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On the Co-polymeric Structure of Dermatan Sulfate from Normal and Transformed 3T3 Fibroblasts in Confluent Cultures

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Radio-labelled glycosaminoglycans were isolated from intra-, peri- and extracellular compartments of confluent 3T3 fibroblasts and their SV40- or polyoma-transformed counterparts after incubation with [^{3}H]glucosamine and $^{35}SO_4^{2-}$.

The various glycans were separated into hyaluronate and sulfated glycosaminoglycans by ion exchange chromatography. The content of heparan sulfate, chondroitin sulfate and dermatan sulfate was assessed after chemical and enzymic degradations. The L-iduronate content and copolymeric structure of dermatan sulfate was determined after periodate oxidation and alkaline scission.

There was increased incorporation of ^{3}H into hyaluronate by transformed cells. The distribution of the three sulfated glycosaminoglycans showed little variation between the various compartments. There was no significant difference in L-iduronate content between the dermatan sulfates isolated from normal and transformed cells. In addition, the sequential arrangements of L-iduronate- and D-glucuronate-containing units appeared to be similar.

It is concluded that transformed cells express little qualitative or quantitative alteration in dermatan sulfate metabolism.

A variety of cell surface properties are modified in tumour cells or transformed cells compared with the normal counterpart.[1] Structural studies on pericellular proteoglycans or their glycosaminoglycan (GAG) prosthetic groups from neoplastic cells or tissues have been performed in many laboratories.[2] Extensive analyses of extracellular GAGs from normal and neoplastic tissues have shown that a relative increase of chondroitin sulfate or of hyaluronate and a decrease of heparan sulfate or of dermatan sulfate is the most frequent alteration in malignant neoplasias.[3] Also tumorigenic mutants of cell lines have increased amounts of chondroitin sulfate in their cell coat.[4,5] Cells from spontaneously occurring human malignant gliomas[6,7] show a great variation in their production of hyaluronate and sulfated proteoglycans. However, most lines produce more hyaluronate and less sulfated proteoglycans than their normal counterpart. Both chemically and virally transformed cells appear to secrete more chondroitin sulfate proteoglycans compared to normal cells.[8,10] As the synthesis of L-iduronate-rich dermatan sulfate appears to be reduced in malignant, tumorigenic or transformed cells, it has been suspected[4,6,11] that these cells have a defect in the C-5 uronosyl epimerase specific for dermatan sulfate biosynthesis.[12] A faulty epimerization of GlcA to IdoA* would also effect the co-polymeric structure of the dermatan sulfate chains produced. Therefore we have examined the chemical structure of dermatan sulfate synthesized by normal and transformed mouse 3T3 fibroblasts.

* Abbreviations: GlcA, D-glucuronic acid; IdoA, L-iduronic acid; GlcN, 2-deoxy-2-amido-D-glucose; GAG, glycosaminoglycan; SV-40, simian virus 40; GalNAc, 2-deoxy-2-acetamido-D-galactose; GlyA, glycuronic (hexuronic) acid; R=remnant of an oxidized and degraded IdoA; 4,5ΔGlyA, 4,5-unsaturated hexuronic acid.

