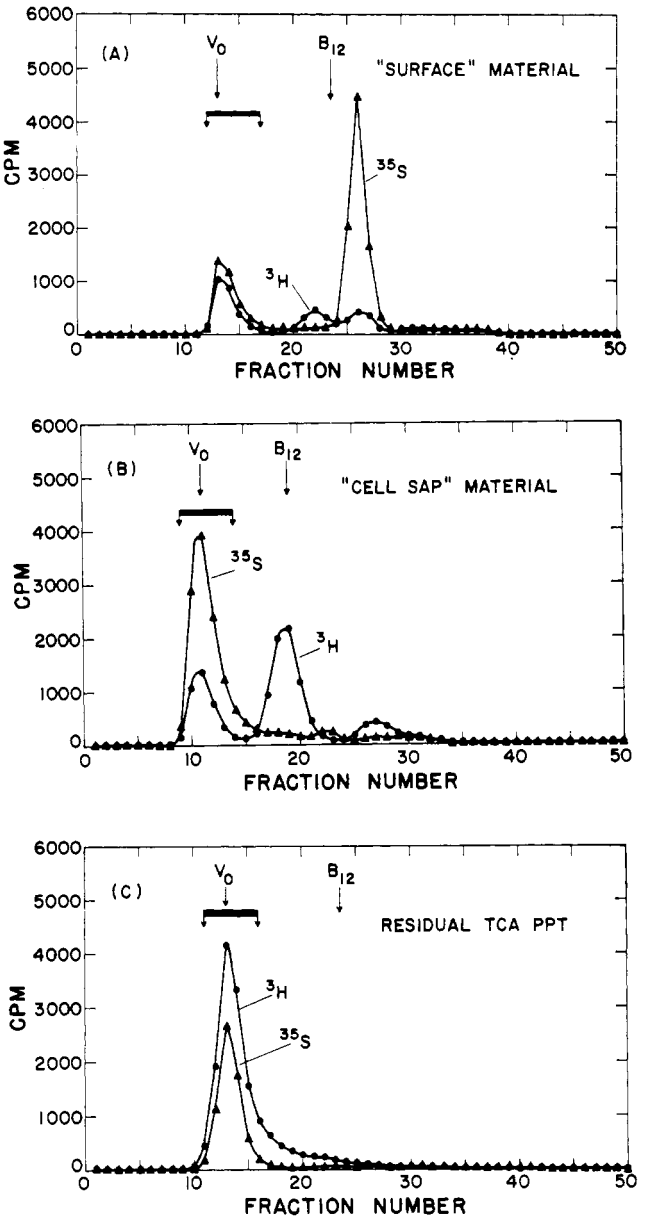

FIGURE 2: Bio-Gel P-2 chromatography profiles of the three acid-soluble cell fractions (see text for details) from 6×10^8 cells grown for one generation in medium with 0.1 μCi/ml of glucosamine-*6-t* and 1.0 μCi/ml of $Na_2{}^{35}SO_4$. Aliquots of 0.1 ml from each 2-ml fraction were counted; then the remainder of those fractions indicated by the bar were pooled and lyophilized.

and almost none of the cell-sap macromolecular material. The distribution of macromolecular radioactivity of the three cell fractions is summarized in Table I. Under these conditions of prolonged labeling, the ^3H distribution is a rough measure of all of the cellular complex carbohydrates, while the ^{35}S distribution represents only the sulfated species.

Isolation of Heparan Sulfate from the Macromolecular Constituents of Cell Fractions. Heparan sulfate was isolated from the material excluded from Bio-Gel P-2 by DEAE-cellulose chromatography. Figure 3 illustrates typical elution profiles of this type for the three cell fractions from a single experiment. As indicated previously (Kraemer, 1968), heparan sulfate constituted the bulk of the acid-soluble macromolecular material of the cell-sap that contains radioactivity from supplied glucosamine-*6-t* and inorganic $^{35}SO_4$ (Figure 3B). The

