Composition and Distribution of Glycosaminoglycans in Cultures of Human Normal and Malignant Glial Cells

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The glycosaminoglycans of human cultured normal glial and malignant glioma cells were studied. [^{35}S]Sulphate or [^3H]glucosamine added to the culture medium was incorporated into glycosaminoglycans; labelled glycosaminoglycans were isolated by DEAE-cellulose chromatography or gel chromatography. A simple procedure was developed for measurement of individual sulphated glycosaminoglycans in cell-culture fluids. In normal cultures the glycosaminoglycans of the pericellular pool (trypsin-susceptible material), the membrane fraction (trypsin-susceptible material of EDTA-detached cells) and the substrate-attached material consisted mainly of heparan sulphate. The intra- and extra-cellular pools showed a predominance of dermatan sulphate. The net production of hyaluronic acid was low. The accumulation of ^{35}S -labelled glycosaminoglycans in the extracellular pool was essentially linear with time up to 72 h. The malignant glioma cells differed in most aspects tested. The total production of glycosaminoglycans was much greater owing to a high production of hyaluronic acid and hyaluronic acid was the major cell-surface-associated glycosaminoglycan in these cultures. Among the sulphated glycosaminoglycans chondroitin sulphate, rather than heparan sulphate, was the predominant species of the pericellular pool. This was also true for the membrane fraction and substrate-attached material. Furthermore, the accumulation of extracellular ^{35}S -labelled glycosaminoglycans was initially delayed for several hours and did not become linear with time until after 24 h of incubation. The glioma cells produced little dermatan sulphate and the dermatan sulphate chains differed from those of normal cultures with respect to the distribution of iduronic acid residues. The observed differences between normal glial and malignant glioma cells were not dependent on cell density; rather they were due to the malignant transformation itself.

Most or all mammalian cells produce and metabolize glycosaminoglycans (Kraemer, 1972). The widespread occurrence of this kind of substance indicates that they may have important biological functions. In particular, the localization of certain glycosaminoglycans in the plasma membrane may suggest their involvement in cellular interaction phenomena, e.g. between individual cells or between cells and compounds of the extracellular space. The possible role of glycosaminoglycans in cell biological phenomena has been studied in cell culture. In such systems, they may influence cell proliferation (see, e.g., Lippman, 1968; Ohnishi *et al.*, 1975), cell adhesion (Roblin *et al.*, 1975; Culp, 1976) or cell aggregation (Burger & Martin, 1972; Pessac & Defendi, 1972; Wasteson *et al.*, 1973b). Many studies dealing with glycosaminoglycans of various non-human, mostly

rodent, cell lines have been published (see, e.g., Roblin *et al.*, 1975, and references quoted therein). In contrast, the structure and metabolism of glycosaminoglycans of human normal cells have only been studied in few types of cells, such investigations being almost exclusively limited to fibroblasts (Malmström *et al.*, 1975; Kleinman *et al.*, 1975).

The importance of glycosaminoglycans in normal and malignant growth control is virtually unknown, although quantitative and qualitative differences in the production and composition of glycosaminoglycans have been revealed in certain virus-transformed cells, compared with their non-transformed counterparts (see, e.g., Cohn *et al.*, 1976, and references quoted therein). The importance of using a strictly normal cell as a reference (Pontén, 1975) in such studies does not seem to have been fully recognized. Also, more information is needed on human cell systems before general conclusions can be made.

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We have initiated an investigation on the structural and metabolic features of glycosaminoglycans produced by human normal and malignant cultured glial cells. By using this cell system strictly normal diploid cells *in vitro* (Pontén, 1975) may be compared with spontaneously transformed neoplastic cells having the same histogenetic origin. The present paper deals with the distribution and composition of glycosaminoglycans in various compartments of the cultures.

Experimental

Chemicals

Inorganic [^{35}S]sulphate (carrier-free) and D- ^{3}H -glucosamine (sp. radioactivity 19 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Cetylpyridinium chloride was provided by AB Recip, Stockholm, Sweden. Sephadex gels were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Whatman DEAE-cellulose (DE-52) was a product of Whatman Biochemicals, Maidstone, Kent, U.K. Bacterial chondroitinases ABC and AC were obtained from Miles Laboratories, Elkhart, IN, U.S.A., and trypsin was from United States Biochemical Corp., Cleveland, OH, U.S.A. Leech hyaluronidase (hyaluronate 3-glycanohydrolase; EC 3.2.1.36) was kindly provided by Dr. E. A. Balazs, Columbia University, New York, NY, U.S.A. Protease type V and soya-bean trypsin inhibitor were provided by Sigma Chemical Co., St. Louis, MO, U.S.A. Reference samples of hyaluronic acid, chondroitin sulphate and heparin were available in our laboratory (Wasteson, 1971). ^{35}S -labelled heparin from mouse mastocytoma was kindly provided by Dr. U. Lindahl of this Institute. ^{14}C -labelled chondroitin sulphate was prepared from embryonic chick cartilage as described by Amadò *et al.* (1974). Unsaturated reference disaccharides from chondroitin sulphate [2-acetamido-2-deoxy-3-O-(β -D-glucopyranuronic acid)-4-O-sulpho-D-galactose and 2-acetamido-2-deoxy-3-O-(β -D-glucopyranuronic acid)-6-O-sulpho-D-galactose] were provided by Miles Laboratories.

Cell lines and routine culture conditions

The normal glial cell line U-787 CG was generated from adult human brain tissue as previously described (Pontén & Macintyre, 1968). The establishment of the glioma line U-251 MG, derived from a spontaneously occurring human malignant glioma, has also been described (Westermarck *et al.*, 1973). Cells were routinely grown in Eagle's minimum essential medium (Eagle, 1959) supplemented with 10% calf serum and antibiotics (100 i.u. of penicillin/ml,

50 μg of streptomycin/ml and 1.25 μg of amphotericin B/ml) (Squibb, Lidingö, Sweden).

Subcultivation was carried out once or twice a week with 0.25% (w/v) trypsin. Line U-251 MG was treated with 0.5 mM-EDTA in phosphate-buffered saline (137 mM-NaCl, 2.7 mM-KCl, 8.1 mM- Na_2HPO_4 , 1.5 mM- KH_2PO_4 , pH 7.3) before trypsin digestion.