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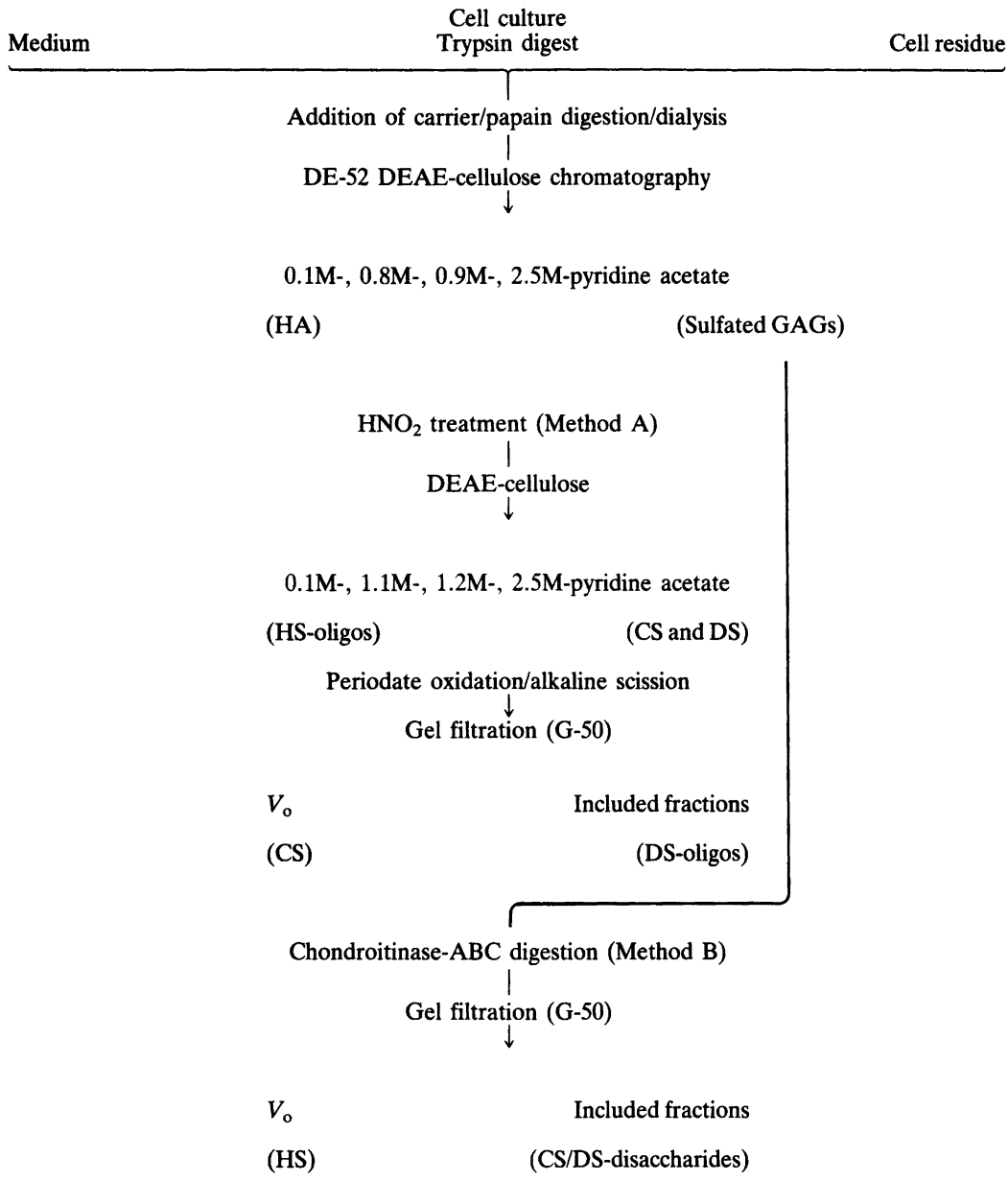

Scheme 1. Flow-chart for the preparation of labelled GAGs from cell cultures. Cells were incubated with $^{35}SO_4^{2-}$ and [^{3}H]GlcN for 48 h. Medium was collected separately and the cells were treated with trypsin. For details see the Experimental section. HA, hyaluronate; GAG, glycosaminoglycan; HS, heparan sulfate; CS, chondroitin sulfate; DS, dermatan sulfate; oligos, oligosaccharides.

EXPERIMENTAL

Materials. Standard GAGs were obtained from sources listed elsewhere.[13,14] Chondroitinase-AC and -ABC were purchased from Miles Laboratories, Elkhart, IN, U.S.A. Scintillators were from Packard AB, Bandhagen, Sweden and gel-chromatographic materials were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. Microgranular DEAE-cellulose (Whatman type DE-52) was used for ion-exchange chromatography. $^{35}SO_4^{2-}$ (carrier-free) and D-[1-^{3}H]glucosamine (120 GBq/mmol) were purchased from Amersham.

Preparation of radiolabelled glycosaminoglycans (GAGs). The cell-lines used were a Balb-3T3 mouse fibroblast line and its SV40- or polyoma-transformed counterparts.[10] Confluent cultures were given ^{3}H GlcN and $^{35}SO_4^{2-}$, respectively, for 48 h and media and cells were collected separately. The cells were digested briefly with trypsin[13] and the material released by this treatment (pericellular) as well as the remaining material (intracellular) was recovered. The various fractions were processed as described in detail elsewhere.[13] In brief, GAGs were released as free chains by papain digestion and the total pool of sulfated GAGs was separated from hyaluronate by ion-exchange chromatography (Scheme 1).