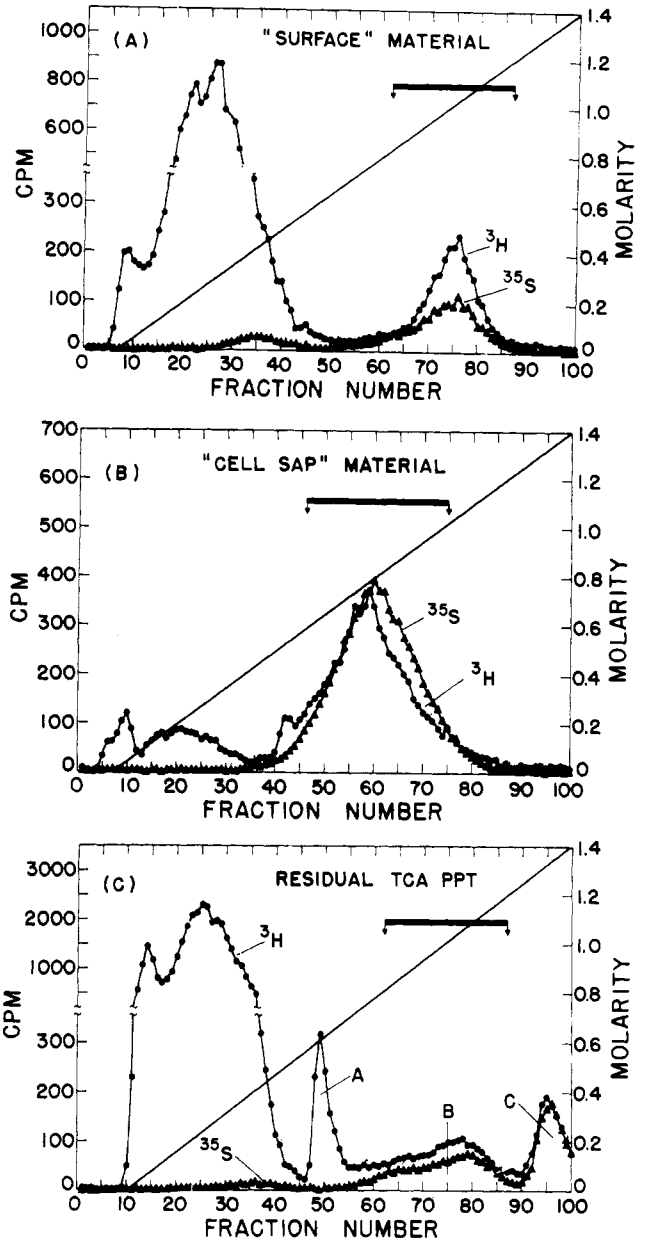

FIGURE 3: DEAE-cellulose chromatography profiles of material excluded from Bio-Gel P-2 and harvested as indicated in Figure 1. Aliquots (0.5 ml) of each 3-ml fraction were counted, and the remainder of fractions covered by the bar were pooled and lyophilized. The harvested material was identified as heparan sulfate.

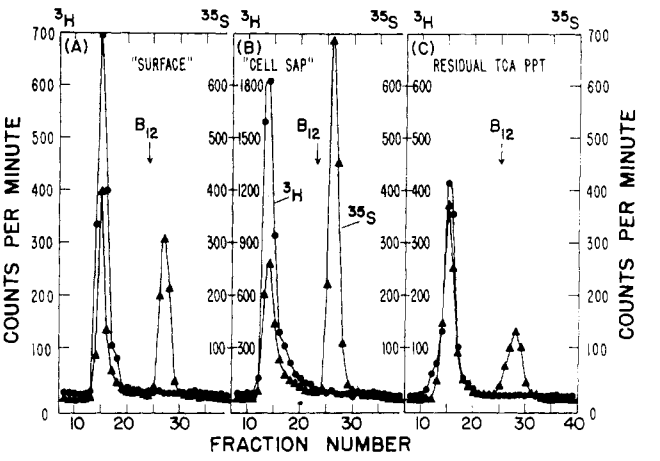

FIGURE 4: Bio-Gel P-2 chromatography profiles of heparan sulfates following treatment with 0.04 N HCl, 100°, 90 min (from an experiment similar to that illustrated in Figures 1 and 3). The material treated with mild acid was harvested from DEAE-cellulose in a similar fashion to that indicated in Figure 3.

doubly labeled heparan sulfate of this cell fraction eluted as a broad peak with 0.5 M to 1.1 M salt. A smaller portion of the ^3H radioactivity eluted with lower salt concentration and appears to be unrelated to the heparan sulfate. By contrast to the cell-sap fraction, material identified (see below) as heparan sulfate of the surface and residual trichloroacetic acid precipitable fractions constituted only a small portion of the total macromolecular ^3H radioactivity. This small portion was also a minor fraction of the total heparan sulfate of the cells. The major ^3H-labeled species of these cell fractions, eluting between 0.01 M and 0.5 M salt and possessing very little ^{35}S radioactivity, have been shown to consist of a large group of glycopeptides (Kraemer, 1969). Because of the predominance of these glycopeptides, the presence of heparan sulfate was not previously detected in these cell fractions (Kraemer, 1968). Nevertheless, in terms of total ^{35}S radioactivity, heparan sulfate-^{35}S constituted a major component. As shown in Figure 3A, the surface material included only a single, highly charged, doubly labeled peak which was identified as heparan sulfate (below). The residual trichloroacetic acid precipitable material included three highly charged macromolecular species, labeled A, B, and C in Figure 3C. Peak B was identified (below) as heparan sulfate. Peak A is evidently nonsulfated, was cleaved by testicular hyaluronidase, was completely excluded from Bio-Gel P-10, and was unaffected by direct nitrous acid treatment. Further studies of this material (which appears to be hyaluronic acid) will be reported separately. Peak C is highly sulfated but, like peak A, was cleaved by testicular hyaluronidase and was unaffected by direct nitrous acid treatment; it is undoubtedly chondroitin sulfate. Thus, all three cell fractions contained heparan sulfate, and further studies and identification were done with pooled fractions harvested as indicated in Figure 3.