Incubation of cells with [^{35}S]sulphate and [^3H]glucosamine

Cells were treated with trypsin, pooled in routine Eagle's medium and seeded at a density of 4×10^5 cells/55 mm Nunc plastic dish. Sparse cultures were used 6–8 h later when cells had settled and spread. Dense cultures were obtained by growing the cells for 10–14 days under routine conditions. Normal cultures had then reached a cell density of about 1.2×10^6 cells/dish and showed a stationary growth phase (Pontén *et al.*, 1969). Growth of the glioma cell line also decreased at high cell densities, although not to the same extent as that of the normal cell line. Dense cultures of glioma cells contained about 15×10^6 cells/dish (Westermarck, 1973). Incubations were started by replacing the medium with 5 ml of F-10 medium (Ham, 1963) supplemented with 10% calf serum and 10 μCi of [^{35}S]sulphate/ml or 25 μCi of [^3H]glucosamine/ml. In experiments with [^{35}S]sulphate the experimental F-10 medium had a lower content of unlabelled inorganic sulphate, MgCl_2 being substituted for MgSO_4 . Morphology and growth behaviour was unaltered in this medium compared with the ordinary F-10 medium.

Fractionation of labelled cells

After incubation with radioactive precursors the medium, representing the extracellular pool, was collected and dishes were gently washed with 3×2 ml of phosphate-buffered saline containing 100 mg of CaCl_2 and 100 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ /litre. Cultures were then treated with trypsin (2 ml of trypsin, 50 μg /ml in phosphate-buffered saline for 60 min at 37°C). The trypsin fraction (pericellular pool) and the cell pellet after two washings with 2 ml of phosphate-buffered saline (intracellular pool) were then collected. No decrease in cell number occurred during the trypsin incubation and all cells (>99%) remained viable, as tested by the Trypan Blue exclusion test. Alternatively, the cell layer was treated with EDTA (0.5 mM in phosphate-buffered saline) for 60 min at 37°C; remaining cells were detached by gentle pipetting. The dispersed cells were centrifuged (180g for 5 min) and the supernatant was collected (EDTA fraction). EDTA was preferred to EGTA, since EDTA detached the glial cells much more efficiently than did EGTA. After treatment with EDTA for 60 min, 5–10% of the cells were stained with Trypan Blue. After one 2 ml wash in phosphate-

buffered saline the cell pellet was treated with trypsin for 60 min, during which time no further significant decrease in cell number or cell viability occurred. Radioactively labelled material liberated by this secondary trypsin treatment was considered as the membrane fraction. Finally, material remaining on the plastic surface after removal of all cells was removed by 1 ml of 0.3 M-NaOH for 60 min at 37°C, or with trypsin, which was as effective as NaOH (substrate-attached material; Terry & Culp, 1974).

Determination of ^{35}S -labelled glycosaminoglycans by precipitation with cetylpyridinium chloride

^{35}S -labelled glycosaminoglycans of medium, trypsin or EDTA fractions were assayed by precipitation with cetylpyridinium chloride on filter paper as described previously (Wasteson *et al.*, 1973a), except that 0.05 M-NaCl rather than 0.3 M-NaCl was used. The cell fractions were analysed in the same way after digestion with a mixture of Protease V (10 mg/ml) and Triton X-100 (0.1 %, v/v) in phosphate-buffered saline for 5 h at 37°C. The NaOH extracts were analysed after neutralization with an equal volume of 0.3 M-HCl.

Radioactivity was measured in an Intertechnique SI 30 liquid-scintillation spectrometer or in a Packard model 2002 liquid-scintillation counter, in Insta-Gel (Packard Instruments International S.A., Zürich, Switzerland) as the scintillation medium. Results (means for duplicate determinations on duplicate dishes) are presented as c.p.m./culture or c.p.m./ μg of cell protein; protein was determined on parallel non-labelled cultures by the method of Lowry *et al.* (1951).

Isolation of glycosaminoglycans

Dense cultures were incubated with [^{35}S]sulphate (sp. radioactivity 25 $\mu\text{Ci/ml}$) or [^3H]glucosamine (sp. radioactivity 50 $\mu\text{Ci/ml}$) for 48 h and harvested as described above.

Isolation was performed by either or both of two methods after digestion with crystalline papain at 55°C, pH 5.5, for 17 h (Lindahl *et al.*, 1973) in the presence of carrier chondroitin sulphate (100 $\mu\text{g/ml}$) (procedure 1) or hyaluronic acid (1 mg), chondroitin sulphate (2 mg) and heparin (4 mg) (procedure 2). The digests were then heat-inactivated at 100°C for 5 min.

In procedure 1, the macromolecular ^{35}S -labelled material was recovered by gel chromatography in 1 M-NaCl on a column (49 cm $\times$ 5 cm or 90 cm $\times$ 2 cm) of Sephadex G-50, and desalted by passage through a column of Sephadex G-50 in 10% (v/v) ethanol. The product was freeze-dried, dissolved in water and stored at -20°C.

In procedure 2, ^3H - or ^{35}S -labelled material was

subjected to ion-exchange chromatography on a column (10 cm $\times$ 0.9 cm) of DEAE-cellulose (Höök *et al.*, 1975). Elution was with a linear gradient of 0.2–1.5 M-LiCl in 0.05 M-sodium acetate buffer, pH 4.0. The various fractions were pooled separately, desalted on a column of Sephadex G-50 in 10% ethanol, freeze-dried, dissolved in water and stored at -20°C. ^3H -labelled material eluted together with reference hyaluronic acid is referred to as ^3H -labelled hyaluronic acid and material eluted later than hyaluronic acid as sulphated ^3H -labelled glycosaminoglycans.

Determination of individual glycosaminoglycans

Hyaluronic acid. The proportion of hyaluronic acid in ^3H -labelled glycosaminoglycans was calculated as the relative amount of ^3H co-migrating with reference hyaluronic acid on DEAE-cellulose chromatography (Fig. 7). This material was completely susceptible to leech hyaluronidase (Wasteson *et al.*, 1973b) and showed glycosamine as the only hexosamine (Lohmander, 1971).

The relative amounts of heparan sulphate, chondroitin sulphate and dermatan sulphate in ^3H - or ^{35}S -labelled glycosaminoglycans were estimated by a combination of treatments with HNO_2 (procedure A; Lindahl *et al.*, 1973) and chondroitinases AC and ABC (Yamagata *et al.*, 1968). The reaction products were chromatographed on a column (130 cm $\times$ 1 cm) of Sephadex G-50, enabling the separation of susceptible (included) from resistant (excluded) fractions.

Heparan sulphate. This was taken as the proportion of labelled material resistant to treatment with chondroitinase ABC but susceptible to subsequent treatment with HNO_2 .

Chondroitin sulphate. Treatment of labelled glycosaminoglycans with chondroitinase AC yielded heterogeneous degradation products, as shown by Sephadex G-50 chromatography (Fig. 8). These products could be further analysed by chromatography on a column (140 cm $\times$ 1 cm) of Sephadex G-15 separating disaccharides from higher oligosaccharides. Chondroitin sulphate was estimated from the proportion of labelled glycosaminoglycans appearing in the disaccharide fraction after treatment of HNO_2 -resistant material with chondroitinase AC.