The proportion of heparan sulfate was determined by quantification either of the nitrous acid-sensitive material (Method A in Scheme 1) or of the chondroitinase-ABC resistant material (Method B). In the former case radiolabelled galactosaminoglycans were isolated after ion-exchange chromatography.[13] The two methods gave values within ±3 %. The amount of chondroitin sulfate was estimated after degradation of dermatan sulfate by periodate oxidation-alkaline elimination (Fig. 1). The void volume fractions obtained by gel chromatography on Sephadex G-50 were digested with chondroitinase-AC and the amount of disaccharide obtained (results not shown) were used to estimate chondroitin sulfate.

Degradative and separatory methods. Oxidations of galactosaminoglycans with periodate were performed at low pH and temperature as described.[15] The IdoA residues thus oxidized were cleaved with alkali[15] and the resulting fragments separated by gel filtration on Sephadex G-50 (see Fig. 1). Oligosaccharides were digested with chondroitinase-AC in 0.5 M Tris/HCl, pH 8.0, at 37 °C overnight.[13]

Evaluation of co-polymeric structures. Periodate oxidation-alkaline elimination of dermatan sulfate gives rise to a homologous series of oligosaccharides of the general carbohydrate structure GalNAc-(GlyA−GalNAc)$_n$-R where R is the remnant of an oxidized and degraded IdoA residue and GlyA is either GlcA or IdoA−SO_4. The smallest fragment, *i.e.* GalNAc−R, is indicated as the component with n=0 in Fig. 1, the next higher saccharide as n=1 and so on. The content of IdoA (x) in dermatan sulfate was calculated by the formula

$$x=\sum_{n=0}^{n=5}\frac{a_n}{n+1}\sum_{n=0}^{n=5}a_n \qquad \text{(Ref. 13)}$$

The symbol a_n denotes total radioactivity in oligosaccharides n=0,1,2... *etc.* Saccharides with n>6 were not included as such peaks were not regularly observed. Furthermore, higher saccharides contribute less to the sum of IdoA compared to smaller saccharides.

RESULTS

Quantification of GAGs from normal and transformed cells. After administration of $^{35}SO_4^{2-}$ and [^{3}H]GlcN to 3T3 cells, the cultures were divided into medium, trypsin-releasable material and cell residue. Papain-solubilised hyaluronate and sulfated GAGs were separated and quantified (Table 1). In general, there was an increased incorporation of ^{3}H into hyaluronate by transformed cells. Most of the hyaluronate (68−80 %) was found in the medium, in particular in the case of polyoma-transformed cells. There was also an increased incorporation of ^{3}H into sulfated GAGs of transformed cells. The incorporation of ^{35}S into the same material was less increased resulting in a considerable change in the ^{3}H/^{35}S ratio of this material. (For example, the medium-derived sulfated GAGs gave the following ratios: normal cells, 0.53; SV40-transformed cells, 0.28; polyoma-transformed cells, 0.09). The distribution of radiolabelled sulfated GAGs showed little variation between the three compartments, except for polyomatransformed cells, where a large part (56−67 %) of the material was in the medium.

The sulfated GAGs consist of heparan sulfate, chondroitin sulfate and dermatan sulfate. These were quantified after nitrous acid degradation or after chondroitinase-ABC digestion and after periodate oxidation-alkaline elimination (see Scheme 1). Heparan sulfate (nitrous acid-sensi-

Table 1. Distribution of labelled GAGs in the various compartments from normal and transformed 3T3 fibroblasts. After administration of $^{35}SO_4^{2-}$ and [^{3}H]GlcN to 3T3 cells, the cultures were treated as outlined in Scheme 1. Hyaluronate and sulfated GAGs were separated by ion exchange chromatography. SV-40, simian virus 40; Py, polyoma-virus; C, cell residue; T, trypsinate; M, medium. The values are expressed as percentages of total radioactivity recovered with the respective polysaccharide pool from the three culture fractions.