Identification and Characterization of the Heparan Sulfates. Essentially all of the radioactivity, both ^3H and ^{35}S, harvested

TABLE 1: Incorporation of ^3H from Glucosamine-*6-t* and Inorganic $^{35}SO_4$ into Bio-Gel P-2 Excluded Material.[a]

	^3H		^{35}S		
Cell Fraction	cpm	%	cpm	%	$^{35}S/^3H$
Surface material	2,648	14	3,660	17	1.4
Cell-sap material	3,994	21	11,800	54	3.0
Residual trichloroacetic acid precipitate	12,304	65	6,501	29	0.5
Total	18,946	100	21,961	100	1.2

[a] Radioactivity incorporation data into Bio-Gel P-2 excluded material from cell fractions, prepared as described in the text, and representing approximately 3×10^7 cells after growth for one generation in medium containing 0.1 μCi/ml of glucosamine-*6-t* and 1.0 μCi/ml of $Na_2{}^{35}SO_4$.

TABLE II: Contribution to Total Macromolecular Radioactivity and Molecular Characteristics of Heparan Sulfates of Cell Fractions.[a]

	Surface		Cell-Sap		Residual Precipitate		Total	
	^3H	^{35}S	^3H	^{35}S	^3H	^{35}S	^3H	^{35}S
Total macromolecular cpm[b]	18,587	2,226	9,539	7,971	40,189	2,864	68,315	13,011
Heparan sulfate cpm[c]	2,880	1,619	7,098	7,626	2,124	1,381	12,102	10,626
Heparan sulfate cpm, % of total macromolecular cpm	15	73	74	96	5	48	18	82
Heparan sulfate distribution (% ^3H)	24		59		17		100	
Heparan sulfate, total sulfation ($^{35}S/^3H$)	0.56		1.07		0.65		0.88	
N-$^{35}SO_4/^3H$[d]	0.29		0.70		0.22			
O-$^{35}SO_4/^3H$[e]	0.27		0.37		0.43			

[a] Radioactivity data represent approximately 10^8 cells after growth for one generation in medium containing 0.1 μCi/ml of glucosamine-*6-t* and 1.0 μCi/ml of Na_2-$^{35}SO_4$. [b] Total Bio-Gel P-2 excluded material put on DEAE-cellulose column (Figure 3). [c] Material harvested as indicated in Figure 3. [d] Calculated from Figure 4, proportion of total incorporated macromolecular ^{35}S retained on Bio-Gel P-2 following treatment with 0.04 N HCl, 100°, 90 min, expressed as a subdivision of the overall $^{35}S/^3H$ ratio. [e] Same as *d* except that value refers to incorporated ^{35}S *not* released with mild acid.

as indicated in Figure 3 for each fraction, migrated as heparan sulfate on cellulose acetate electrophoresis. Aliquots of each fraction were run with known standards (hyaluronic acid, chondroitin sulfate, bovine heparan sulfate, heparin), and in each case runs were made with each of the three buffer systems described in Materials and Methods.

Table II presents data from the isolated heparan sulfate of the three cell fractions harvested as illustrated in Figure 3. In percentage and ratio terms, the same trends have been determined in three other similar experiments not illustrated. In terms of total cellular macromolecular radioactivity derived from supplied radioactive glucosamine, the heparan sulfate fractions have, in various experiments, accounted for 10–20% while, in terms of total incorporated ^{35}S, heparan sulfate is by far the predominant species in the CHO cell line (82% in Table II). About two-thirds of the total heparan sulfate (as estimated from ^3H radioactivity) of the cells has consistently been found in the direct trichloroacetic acid soluble or trichloroacetic acid soluble cell-sap fractions. The other third was variably distributed between surface and residual trichloroacetic acid precipitable cell fractions, generally about half and half.

The possibility that the heparan sulfate of the surface and residual trichloroacetic acid precipitable fractions represented contamination and/or leaching out of cell-sap material appears unlikely, since the three isolated heparan sulfates had quite different molecular characteristics. In every experiment done so far, the cell-sap heparan sulfate has had a higher $^{35}S/^3H$ than the other two varieties. Whether this indicates that the cell-sap heparan sulfate was more highly sulfated than the other varieties must await specific activity data; however, the other two heparan sulfates consistently eluted somewhat later in DEAE-cellulose salt-gradient chromatography, suggesting that they had a higher negative charge. Hence, it seems likely that other differences besides extent of sulfation existed between these varieties. One such possible difference, now under investigation, is the question of residual peptides on the molecules.