Dermatan sulphate. This was taken as the proportion of labelled material resistant to treatment with HNO_2 and chondroitinase AC, but subsequently susceptible to chondroitinase ABC. Intermediary-size products, arising after treatment with chondroitinase AC, i.e. fragments being included on Sephadex G-50 but having a size larger than that of disaccharides, were ascribed to dermatan sulphate rather than chondroitin sulphate, since they should contain a maximum of one glucuronic acid residue per fragment.

Simplified assay for heparan sulphate, chondroitin sulphate and dermatan sulphate

A simplified method to determine the susceptibility of ^{35}S -labelled glycosaminoglycans to treatment with HNO_2 and chondroitinases was also developed. Culture media were prepared for HNO_2 treatment by digestion with Protease V (5 mg/ml) for 3 h at 37°C to avoid precipitation of proteins in the acidic reaction mixture. All fractions containing trypsin were prepared for chondroitinase treatment by the addition of soya-bean trypsin inhibitor (100 $\mu\text{g}/\text{ml}$).

Heparan sulphate. Samples (100 μl) of culture fluids were mixed with 100 μl of 5.7 M-acetic acid and 100 μl of 0.75 M- NaNO_2 and left at room temperature (22°C) for 80 min. The reaction was interrupted by the addition of 100 μl of 0.1 M-ammonium sulphamate. Blanks were prepared by adding the test sample only after inactivation of the reaction mixture with sulphamate. Samples of the reaction mixtures were spotted on filter-paper strips and the strips treated with cetylpyridinium chloride in 0.3 M- NaCl as described by Wasteson *et al.* (1973a). In this procedure macromolecular ^{35}S -labelled glycosaminoglycans remain on the filter paper, whereas low-molecular-weight degradation products, such as inorganic sulphate or small oligosaccharides, may be washed away. The difference in radioactivity between HNO_2 -treated samples and blanks was taken to represent heparan sulphate.

Chondroitin sulphate. Samples (100 μl) of culture fluids were mixed with 0.1 unit (where 1 unit of activity is that amount of enzyme that converts 0.1 μmol of substrate/min) of chondroitinase AC in 300 μl of 0.08 M- NaCl /0.06 M-sodium acetate/0.01 M-Tris/HCl, pH 8.0, containing 170 μg of bovine serum albumin/ml. Blanks were obtained by using heat-inactivated enzyme. The reaction mixtures were incubated at 37°C for 6 h; they were then subjected to cetylpyridinium chloride precipitation as described above. The difference in radioactivity between

chondroitinase AC-treated samples and blanks was taken to represent chondroitin sulphate.

Dermatan sulphate. Samples (100 μl) of culture fluids were incubated with 0.1 unit of chondroitinase ABC, as described for chondroitinase AC, and subsequently treated with cetylpyridinium chloride (see above). The difference in radioactivity between chondroitinase ABC-treated samples and blanks was taken to represent chondroitin sulphate plus dermatan sulphate; dermatan sulphate was calculated by subtracting the value obtained for chondroitin sulphate (see above).

In control experiments authentic ^{35}S -labelled heparin and ^{14}C -labelled chondroitin sulphate showed an expected susceptibility to the various treatments under the experimental conditions used (Table 1).

Results

Kinetics of ^{35}S incorporation into glycosaminoglycans of different culture compartments

Extracellular pools. In both sparse growing and dense stationary cultures of the normal glial line the amount of extracellular ^{35}S -labelled glycosaminoglycans increased virtually linearly with time up to 72 h (Fig. 1). With short-time incubations a slight lag was revealed in the extracellular accumulation; however, the lag phase was consistently shorter than 1 h.

In contrast with the almost constant incorporation rate in normal glial cells, glioma cells showed a prolonged delay in the incorporation of ^{35}S into the extracellular pool, with a continuous increase in the incorporation rate up to about 24 h (Fig. 2). Also the release of ^{35}S -labelled glycosaminoglycans to the medium was less in glioma cultures than in normal cultures, with respect to both the c.p.m. incorporated per μg of cellular protein (Table 2) and with respect to the relative amounts of ^{35}S -labelled glycosaminoglycans present in the extracellular pool. After 1 h of incubation the extracellular pool of the glioma

Table 1. *Susceptibility of [^{35}S]heparin and [^{14}C]chondroitin sulphate to treatment with nitrous acid or chondroitinase ABC*
Samples of [^{35}S]heparin or [^{14}C]chondroitin sulphate dissolved in growth medium (Ham's F-10, supplemented with 10% calf serum) or in trypsin (50 $\mu\text{g}/\text{ml}$) were treated as described in the Experimental section and precipitated on filter paper with cetylpyridinium chloride. The cetylpyridinium chloride-precipitable (non-susceptible) radioactivity is recorded for incubations with active (sample) and inactivated (control) reaction mixtures.

Glycosaminoglycan	Dissolved in	Precipitated glycosaminoglycan (c.p.m.) after treatment with:			
		Chondroitinase ABC		HNO_2	
		Sample	Control	Sample	Control
[^{35}S]Heparin	Growth medium	940	960	10	840
	Trypsin	1120	1170	70	1030
[^{14}C]Chondroitin sulphate	Growth medium	20	1010	1000	1080
	Trypsin	40	990	930	1040

Fig. 1. Time course of incorporation of [³⁵S]sulphate into glycosaminoglycans in normal glial-cell cultures
 Sparse (a) and dense (b) cultures were incubated with 10 μCi of [³⁵S]sulphate/ml for the indicated times. The extracellular (■), pericellular (○) and intracellular (Δ) pools were recovered as described in the Experimental section. Results are expressed as cetylpyridinium chloride-precipitable ³⁵S radioactivity (c.p.m./μg of cell protein; average protein concentration during the incorporation period).