Cell line	Fraction	Hyaluronate ^{3}H $10^{-4}\times$dpm	%	Sulfated GAGs ^{3}H $10^{-4}\times$dpm	%	^{35}S $10^{-4}\times$dpm	%
3T3	C	3.36	15	5.95	37	1.83	23
	T	3.88	17	3.19	20	2.24	29
	M	15.83	68	7.05	43	3.74	48
SV-40 3T3	C	7.58	16	17.83	40	1.28	17
	T	6.58	15	11.13	25	2.21	29
	M	32.17	69	15.15	34	4.22	54
Py 3T3	C	20.06	8	31.60	19	1.82	13
	T	27.66	12	43.06	25	2.62	20
	M	191.91	80	96.09	56	8.96	67

tive and chondroitinase-ABC resistant material) was generally concentrated in the pericellular pool but dominated also other fractions (Table 2). There were no significant quantitative differences between normal and transformed cells in terms of heparan sulfate distribution. In contrast, there are significant qualitative differences between the heparan sulfates of normal and transformed cells.[14] The amount of chondroitin sulfate (chondroitin-AC-sensitive, but nitrous acid- and periodate-resistant material) was low in general but appeared slightly increased in all compartments of transformed cells. The proportions of dermatan sulfate (nitrous acid-resistant, but periodate-sensitive material) were not significantly altered.

The copolymeric structure of dermatan sulfate. The sulfated GAGs from the various compartments of normal and transformed cell cultures were subjected to periodate oxidation-alkaline elimination followed by gel filtration on Sephadex G-50 (Fig. 1). The void volume fractions are taken to represent chondroitin sulfate, whereas the included saccharides are the de-

Table 2. Proportions of heparan sulfate, chondroitin sulfate and dermatan sulfate in the various compartments from normal and transformed 3T3 fibroblasts. The values are expressed as (a) percentages of total radioactive [^{35}S]GAG in each culture fraction or (b) percentages of total radioactive [^{35}S]GAG in all culture fractions. The values vary within ±5 % between three different experiments. HS, heparan sulfate; CS, chondroitin sulfate; DS, dermatan sulfate; see also Table 1.

Cell line	Fraction	HS a	HS b	CS a	CS b	DS a	DS b
3T3	C	74	17	5	1	21	5
	T	89	25	3	1	8	2
	M	67	32	8	4	25	12
SV40-3T3	C	52	9	10	2	38	6
	T	88	25	5	1	7	1
	M	62	34	13	7	25	14
Py-3T3	C	57	8	10	1	33	4
	T	78	15	8	2	14	3
	M	67	45	15	10	18	12