The highly characteristic lability of the *N*-sulfate groups of

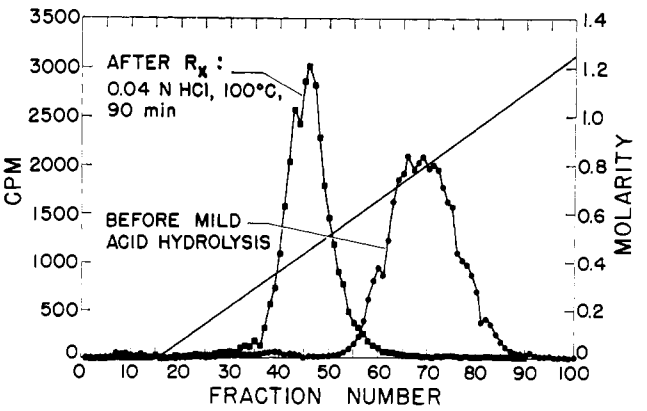

FIGURE 5: DEAE-cellulose chromatography of cell-sap heparan sulfate, before and after mild acid hydrolysis, from cells grown with glucosamine-*1*-^{14}C.

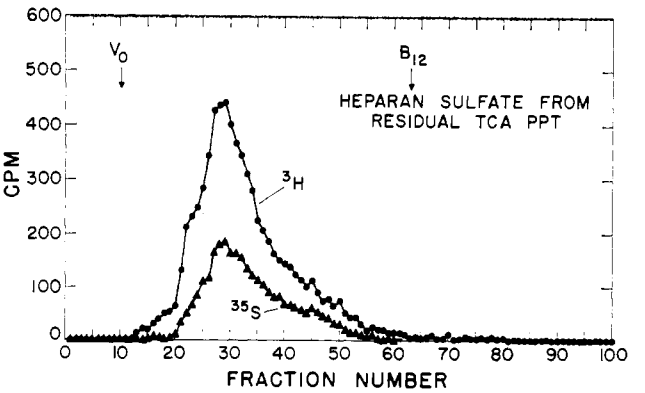

FIGURE 6: Bio-Gel P-10 chromatography profile of doubly labeled heparan sulfate from the residual trichloroacetic acid precipitate isolated in a manner similar to that illustrated in Figure 3C.

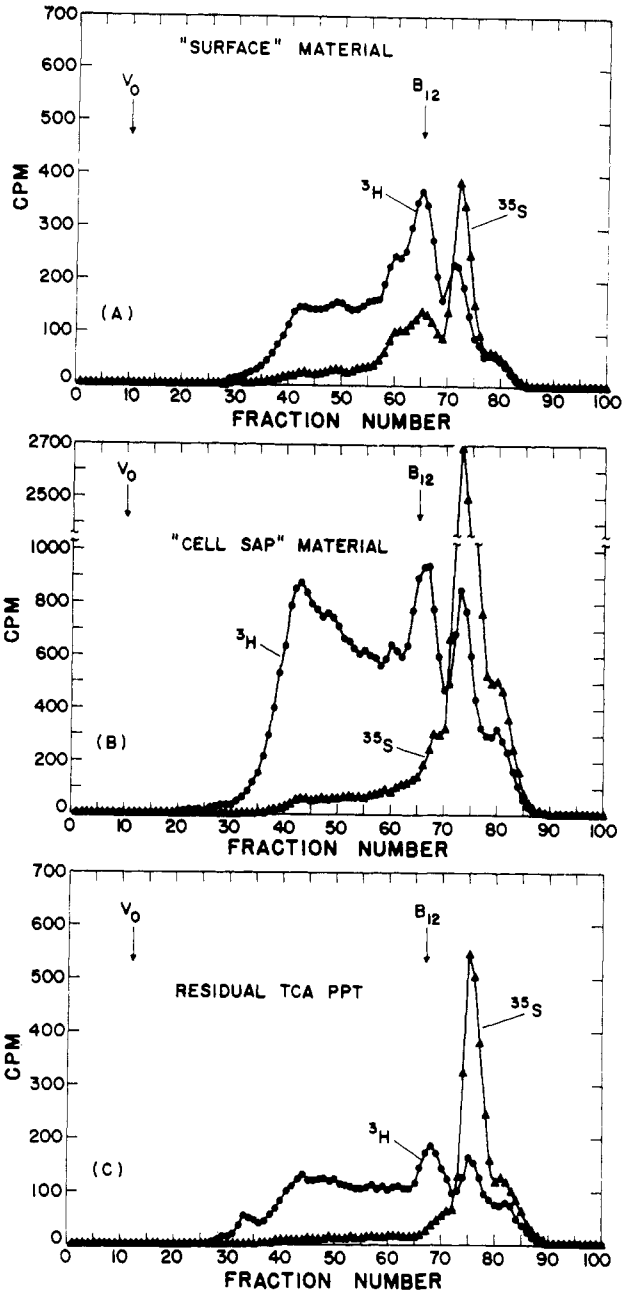

FIGURE 7: Bio-Gel P-10 chromatography profiles of doubly labeled heparan sulfates (harvested as indicated in Figure 3) after direct nitrous acid treatment.

heparin and heparan sulfate was employed to determine the presumptive ratio of *N*- and *O*-sulfates in the molecules. This was done (Figure 4) by determining the proportion of ^{35}S that was retained on a Bio-Gel P-2 column following treatment of the double-labeled heparan sulfate with 0.04 N HCl, 100°, 90 min. As illustrated in Figure 4, all of the ^3H radioactivity continued to elute as macromolecular material following mild acid hydrolysis. Such experiments have been done several times and have consistently shown that not only did the cell-sap heparan sulfate have a higher $^3H/^{35}S$ ratio than the membrane-associated species but that the higher ratio is largely accountable as increased proportion of labile *N*-sulfates.