Fig. 2. Time course of incorporation of [³⁵S]sulphate into glycosaminoglycans in glioma-cell cultures
³⁵S-labelled glycosaminoglycans were recovered from the extracellular (■), pericellular (○) and intracellular (Δ) pools of sparse (a) and dense (b) cultures. See also the legend to Fig. 1. Note the delay in the accumulation of ³⁵S-labelled glycosaminoglycans in the extracellular pool.

cultures comprised 10–15% and after 48 h 60–70% of total ³⁵S-labelled glycosaminoglycans; in the normal glial-cell cultures the corresponding percentages were 50% (1 h) and 80% (48 h) (Figs. 1 and 2; Table 2).
Pericellular pool. A major part of the trypsin-labile glycosaminoglycans was released from ³⁵S-labelled glial or glioma cells within 15 min of incubation with a dilute trypsin solution (50 μg/ml) (Fig. 3). A trypsin

Table 2. Production of ³⁵S-labelled glycosaminoglycans by sparse and dense cultures of normal and malignant glial cells
 Results are means for duplicate dishes from one typical experiment after incubation with 10 μCi of [³⁵S]sulphate/ml for 48 h. The average protein concentrations (μg/dish) were as follows: sparse (120) and dense (270) normal and sparse (180) and dense (2220) malignant cultures respectively. Total radioactivity refers to the sum of the extra-, peri- and intra-cellular pools. The 'estimated membrane fraction' denotes trypsin-sensitive material from the EDTA-dispersed cells; the 'calculated membrane fraction' refers to the numerical difference between the pericellular pool and the sum of the EDTA fraction + substrate-attached material. See the Experimental section for a fuller definition of compartments. NT = not tested.

Compartment	Normal glial cells		Malignant glioma cells	
	Sparse		Dense	
	(c.p.m./μg of protein) (% of pericellular pool)	(% of total cellular pool)	(c.p.m./μg of protein) (% of pericellular pool)	(% of total cellular pool)
Extracellular	542	80	366	79
Pericellular	124	18	82	18
EDTA fraction	21	14	15	1
Substrate-attached material	14	13	1	NT
Estimated membrane fraction	69	64	NT	81
Calculated membrane fraction	90	73	66	3
Intracellular	9	2	13	100
Total	675	100	461	100

treatment time of 60 min was chosen for all later experiments.

The pool of glycosaminoglycans liberated by trypsin (pericellular pool) increased rapidly during the initial period of incubation, as shown in Fig. 1 (glial cells) and Fig. 2 (glioma cells). The fact that the incorporation of ^{35}S into glycosaminoglycans of the pericellular pools started promptly at zero time, without any detectable delay, seems to indicate that the delay in the extracellular accumulation of ^{35}S -labelled glycosaminoglycans was not due to diffusion-restricted access of the radioactive precursor. The accumulation of ^{35}S -labelled glycosaminoglycans in the pericellular pool decreased with time, in both glial- and glioma-cell cultures, approaching a constant value at which the amount of ^{35}S -labelled glycosaminoglycans entering and disappearing from the pool should be equal. Although the pericellular pool of glioma cultures constituted a larger proportion of the total incorporated radioactivity than that of the glial cultures, the absolute pool size (c.p.m./ μg of protein) was smaller in glioma than in normal glial cultures (Table 2).

Intracellular pool. The fraction of ^{35}S -labelled glycosaminoglycans remaining cell-associated after trypsin digestion showed a low rate of incorporation in both glial and glioma cultures (Figs. 1 and 2). After about 24 h of incorporation a constant amount of ^{35}S -labelled glycosaminoglycans was obtained. At 48 h it constituted about 13% of the total cell-associated ^{35}S -labelled glycosaminoglycans with no significant difference between the cell lines (Fig. 4).

Subdivision of the pericellular pool

After incubation of normal glial cells with EDTA for 60 min, the cells rounded up but only about 10% of

Fig. 3. Removal of ^{35}S -labelled glycosaminoglycans from dense normal (a) and malignant (b) glial-cell cultures by trypsin (○) or EDTA (●).

Cultures labelled with ^{35}S sulphate for 48 h were exposed to trypsin (50 $\mu\text{g}/\text{ml}$) or EDTA (0.5 mM) for various periods of time at 37°C. The incubation solutions were removed together with spontaneously detached cells and centrifuged (180g for 5 min). ^{35}S radioactivity was measured in the supernatants after precipitation with cetylpyridinium chloride as described in the Experimental section.

Fig. 4. Subdivision of cell-associated ^{35}S -labelled glycosaminoglycans in dense and sparse cultures of normal (a; U-787 CG) and malignant (b; U-251 MG) glial cells.

Cultures labelled with ^{35}S sulphate for 48 h were exposed either to trypsin alone or to a sequential treatment with EDTA and trypsin as described in the Experimental section. Results (means $\pm$ S.D.; $n = 5-7$) are expressed as percentages of total cell-associated ^{35}S -labelled glycosaminoglycans, i.e. the sum of trypsin-releasable glycosaminoglycans (pericellular pool; P) and glycosaminoglycans associated with the washed cell pellet after trypsin treatment (intracellular pool; C_T). No material was recovered from the culture dish after the trypsin treatment. Alternatively, ^{35}S -labelled glycosaminoglycans were determined in the soluble phase after incubation with EDTA and dispersal of the cells (EDTA fraction: E), followed by trypsin treatment of the detached cells (membrane fraction; M), and of the culture dish (substrate-attached material; S). The combined treatment gave a somewhat lower total recovery; this discrepancy is indicated in the Figure by dashed lines. The slightly less (about 20%) amount of ^{35}S -labelled glycosaminoglycans associated with the cells after the sequential treatment (C_{E+T}) compared with trypsin treatment alone (C_T) is not separately indicated.

them detached. If the EDTA solution was gently removed from the dish together with the spontaneously detached cells, 11% (range 8-13%) of total cell-associated ^{35}S -labelled glycosaminoglycans was found in the supernatant from both dense and sparse cultures (cultures labelled with ^{35}S sulphate for 48 h). After treatment with phosphate-buffered saline containing Ca^{2+} and Mg^{2+} , which did not cause any morphological effects, 7% (range 5-12%) was found in the supernatant; consequently very little, if any, ^{35}S -labelled glycosaminoglycans was specifically removed by EDTA. If, on the other hand, all cells were removed from the Petri dish by slight pipetting, more ^{35}S -labelled glycosaminoglycans, 17% (13-20%) in sparse and 20% (15-27%) in dense cultures respectively, was found in the EDTA fraction (Fig. 4). Thus a small fraction of ^{35}S -labelled glycos-

aminoglycans was liberated when contacts between cells and the Petri dish were broken. However, it could not be excluded that part of this material was caused by a release from non-viable cells or caused by liberated proteinases from damaged cells.

The EDTA-susceptible portion of ^{35}S -labelled glycosaminoglycans was considerably larger in glioma than in glial cultures, amounting to 31% (26–40%) and 60% (51–67%) of cell-associated ^{35}S -labelled glycosaminoglycans in sparse and dense cultures respectively (Fig. 4). Incubation with phosphate-buffered saline alone liberated only 7% (5–14%); therefore, in contrast with the glial cells, the glioma cells showed a significant portion of specifically EDTA-susceptible ^{35}S -labelled glycosaminoglycans.