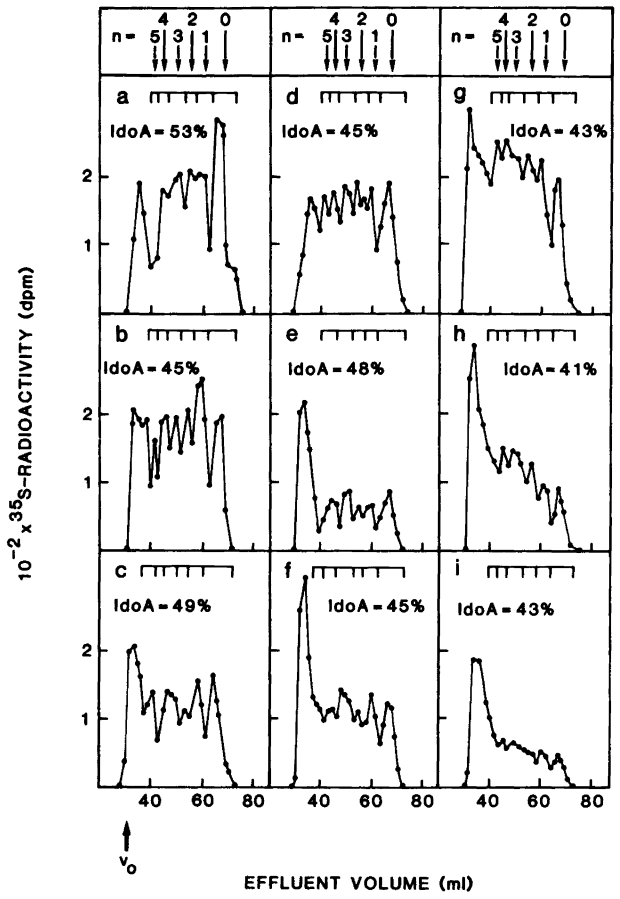

Fig. 1. Gel chromatography of degradation products obtained after periodate oxidation-alkaline elimination of ^{35}S galactosaminoglycans from the intracellular (a,d,g), pericellular (b,e,h) and extracellular pools (c,f,i) of normal 3T3 cells (a–c), SV-40 transformed cells (d–f) and polyoma-transformed cells (g–i). Column: Sephadex G-50, superfine (8×1600 mm) eluted with 0.2 M pyridine acetate, pH 5.0, at a rate of 6 ml/h. Fractions were pooled as indicated by horizontal bars. The calculated IdoA-content is given in each panel.

gradation products of dermatan sulfate[general carbohydrate structure GalNAc-(GlyA-GalNAc)$_n$-R]. The distribution of radioactivity between the various saccharides (n=0–5) can be used to quantify IdoA in dermatan sulfate. As indicated in Fig. 1 there was no significant difference in IdoA-content between the dermatan sulfates isolated from corresponding compartments of normal and transformed cells. The oligosaccharide profiles were also similar except in the case of dermatan sulfate from the peri- and extracellular pools of polyoma-transformed cells (Fig. 1h and 1i), where the longer saccharides (n=3–5) appeared to be more prominent.

The fragments obtained by oxidation and scission of dermatan sulfate have the general structure

GalNAc-[GlcA/IdoA(−SO_4)−GalNAc]$_n$-R

Oligomers containing only GlcA or copolymeric variants with both of the GlyA residues can be totally or partially degraded by chondroitinase-AC treatment as shown previously.[16,17] As the GalNAc→GlcA bond is sensitive to chondroitinase-AC, saccharides obtained after consecutive

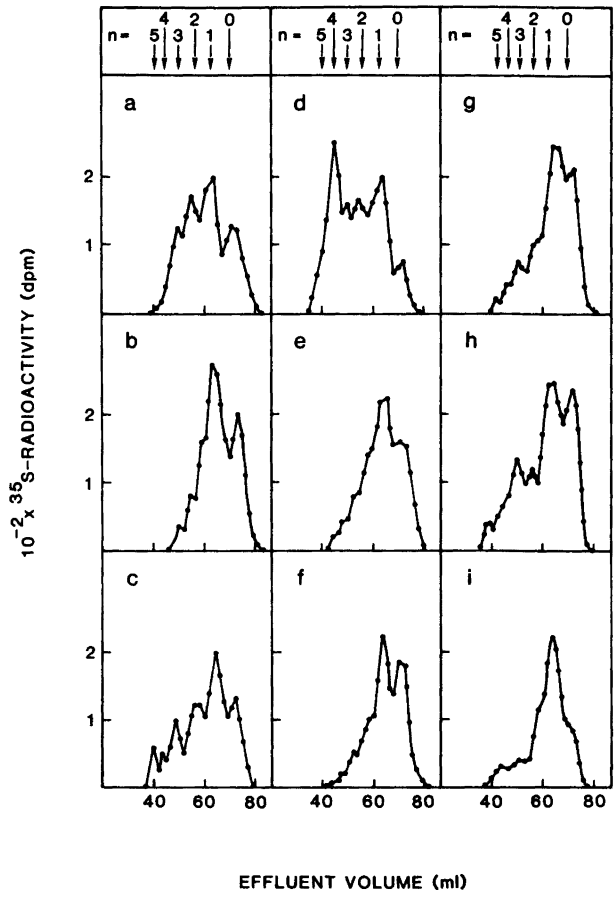

Fig. 2. Gel chromatography of degradation products obtained after chondroitinase-AC digestion of oligosaccharides (n=1–5) produced by periodate oxidation-alkaline elimination of ^{35}S dermatan sulphate from the intracellular (a,d,g), pericellular (b,e,h) and extracellular pools (c,f,i) of normal 3T3 cells (a–c), SV40-transformed cells (d–f) and polyoma-transformed cells (g–i). The column was the same as the one used in Fig. 1.