The relationship of the *N*-sulfate groups to DEAE-cellulose chromatography behavior is suggested by the experiment illustrated in Figure 5. In this experiment, cell-sap heparan sulfate isolated from cells grown with glucosamine-*1*-^{14}C was run on DEAE-cellulose, harvested, treated by the very mild acid hydrolysis method to de-*N*-sulfate the molecules, then rerun on a similar DEAE-cellulose column. The results indicate that this treatment resulted in a loss of negative charge as well as decreased molecular heterogeneity. No obvious molecular size change was evident when such de-N-sulfated heparan sulfates were rerun on Bio-Gel P-10.

The molecular weight of the cell-sap heparan sulfate was previously (Kraemer, 1968) estimated to be about 10,000 on the basis of partial retention on Bio-Gel P-10 between the void volume and the vitamin B_{12} marker (mol wt 1357). Since the membrane-associated varieties reported here were the degradation products of proteolytic digestion and also had different DEAE-cellulose chromatography behavior, it seemed conceivable that their behavior on Bio-Gel P-10 might be different. This was not the case. Figure 6 illustrates the Bio-Gel P-10 elution profile of heparan sulfate from the residual trichloroacetic acid precipitate; all three varieties behaved similarly on Bio-Gel P-10, identically with that previously reported (Kraemer, 1968), and similar to bovine heparan sulfate with molecular weight 12,000. If the membrane-associated varieties do have a different amount of residual peptide, the difference is not distinguishable by this means.

Nitrous Acid Degradation of Isolated Heparan Sulfates. Deaminative cleavage by direct nitrous acid treatment is specific for N-sulfated glycosaminoglycans (Cifonelli, 1968a, b; Shively and Conrad, 1970) and was used in the present study to confirm the identification of the heparan sulfates as well as to examine the characteristics of the fragments. The products of direct nitrous acid treatment of the isolated heparan sulfates were examined by Bio-Gel P-10 chromatography as illustrated in Figure 7. Prior to treatment, all three behaved similarly to that illustrated in Figure 6.

It is apparent from the ^3H radioactivity profiles that all three varieties of heparan sulfate were cleaved to a mixture of smaller fragments. In itself, this result confirms the identity, as heparan sulfate, of these species. The largest cleavage products (*i.e.*, ^3H radioactivity eluting first) appear to overlap the elution region of the smallest untreated chains, raising the possibility that some of the smaller molecules were not cleaved at all. However, this seems unlikely on the following basis. If de-N-sulfated heparan sulfate (such as that illustrated in Figure 5) is treated with nitrous acid and then rerun on DEAE-cellulose, all of the glucosamine-derived radioactivity elutes slightly ahead. Thus, we interpret the ^3H radioactivity profiles of direct nitrous acid treated heparan sulfate on Bio-Gel P-10 as representing a spectrum of size of fragments where fragment size is inversely proportional to the frequency of *N*-sulfate groups in the original molecule. The ^3H radioactivity that eluted early in the Bio-Gel P-10 run, therefore, would represent extensive regions of the sugar chain lacking *N*-sulfate groups. By similar argument, the smaller fragments, eluting from just before vitamin B_{12} to the end, were smaller for the reason that the undegraded molecule possessed regions with frequent *N*-sulfate groups. Judging from the estimated molecular weight spectrum of the undegraded molecules and the elution position of the nitrous acid cleavage products, one would suppose that the fragments range from dissacharides to pieces possibly as long as 10 or 12 sugars. In fact, the fragment elution profiles do suggest that they represent a small finite number of discrete size classes, as expected, rather than a continuum of breakdown products.

Consideration of the ^{35}S radioactivity profiles of the nitrous acid treatment products (Figure 7) strongly suggests that the

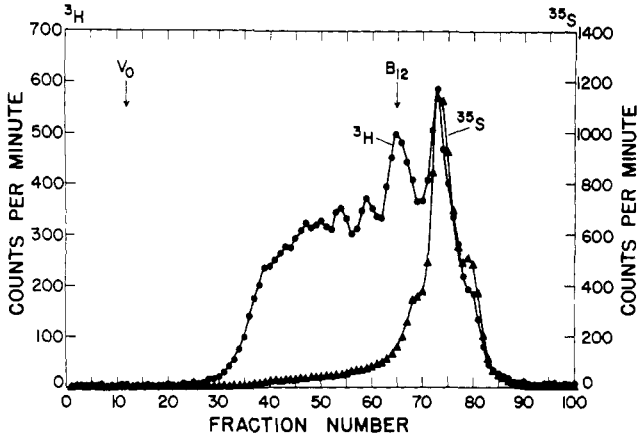

FIGURE 8: Bio-Gel P-10 chromatography profile of doubly labeled cell-sap heparan sulfate first de-N-sulfated as illustrated in Figure 4, then treated with nitrous acid.