After removal of cells with trypsin, no ^{35}S -labelled glycosaminoglycans could be recovered from the plastic dish by subsequent treatment with alkali. In contrast, detachment of the cells with EDTA rather than trypsin left significant amounts of ^{35}S -labelled glycosaminoglycans associated with the plastic surface (Fig. 4). It is noteworthy that this fraction was both relatively (Fig. 4) and absolutely (Table 2) considerably greater in sparse cultures [glial 14% (range 7–16%); glioma 21% (range 15–29%)] than in dense cultures (1–3%). Subcultivation and continuous labelling with [^{35}S]sulphate of both glial and glioma cultures over an extended period of time revealed that this pool increased with time in non-confluent cultures, but remained constant after confluency was reached (Fig. 5). This finding, in conjunction with the data in Fig. 4, indicates that no sulphated glycosaminoglycans are deposited into substrate-attached material in confluent cultures. At confluence, ^{35}S -labelled substrate-attached material was 10% and 20% of total cell-associated ^{35}S -labelled glycosaminoglycans in normal cultures and glioma cultures respectively.

Much of the ^{35}S -labelled glycosaminoglycans remaining with the cell pellet after treatment with EDTA was solubilized by subsequent trypsin treatment (designated membrane fraction). As shown in Fig. 4, glial-cell cultures showed a higher proportion of the membrane fraction than did the glioma-cell cultures. Since the absolute pool size (c.p.m./ μg of protein) of cell-associated ^{35}S -labelled glycosaminoglycans was greater in glial cells, the total amount of ^{35}S -labelled glycosaminoglycans in the membrane fraction was also greater (2–3 times) than in glioma-cell cultures. Slightly less ^{35}S -labelled glycosaminoglycans was associated with the cells after a sequential treatment with EDTA and trypsin compared with trypsin treatment alone. Also, the combined treatment gave a somewhat lower total recovery of ^{35}S -labelled glycosaminoglycans (6–21% lower than the procedure involving trypsin only). This discrepancy may be due to losses during extra washings and centrifugations. In addition, intra-

Fig. 5. Incorporation of [^{35}S]sulphate into substrate-attached material

Normal glial cells (CG; ●) and glioma cells (MG; ○) were pooled in the experimental F10 medium (MgCl_2 substituted for MgSO_4) containing $20\mu\text{Ci}$ of [^{35}S]sulphate/ml and seeded at a density of 10^5 cells/35mm culture dish. The cells were then grown in medium containing the same amount of [^{35}S]sulphate. ^{35}S -labelled glycosaminoglycans attached to the culture dish (recovered by trypsin) after detachment of all cells with EDTA were measured at the indicated times.

cellular degradation of ^{35}S -labelled glycosaminoglycans during the incubation with EDTA may be a contributing factor (Glimelius *et al.*, 1977).

Nature of glycosaminoglycans of glial and glioma cultures

Isolation of glycosaminoglycans. After 48h of incubation, labelled material from the various fractions was isolated by gel chromatography on Sephadex G-50 (Fig. 6) or by ion-exchange chromatography on DEAE-cellulose (Fig. 7). When the amounts of ^{35}S -labelled glycosaminoglycans recovered from the individual compartments by any of the methods were compared with the original amounts of cetyl-pyridinium chloride-precipitable ^{35}S -labelled glycosaminoglycans in the respective fractions, recoveries of 90–100% were found (results not shown). It is reasonable to believe that the recovery of ^3H -labelled glycosaminoglycans was similar; this entity may not be readily assessed.

The separation of macromolecular ^{35}S -labelled glycosaminoglycans from low-molecular-weight components on Sephadex G-50 was clear-cut in the extracellular and pericellular pools. However, the intracellular pools showed partial overlap between excluded and retarded material (Fig. 6). Besides, in all the glioma fractions the gel chromatography pattern showed a peak of included material with K_{av} about 0.4, amounting to approx. 15–20% of incorporated ^{35}S . This included component (operationally designated fraction B) was apparent only after

Fig. 6. Isolation of ^{35}S -labelled glycosaminoglycans by gel chromatography on Sephadex G-50. Dense normal (a-c) and malignant (d-f) glial-cell cultures were labelled with $25\mu\text{Ci}$ of ^{35}S sulphate/ml for 48 h and divided into individual compartments [extracellular (a, d), pericellular (b, e) and intracellular (c, f) pools]. After digestion with papain the material was applied to columns $[40\text{cm} \times 5\text{cm}$ (a) or $90\text{cm} \times 2\text{cm}$ (b-f)] of Sephadex G-50 as described in the Experimental section. Macromolecular material was pooled according to horizontal arrows.

Fig. 7. Ion-exchange chromatography on DEAE-cellulose of samples from the extracellular (a, d), pericellular (b, e) and intracellular (c, f) pools from normal (a-c) and malignant glial cells (d-f).

Dense cultures were labelled with $[^3\text{H}]$ glucosamine ($50\mu\text{Ci}/\text{ml}$) and ^{35}S sulphate ($10\mu\text{Ci}/\text{ml}$) for 48 h. Elution from the cellulose was made with a linear gradient of LiCl as described in the Experimental section. Standards of hyaluronic acid (HA), chondroitin sulphate (CS) and heparin (Hep) were eluted from the column as indicated by the arrows. The fraction volume was 3.0ml .

proteolysis (e.g. with papain). Its presence was not dependent on the cell density of the culture from which it was derived; it was demonstrable in sparse glioma but not sparse glial-cell cultures (results not shown). Fraction B was pooled separately from the excluded macromolecular ^{35}S -labelled glycosaminoglycans (fraction A).

The ^{35}S -labelled material retained on DEAE-cellulose was eluted from the ion-exchanger along with and slightly after the reference sample of monosulphated chondroitin sulphate (Fig. 7). Over-sulphated species, i.e. carrying more than one sulphate residue per disaccharide unit, were present in both glial and glioma glycosaminoglycans. The pericellular glycosaminoglycans of glial cultures differed from those of glioma cultures in that a major part co-migrated with the chondroitin sulphate standard. DEAE-cellulose chromatography of $[^3\text{H}]$ -glucosamine-labelled material showed similar patterns and, in addition, a distinct component co-migrating with standard hyaluronic acid. The latter was identified as hyaluronic acid as described in the Experimental section. No radioactivity was found at the elution position of standard heparin.

Identification of glycosaminoglycans

Analysis of labelled glycosaminoglycans showed marked differences between glial and glioma cultures and between different compartments of individual cultures (Tables 3-5). Analysis of isolated ^{35}S -labelled glycosaminoglycans gave results similar

Table 3. Composition of ³H-labelled glycosaminoglycans derived from various compartments of dense cultured normal glial and malignant glioma cells

Cultures were labelled with 50 µCi of [³H]glucosamine/ml for 48 h. ³H-labelled glycosaminoglycans were isolated by ion-exchange chromatography on DEAE-cellulose; individual species of glycosaminoglycans were quantified as described in the Experimental section. Resistant ³H-labelled glycosaminoglycans retained macromolecular size on treatment with HNO₂ and chondroitinases. For definition of glucuronic acid-poor and -rich ³H-labelled dermatan sulphate respectively, see the legend to Fig. 8. Abbreviations used: HS, heparan sulphate; CS, chondroitin sulphate; DS, dermatan sulphate; GA, glucuronic acid.