chemical and enzymic degradation have the general structure GalNAc-[IdoA(−SO_4)−GalNAc]$_n$-R. When oligomers with n=1–5 were digested and re-chromatographed in the same column the results shown in Fig. 2 were obtained. The most retarded peak contains GalNAc−SO_4 and the preceding peak contains the disaccharide[4,5] ΔGlyA−GalNAc(−SO_4) which results from cleavage of a GalNAc→GlcA bond by the eliminase.[16] The ratio between the radioactivity of these two peaks is a measure of the average size of the original GlcA-containing segment. However, one must consider whether the presence of copolymeric saccharides containing both GlcA and IdoA−SO_4 can disturb this assessment. A fragment of the structure GalNAc−IdoA(−SO_4)−GalNAc can conceivably be produced after enzymic degradation.[17] Such a fragment would have an elution position that partially overlaps with that of disaccharides. Significant quantities of higher oligomers containing IdoA−SO_4 were obtained from dermatan sulfates of the intracellular pool of SV40-transformed cells (Fig. 2d) and, to a lesser degree, from the intracellular pool of regular 3T3 cells

(Fig. 2a), from the pericellular pool of polyoma-transformed cells (Fig. 2h) and from the extracellular pool of nontransformed cells (Fig. 2c). Assessment of the length of GlcA-containing segments in the dermatan sulfates from the intracellular pools of normal and transformed cells (Fig. 2a,d,g), using the above-mentioned parameters, suggested values of n=2,3 and 1, respectively. As the former two values could have been increased by the presence of small IdoA($-SO_4$)-containing saccharides it is uncertain if the observed differences are significant. The dermatan sulfates of the pericellular pools appeared to contain $(GlcA-GalNAc)_n$-segments with n=1–2 in all three cases (Fig. 2b,e,h). Similar results were obtained with the three medium-derived dermatan sulfates (Fig. 2c,f,i) except for the polyoma-case (Fig. 2i) where n>2.

DISCUSSION

Qualitative or quantitative changes in the structure and composition of GAGs have frequently been observed in conjunction with transformation of fibroblast-like cells. These changes may be the result of transformation *per se* or they may be due to an increased growthrate. Dietrich and collaborators[18] have recently reported that exponentially growing 3T3 cells synthesized both chondroitin sulfate and dermatan sulfate, retaining the former in the pericellular pool and releasing the latter into the medium. Upon reaching quiescence, these cells switched to retention of dermatan sulfate and release of chondroitin sulfate. SV40 transformed cells always retained chondroitin sulfate irrespective of the growthphase. Hence, the increased amounts of radiolabelled chondroitin sulfate found in transformed cells in the present study, may be a transformation-dependent phenomenon. As proteoglycans carrying chondroitin sulfate have different core proteins than those carrying dermatan sulfate,[19,20] their rates of synthesis could be under separate control.

The dermatan sulfate side-chains of proteoglycans synthesized by transformed or non-transformed 3T3 cells had quite similar co-polymeric structures. The heparan sulfates isolated from these very same cells showed much more extensive structural variation as reported earlier.[14] Most of the dermatan sulfate synthesized by 3T3 cells was either retained within the cells or released into the medium. These chains were co-polymeric in the sense that both IdoA–GalNAc–SO_4, GlcA–GalNAc–SO_4 and IdoA($-SO_4$)–GalNAc repeating disaccharides were present as building blocks. Whereas the IdoA($-SO_4$)-containing units were slightly less common in dermatan sulfates from transformed cells, the proportion and sequential arrangements of GlcA–GalNAc–SO_4 units were similar in all cases. Therefore, the epimerization of GlcA to IdoA during dermatan sulfate biosynthesis appears to be quantitively and qualitatively unaffected by viral transformation. This is in keeping with the notion that proteodermatan sulfate has little connection with cell social behaviour but is rather engaged in collagen fibrillogenesis in the extracellular space.

Polyoma-transformed cells incorporated much more [^{3}H]GlcN relative to $^{35}SO_4^{2-}$ into sulfated GAGs, compared with the other cell lines. This could be due to increased degradation with preferential re-utilisation of [^{3}H]hexosamine or to a smaller UDP-hexosamine pool in polyoma-transformed cells, resulting in higher specific activity of the sugar precursors.

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