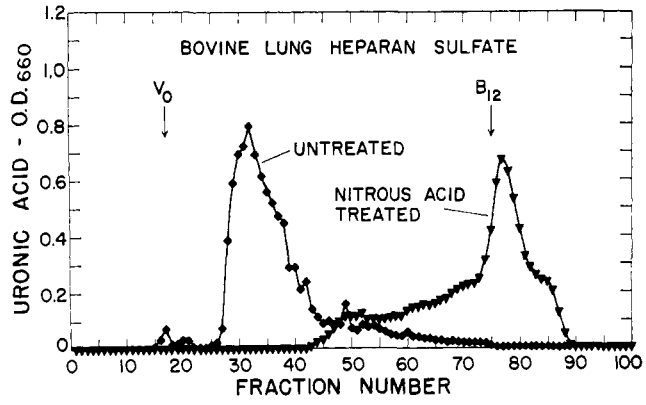

FIGURE 9: Bio-Gel P-10 chromatography profiles of 1-mg aliquots of authentic bovine heparan sulfate either untreated or direct nitrous acid treated. Each fraction (2-ml) was assayed for uronic acid by the orcinol method.

larger fragments possess less residual *O*-sulfates than the smaller ones. Since the larger fragments represent portions of the molecules that were sparse in *N*-sulfates, this suggests that the highest frequency of both *N*- and *O*-sulfates is in the same portion of the molecules. Detailed interpretation of the ^{35}S profiles in Figure 7, however, is complicated by the fact that inorganic sulfate (*e.g.*, $^{35}SO_4$ released from *N*-sulfates) elutes as if it were larger than it is, coeluting, in fact, with the next to the last ^3H peak. This inorganic sulfate could not be selectively removed with $BaCl_2$ (*i.e.*, could not be precipitated without concomitant loss of ^3H radioactivity); hence, further experiments were done in which the doubly labeled heparan sulfate was first de-N-sulfated (as illustrated in Figure 4), then treated with nitrous acid, and chromatographed on Bio-Gel P-10. Such a Bio-Gel P-10 profile for cell-sap heparan sulfate is illustrated in Figure 8; in this case, all of the ^{35}S radioactivity presumably represents residual *O*-sulfates attached to the cleavage products. (In the particular experiment illustrated, the material treated with HNO_2 was from fractions 12–20 of the Bio-Gel P-2 run illustrated in Figure 4B.) In general, depolymerization of various heparan sulfate preparations has been the same either following direct HNO_2 treatment or HNO_2 treatment of de-N-sulfated material, as indicated by the Bio-Gel P-10 elution profiles of radioactivity from precursor glucosamine-*6-t*. In addition, data such as illustrated in Figures 7 and 8, taken together, indicate that the molecules have particular regions of higher than average sulfate content and that such regions are high in both *O*- and *N*-sulfate residues.

Comparative Results with Heparan Sulfate from Bovine Lung. Further confirmation of the identity of heparan sulfates of cultured CHO cells was provided by comparisons, using the same general procedures, with the characteristics of authentic bovine heparan sulfate. In this case, uronic acid (orcinol) assays were used as the parameter for sugar chain characteristics. Figure 9 illustrates Bio-Gel P-10 profiles of bovine heparan sulfate, untreated and following direct nitrous acid treatment. All of the major characteristics are the same as for those reported above. The untreated material shows similar partial retention on Bio-Gel P-10, hence has a similar apparent molecular size (compare with Figure 6). Following direct nitrous acid treatment, cleavage products are formed which behave on Bio-Gel P-10 similarly to those from the heparan sulfate of cultured CHO cells. The striking similarities of the materials from two completely different sources lead one to expect that other mammalian heparan sulfates would also behave in similar fashion when these procedures are used.

Metabolic Relationship of the Heparan Sulfate Species. Preliminary experiments to determine the metabolic interrelationship between the various cellular heparan sulfate varieties were done using a tandem-labeling method. As applied here, the cells were grown for three generations in the presence of 2×10^{-8} M glucosamine-*6-t* sp act. 1300 then, for a final 4 hr, 10^{-5} M glucosamine-*1*-^{14}C sp act. 51.4 was added. The cells were then harvested, washed, and processed as before, and the $^3H/^{14}C$ ratios of the various fractions were determined as an inverse measure of the metabolic activity of the fraction (Table III).

The isolated UDP-*N*-acetylhexosamine, as expected, had the lowest ratio (1.08) of any of the major fractions; the various complex carbohydrate materials had ratios that ranged from 1.50 to 6.26. Surface glycopeptides did not differ from the glycopeptides of the residual precipitate, ranging between 2.8 and 3.6 across the DEAE-cellulose profile, and averaged 3.26 for surface and 3.21 for residual precipitate. The other glycosaminoglycans, hyaluronic acid and chondroitin sulfate, had ratios of 5.45 and 3.98, respectively.