Cells	Compartment	Total ³ H-labelled glycosaminoglycans		Sulphated ³ H-labelled glycosaminoglycans				³ H-labelled DS	
		Hyaluronic acid (%)	Sulphated (%)	HS (%)	CS (%)	DS (%)	Resistant (%)	GA-poor (%)	GA-rich (%)
Normal glial cells	Extracellular	11	89	17	18	59	6	80	20
	Pericellular	3	97	76	13	10	1	70	30
	Intracellular	2	98	12	22	49	17	87	13
	Total	9	91	34	17	49	6	79	21
Malignant glioma cells	Extracellular	88	12	14	71	6	5	26	74
	Pericellular	64	36	36	50	5	9	50	50
	Intracellular	57	43	18	49	4	29	64	36
	Total	85	15	24	69	6	10	35	65

Table 4. Composition of ³⁵S-labelled glycosaminoglycans derived from various compartments of normal glial and malignant glioma-cell cultures labelled with [³⁵S]sulphate for 48 h

Cultures were labelled with [³⁵S]sulphate for 48 h. Heparan sulphate (HS), chondroitin sulphate (CS) and dermatan sulphate (DS) were estimated by treatment with HNO₂ and chondroitinases respectively, either by using isolated ³⁵S-labelled glycosaminoglycans and quantification of degradation products by gel chromatography on Sephadex G-50, or by using culture fluids directly and quantification of susceptibility by a cetylpyridinium chloride precipitation (CPC) procedure. Results are means±s.d. for 3–12 separate experiments; when no s.d. is indicated, results are means from one experiment with duplicate dishes. NT = not tested. See the Experimental section for a definition of compartments.

Cells	Compartment	Dense cultures								
		Isolated ³⁵ S-labelled glycosaminoglycans			CPC procedure			Sparse cultures (CPC procedure)		
		HS (%)	CS (%)	DS (%)	HS (%)	CS (%)	DS (%)	HS (%)	CS (%)	DS (%)
Normal glial cells	Extracellular	24	26	49	26±2	26±3	51±7	25±3	34±6	41±5
	Pericellular	80	13	7	79±4	14±4	7±1	81±5	16±3	7±3
	EDTA fraction	31	6	63		NT			NT	
	Substrate-attached material		NT			NT		75±9	19±9	4±4
	Membrane fraction		NT		85	11	3	86±3	10±3	4±2
	Intracellular	36	18	57		NT			NT	
Malignant glioma cells	Extracellular	12	79	2	17±4	81±4	4±2	14±6	84±5	1±2
	Pericellular	30	61	10	28±8	72±8	1±0	33±10	64±9	3±1
	EDTA fraction		NT		16±6	83±6	1±0	19±7	86±7	
	Substrate-attached material		NT			NT		1±0	97±1	1±0
	Membrane fraction		NT		38		69	42±9	59±9	
	Intracellular	23	77	0		NT			NT	

to those for ³H-labelled glycosaminoglycans, irrespective of whether DEAE-cellulose chromatography or Sephadex G-50 chromatography (fraction A) was used for the preparation of ³⁵S-labelled glycosamino-

glycans (results not shown). Fraction B, being almost completely resistant to HNO₂ and chondroitinase ABC, escaped identification by the methods used in the present study.

Table 5. Production of ^3H -labelled glycosaminoglycans by sparse cultures of normal glial and malignant glioma cells. Cultures were labelled with $25\text{ }\mu\text{Ci}$ of $[^3\text{H}]\text{glucosamine/ml}$ for 48 h. Corresponding fractions from duplicate dishes were pooled before analysis. The protein concentrations ($\mu\text{g/dish}$) were as follows: normal (135) and malignant (170) cultures respectively. See also the legend to Table 2.

Cells	Compartment	Hyaluronic acid			Sulphated glycosaminoglycans			Hyaluronic acid (% of total glycosamino- glycans)
		(c.p.m./ μg of protein)	(% of total)	(% of peri- cellular pool)	(c.p.m./ μg of protein)	(% of total)	(% of peri- cellular pool)	
Normal glial cells	Extracellular	62	89		1408	79		4
	Pericellular	7	10	100	331	19	100	2
	EDTA fraction	4		51	51		15	8
	Substrate-attached material	3		44	38		11	7
	Calculated mem- brane fraction	0		5	250		74	0
	Intracellular	1	1		37	2		3
	Total	70	100		1780	100		4
Malignant glioma cells	Extracellular	1633	90		401	60		80
	Pericellular	168	9	100	237	36	100	42
	EDTA fraction	106		63	48		20	69
	Substrate-attached material	20		12	74		31	21
	Calculated mem- brane fraction	42		25	114		49	27
	Intracellular	26	1		25	4		51
	Total	1827	100		663	100		73

The composition of ^{35}S -labelled glycosaminoglycans was also determined by a simplified procedure, enabling analysis of culture fluids without prior isolation of glycosaminoglycans (Table 4). The results were compared with those obtained by a standard procedure; good agreement between the two procedures was noted. In this experimental protocol the susceptibility of isolated ^{35}S -labelled glycosaminoglycans (from dense cultures) to HNO_2 and chondroitinases (see the Experimental section) was monitored by Sephadex G-50 chromatography; the relative amounts of heparan sulphate, dermatan sulphate and chondroitin sulphate were calculated according to the same principles as those used for the cetylpyridinium chloride procedure. It is noteworthy that the cetylpyridinium chloride technique, although much simpler and more rapid than the Sephadex G-50 method, is inevitably less informative with respect to the nature of the degradation products.

Normal glial cells. The extracellular pool of glial cultures showed a predominance of dermatan sulphate (about 55% of the pool), the remainder being made up by heparan sulphate and chondroitin sulphate in approximately equal proportions (about 20% each) (Tables 3 and 4). A major proportion (about 80%) of dermatan sulphate resisted treatment with chondroitinase AC, in that it showed an unchanged elution with the void volume on Sephadex G-50 chromatography (Fig. 8). The remainder (about 20%) was degraded to smaller fragments by

chondroitinase AC, which thus represented relatively short blocks of iduronic acid-containing units. Hyaluronic acid was demonstrable in the extracellular pool but was only a minor component (10% of the total glycosaminoglycans). Most of the hyaluronic acid (over 90%) of glial cell cultures was found in the extracellular pool (Table 5).