As isolated from DEAE-cellulose, surface and cell-sap heparan sulfate had distinctly different $^3H/^{14}C$ ratios (1.61 and 6.26, respectively), and each had a fairly uniform ratio across the fractions harvested, as well as across the Bio-Gel P-10 column fractions that were run subsequently with the material pooled from DEAE-cellulose. The striking difference between surface and cell-sap heparan sulfate also provided confirmation of the conclusion that the surface material did not merely represent leaching out of cell-sap material during the trypsin treatment.

By contrast with surface and cell-sap heparan sulfate, heparan sulfate from the residual precipitate clearly had more than one component as judged by the $^3H/^{14}C$ ratios: as eluted from DEAE-cellulose, the heparan sulfate coming off first had a higher ratio than that eluting later. That is, the heparan sulfate that eluted similarly to heparan sulfate of cell-sap had a $^3H/^{14}C$ ratio similar to cell-sap, while that which eluted similarly to surface material had a lower ratio. When the total residual precipitate heparan sulfate was run on Bio-Gel P-10, the heparan sulfate with the low $^3H/^{14}C$ ratio eluted at the beginning of the major peak; on this basis, the heparan sulfate of the residual precipitate was divided into "A" and

TABLE III: Radioactivity[a] of Isolated Fractions from Cells Tandem Labeled with Glucosamine-*6-t* and Glucosamine-*1*-^{14}C.

Cell Fraction	Total cpm – Column ^3H	Total cpm – Column ^{14}C	Column $^3H/^{14}C$	Electrophoretic $^3H/^{14}C$[e]
Surface glycopeptides[b]	228,840	70,100	3.26	
Surface heparan sulfate	58,300	36,120	1.61	1.52
Cell-sap UDP-*N*-acetylhexosamine[c]	337,120	312,140	1.08	
Cell-sap heparan sulfate[b]	205,340	32,780	6.26	4.35
Residual precipitate – glycopeptides[b]	1,054,040	327,880	3.21	
Residual precipitate – hyaluronic acid[b]	14,880	2,730	5.45	
Residual precipitate – heparan sulfate A[d]	30,440	20,240	1.50	1.48
Residual precipitate – heparan sulfte B[d]	117,600	22,960	5.12	3.77
Residual precipitate – chondroitin SO_4[b]	116,250	29,210	3.98	

[a] The cpm represent material isolated from approximately 5×10^9 cells after growth for three generations with 0.025 μCi/ml of glucosamine-*6-t* (sp act. 1300 cpm) and for the final 4 hr before harvest with additional 0.50 μCi/ml of glucosamine-*1*-^{14}C (sp act. 51.4). [b] Pooled fractions from DEAE-cellulose chromatography. [c] Pooled fractions from 1° Bio-Gel P-2 chromatography. [d] The residual precipitate A fraction could be separated from the remainder of the residual precipitate fraction (precipitate B) since it eluted earlier on Bio-Gel P-10. [e] Aliquots of the heparan sulfates pooled from column fractions were run on cellulose acetate electrophoresis; the isolated spots were cut out and counted.

"B" subfractions with average ratios of 1.50 and 5.12, respectively. Thus, a total of four heparan sulfate varieties were defined, two of which represented highly active metabolic compartments. As indicated in Table III, the distinctly different $^3H/^{14}C$ ratios of these four varieties were confirmed with electrophoretically purified heparan sulfate fractions.

Discussion

When exponentially growing CHO cells were supplied with radioactive glucosamine and inorganic sulfate for about one generation, radioactivity from both sources was found in the cells in a variety of complex carbohydrates including heparan sulfate. Almost 60% of the radioactivity identified as heparan sulfate appeared to be free in the cytoplasm and directly acid soluble, while the remainder appeared to be membrane associated, requiring proteolysis for release in acid-soluble form. A portion of the latter could be removed from the cell surface with trypsin under conditions that prevented irreversible cell damage and that were unaccompanied by leaching out of the free cytoplasmic heparan sulfate. In addition to the heparan sulfate associated with the cell surface, internal cellular particulates contained two heparan sulfate varieties. The possibility of a fifth metabolic variety (namely, heparan sulfate secreted into the medium) has not yet been investigated (the high serum content in the medium required by CHO cells for large suspension cultures makes this question technically much more difficult to attack).

The four metabolic varieties could be chemically distinguished by their behavior on DEAE-cellulose and Bio-Gel P-10, by their $^3H/^{35}S$ ratios when labeled from glucosamine-*6-t* and $Na_2{}^{35}SO_4$, and by their $^3H/^{14}C$ ratios when tandem labeled with glucosamine-*6-t* and glucosamine-*1*-^{14}C. Assuming that there is a single overall biosynthetic mechanism for heparan sulfate production in these cells, one might suppose, therefore, that one of the isolated forms is precursor to the others. For instance, since it is now generally believed that glycosaminoglycans are synthesized by the building up of polysaccharide chains onto a polypeptide core (recently reviewed by Ginsburg, 1969), one might suppose that the residual acid precipitable heparan sulfate requiring proteolysis for release would include this newly synthesized material. Such newly synthesized heparan sulfate might remain attached near the biosynthetic site, as Silbert (1967a) found for heparin biosynthesis in mouse mast cell tumors, and then a free cytoplasmic material formed by intracellular cleavage of a protein-bound form. Results of the tandem-label experiments reported here are compatible with this notion, since the rank of the $^3H/^{14}C$ ratios was in the order residual precipitate "A," residual precipitate "B," then cell-sap. The apparently greater number of *N*-sulfate residues on the cell-sap form might also indicate its older "age," since studies with mouse mast cell tumor material have shown that an N-acetylated precursor of heparin is N-sulfated concomitant with loss of *N*-acetyl groups (Silbert, 1967b).

Regardless of the above, however, the present data indicate that heparan sulfate is also a cell surface component and that the cell surface heparan sulfate represents a metabolically distinct compartment. The tandem-label experiments suggest that the heparan sulfate of the cell surface is derived directly from a small intracellular membrane-associated compartment (residual precipitate A) rather than from the major precipitable and cell-sap intracellular species. The present data also suggest that this feature is distinctly different from the metabolism and transit to the cell surface of glycoproteins and is also different from that of other glycosaminoglycans which do not assume stable cell surface residency at all in this cell line.

The surface of mammalian cells, including cultured cells, is known to possess a wide variety of complex carbohydrates (glycoproteins, glycolipids, and glycosaminoglycans) and, in fact, the cell surface is enormously enriched in these materials (reviewed by Kraemer, 1971). It appears that heparan sulfate can now be added to this rapidly expanding list of surface complex carbohydrate species and is of particular interest since its ubiquity in mammalian organs and tissues (Engelberg, 1963) probably precludes an antigenic or intercellular recognition role. Further evidence for a general cellular role for heparan sulfate will be considered in part II of this series.

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Heparan Sulfates of Cultured Cells. II. Acid-Soluble and -Precipitable Species of Different Cell Lines*

Paul M. Kraemer

ABSTRACT: Six established mammalian cell lines, all adapted to growth in suspension culture, as well as a diploid mouse embryo lung strain grown as monolayers on glass, have now been studied for their capacity to synthesize heparan sulfate. The established cell lines are CHO, Don C, L5178Y, BHK-C13, L-929, and HeLa, and they differ in their species of origin, their morphology on glass, and their ability to synthesize differentiated cell products. Each was examined for both directly acid-soluble heparan sulfate and heparan sulfate rendered acid-soluble by papain digestion of the acid precipitate. All seven cell types synthesized heparan sulfate during exponential cell growth. In each case, heparan sulfate appeared in both directly acid-soluble and precipitable cell fractions. In fact, similar amounts of radioactive precursors were incorporated into the acid precipitate in all cells studied. The results suggest that heparan sulfate is a general cellular constituent rather than a differentiated cell product. The possibility is raised that all mammalian cells can synthesize this material and that it is vital to some general life process at the cellular level.

The term heparan sulfate designates mammalian glycosaminoglycans that structurally resemble heparin but which are less highly sulfated and lack significant anticoagulant activity (Brimacombe and Webber, 1964). That is, the sugar polymer chain consists largely of alternating glucosamine and glucuronic acid units; both linkages are $\alpha 1 \rightarrow 4$; the molecules contain both *O*-sulfate and *N*-sulfate residues, and in their native state are covalently linked to polypeptide by means of a galactosyl–galactosyl–xylosyl–*O*-serine linkage region (Knecht *et al.*, 1967). It appears that heparin and heparan sulfate are the only mammalian complex carbohydrates that contain N-sulfated hexosamine residues; hence, the pronounced lability of these *N*-sulfate groups can be exploited in a number of ways for studies of the N-sulfated glycosaminoglycans in the presence of large amounts of other complex carbohydrate species.

The structural distinctions between heparan sulfate and heparin appear to be solely quantitative rather than qualitative [*e.g.*, it now seems clear that both types contain at least some N-acetylated hexosamine units (Linker *et al.*, 1958; Jaques *et al.*, 1966; Lindahl, 1966; Cifonelli and King, 1970)], raising the possibility that animal tissues contain a continuum of N-sulfated glycosaminoglycan species. If this is true, the further possibility must be considered that all of these species are metabolically and functionally related and that the anticoagulant activity of the more highly sulfated varieties is essentially fortuitous and physiologically irrelevant.

The N-sulfated glycosaminoglycans are widely distributed in animal tissues (Brimacombe and Webber, 1964); however, this tissue ubiquity leaves unanswered the question of which cells possess synthetic capability. Indeed, it has been widely believed that the synthesis of these molecules is restricted to certain highly differentiated cells, especially mast cells. Thus, the previous report from this laboratory that demonstrated heparan sulfate production by an established line of Chinese hamster fibroblasts (Kraemer, 1968) encouraged a reexamination of this question. In the following report, evidence is pre-

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