The pericellular pool showed a typical composition of glycosaminoglycans, heparan sulphate being by far the most abundant species (75–80% of glycosaminoglycans). Dermatan sulphate and chondroitin sulphate were only minor components (about 10% each) and hyaluronic acid was present only in trace amounts.

The intracellular glycosaminoglycans resembled extracellular glycosaminoglycans, dermatan sulphate being the predominant species. A significant portion of the intracellular pool (17%) was not susceptible to the combined treatment with HNO_2 and chondroitinase ABC (Table 3).

Great differences were found between the compositions of the various subfractions of the pericellular pool. The EDTA fraction, like the extra- and intracellular pools, showed a predominance of dermatan sulphate, whereas the other subfractions were mainly composed of heparan sulphate (Table 4). In the membrane fraction, almost 90% of glycosaminoglycans was heparan sulphate, chondroitin sulphate was a minor component and dermatan sulphate and hyaluronic acid were present only in trace amounts.

Fig. 8. Gel chromatography on Sephadex G-50 of ^3H -labelled galactosaminoglycans digested with chondroitinase AC

^3H -labelled HNO_2 -resistant glycosaminoglycans from the extracellular pools of dense normal (a) and malignant (b) glial cell cultures were incubated with chondroitinase AC; the reaction mixtures were analysed by gel chromatography on a column (130 cm $\times$ 1 cm) of Sephadex G-50. The glial galactosaminoglycans yielded a higher ratio of macromolecular (elution volume 43–54 ml) to intermediate size (elution volume 54–92 ml) than did the glioma galactosaminoglycans. These two types of products were taken to represent glucuronic acid-poor and glucuronic acid-rich ^3H -labelled dermatan sulphate respectively; see also the text and Table 3. V_0 and V_t of the column were determined by chromatography of ^{35}S -labelled chondroitin sulphate isolated from chick cartilage (M_w 28000) and inorganic [^{35}S]-sulphate respectively.

Glioma cells. All the compartments of glioma cultures contained a high proportion of hyaluronic acid, in contrast with the corresponding fractions of glial cultures (Table 3). This was particularly striking in the extracellular pool, where about 90% of total glycosaminoglycans was hyaluronic acid. Among the sulphated glycosaminoglycans of the extracellular pool glioma and glial cultures displayed similar ratios of HNO_2 -susceptible to chondroitinase ABC-susceptible glycosaminoglycans. However, in glioma cultures, the latter pool consisted mostly of chondroitin sulphate, whereas in glial cultures dermatan sulphate was predominant. The size distribution of the products obtained after treatment with chondroitinase AC showed that, not only were iduronic acid residues more scarce in glycosaminoglycans of the glioma than of the glial cells, but also blocks consisting of consecutive iduronic acid-containing disaccharides were shorter in glycosaminoglycans of glioma than of glial cells (Fig. 8; Table 3). The pericellular pool consisted, apart from hyaluronic acid, mainly of chondroitin sulphate and heparan sulphate; the predominance of heparan sulphate, typical of the pericellular pool of glial cultures, was not observed in the glioma-derived material (Tables 3 and 4). The

intracellular pool, like the extracellular pool, showed chondroitin sulphate as the most abundant sulphated glycosaminoglycan; iduronic acid-containing disaccharides were rare (Table 3). The amount of sulphated glycosaminoglycans resisting the combined action of HNO_2 and chondroitinase ABC was considerable (about 30%); as shown above the glial intracellular pool contained similarly a significant amount of resistant material. The identity of the resistant glycosaminoglycans was not resolved; however, a possible candidate would be *N*-acetylated heparan sulphate, which has previously been demonstrated in brain tissue (Margolis & Atherton, 1972).

Material released by EDTA from the glioma cells showed a higher proportion of hyaluronic acid and a slightly lower proportion of heparan sulphate than the whole pericellular pool; the differences were, however, much smaller than for the normal cells (Tables 4 and 5). The membrane fraction was enriched in heparan sulphate, compared with the whole pericellular pool; a similar finding was made with normal glial-cell cultures. However, the proportion of heparan sulphate in the membrane fraction of normal cells was consistently higher (86%; range 81–92%) than in glioma cells (42%; range 36–57%). Substrate-attached material of glioma cultures did not show any detectable heparan sulphate, whether examined after labelling with [^{35}S]sulphate (Table 4) or [^3H]glucosamine (results not shown); rather, the only sulphated glycosaminoglycan was chondroitin sulphate. The composition of glioma substrate-attached material was therefore markedly different from that of normal glial substrate-attached material, in which heparan sulphate was the predominant species.

Effects of cell density on synthesis of glycosaminoglycans

The total net incorporation of [^{35}S]sulphate or [^3H]glucosamine into glial and glioma glycosaminoglycans after 48 h of radioactive labelling is shown in Tables 2 and 5 respectively. Glial cells showed a higher rate of production of sulphated glycosaminoglycans (c.p.m./ μg of protein) than did glioma cells. Furthermore, sparse cultures consistently produced slightly higher amounts of sulphated glycosaminoglycans (c.p.m./ μg of protein) than did dense cultures (Table 2). Normal glial cultures showed approximately the same ratio of extracellular to cell-associated ^{35}S -labelled glycosaminoglycans (4:1) irrespective of cell density. In contrast, glioma cultures showed more cell-associated ^{35}S -labelled glycosaminoglycans under dense (about 40%) than under sparse conditions (about 30%). This phenomenon may reflect a passive entrapment of glycosaminoglycans between cells of the crowded multilayer glioma cultures, or may alternatively represent a specific formation of intercellular matrix.

Normal glial cells produced little hyaluronic acid (less than 10% of total glycosaminoglycans) (Table 5). Sparse and dense cultures were roughly equal in this respect. Unlike normal glial cells, glioma cells showed hyaluronic acid as the major glycosaminoglycan, sparse cultures producing almost twice as much hyaluronic acid (c.p.m./ μ g of protein) as dense cultures (results not shown). The proportions of heparan sulphate/dermatan sulphate/chondroitin sulphate were constant in both types of cells irrespective of cell density (Table 4).

Discussion

A number of cultured cells of various origins have previously been shown to produce glycosaminoglycans. Previous observations in our laboratory indicated that cultured cells of glial origin manufactured both sulphated and non-sulphated glycosaminoglycans (Wasteson *et al.*, 1973a,b; Westermark & Wasteson, 1973; Wasteson *et al.*, 1975; Norling *et al.*, 1976). The present results confirm and extend these findings, demonstrating the ability of human normal and malignant glial cells in culture to produce and secrete three species of sulphated glycosaminoglycans, i.e. heparan sulphate, dermatan sulphate and chondroitin sulphate, and the non-sulphated glycosaminoglycan hyaluronic acid. Dorfman & Ho (1970) isolated heparan sulphate, chondroitin sulphate and hyaluronic acid from a cell line derived from an experimentally induced mouse glial tumour; the proportions of individual glycosaminoglycans were similar to those displayed by the human glioma line used in the present study. More recently, Fluharty *et al.* (1975) observed the incorporation of [35 S]sulphate into glycosaminoglycans of human cultured glial cells; in this study, the ratio of chondroitinase ABC-resistant to chondroitinase ABC-susceptible material in the extracellular pool was similar to the ratio of heparan sulphate to dermatan sulphate plus chondroitin sulphate found in the present investigation (Table 4). Glycosaminoglycans have previously been isolated from various kinds of brain tissue, including purified astrocytes (Margolis & Margolis, 1974). Therefore the ability to produce this kind of substance may reflect a physiological property of glial cells rather than being a product of the conditions under which the cells were maintained *in vitro*. The functional significance of glycosaminoglycans in the nervous system is still unclear (Margolis & Margolis, 1977).

In the present study [35 S]sulphate and/or [3 H]-glucosamine were used as markers of newly formed glycosaminoglycans. The kinetics of accumulation of 35 S-labelled glycosaminoglycans in the extracellular pool (Figs. 1 and 2) suggest that glycosaminoglycans of glioma cells may enter this compartment via routes different from those used by glial glycos-

aminoglycans. The results presented here together with pulse-chase experiments (B. Glimelius, B. Norling, B. Westermark & Å. Wasteson, unpublished work) indicate that in glioma cultures most 35 S-labelled glycosaminoglycans found in the extracellular pool are retained at the cell surface for a considerably longer time than in normal glial cultures.

Glycosaminoglycans liberated from their cell-associated state by treatment with a dilute trypsin solution were considered as the pericellular pool. Since the use of trypsin for this purpose was introduced by Kraemer (1971a) it has been generally recognized and adapted in several investigations (e.g. Underhill & Keller, 1976). However, by use of trypsin only, no distinction can be made between the intercellular matrix and the cell surface (plasma membrane) (Chiarugi *et al.*, 1974; Kleinman *et al.*, 1975). Such a distinction is critical to the understanding of the role of cell-surface-associated glycosaminoglycans in cellular-interaction phenomena (Vaheri, 1978), but, since methods for a direct analysis of glycosaminoglycans at different localities of a cell culture do not exist, we are left to operational definitions. In the present investigation attempts at a subdivision of cell-surface-associated glycosaminoglycans were performed by treatment of the cells with EDTA before trypsin treatment. The major drawback of EDTA treatment (Culp & Black, 1972) but also of treatment with collagenase (Kleinman *et al.*, 1975) is decreased viability of the cells. However, the finding that EDTA liberated only slightly more glycosaminoglycans than treatment with phosphate-buffered saline indicates that a major part of glycosaminoglycans solubilized by EDTA was not due to a release from damaged cells. Rather, the EDTA fraction may consist of material from other compartments including entrapped material from the medium and matrix components. The fraction obtained from the EDTA-dispersed cells or from the intact glial-cell layer by treatment with trypsin was markedly homogeneous; 85 and 80% respectively consisted of heparan sulphate. Most of this heparan sulphate could be released from the cells by treatment with a heparan sulphate-degrading endoglycosidase (Wasteson *et al.*, 1977). The observations favour the view that mild trypsin treatment as used in the present investigation, without any pretreatment, yields a fraction representative of cell-surface-associated glycosaminoglycans with slight admixture of intracellular glycosaminoglycans and entrapped material. The trypsin treatment of the EDTA-dispersed cells yields a fraction enriched in plasma-membrane components; contamination from intracellular leakage or remaining matrix components could, however, not be excluded.

In glioma cultures, unlike normal glial cultures, considerably more cell-associated sulphated glycos-

aminoglycans were liberated by incubation with EDTA (26–40% in sparse and 51–67% in dense cultures; Fig. 4) than with phosphate-buffered saline (5–14% in sparse or dense cultures). This may indicate that glycosaminoglycans of the two types of cultures have different modes of association with the cell surface. The relatively large amount of EDTA-susceptible glycosaminoglycans in dense as compared with sparse glioma cultures (Fig. 4) was ascribed to a greater intercellular pool (entrapment?) of glycosaminoglycans in the crowded multicellular layer of the dense cultures. Similar observations were not made in the normal glial cultures, in which growth ceases when cells have formed a single layer.

The substrate-attached fraction of glycosaminoglycans showed a saturability resembling that reported for 3T3 cell cultures (Culp *et al.*, 1975). This helps explain why much less radioactivity was incorporated into this fraction in dense cultures than in sparse cultures. The composition of substrate-attached glycosaminoglycans of both glial and glioma cells (Tables 4 and 5) differed from that of 3T3 cells. Previous reports have also indicated higher amounts of substrate-attached glycosaminoglycans in non-transformed than in virus-transformed 3T3 cells (Terry & Culp, 1974; Roblin *et al.*, 1975). Analogous comparison between normal glial and malignant glioma cells, however, yielded opposite results. In conclusion, these findings indicate that the results obtained with the pseudo-normal 3T3 cell line are not generally applicable to human cells. Whether substrate-attached glycosaminoglycans play a role in density-dependent inhibition of growth, as has been suggested (Roblin *et al.*, 1975; Culp, 1976), remains unclear.

The high proportion of heparan sulphate in the pericellular pool of normal glial cells agrees with previous reports demonstrating the predominance of heparan sulphate on the cell surface of certain established rodent cell lines (Kraemer, 1971*b*). Similarly, the pericellular sulphated glycosaminoglycans of human endothelial cells is almost exclusive heparan sulphate (Wasteson *et al.*, 1977). In contrast, previous experiments with human skin fibroblasts have shown a significantly lower proportion of heparan sulphate (Malmström *et al.*, 1975). Pre-treatment of skin fibroblasts with collagenase markedly increased the proportion of heparan sulphate in the remaining cell-surface fraction (recovered by trypsin) (Kleinman *et al.*, 1975); this finding clearly indicates that admixture of deposited intercellular material distorts the estimated proportions of individual glycosaminoglycans in the cell-surface fraction. In glioma-cell cultures hyaluronic acid, rather than heparan sulphate, was the predominant species among pericellular glycosaminoglycans (Table 3). Similarly, Vannucchi & Chiarugi (1977) demonstrated more hyaluronic acid and less

heparan sulphate in a cell-surface-associated fraction of growing or virus-transformed 3T3 cells compared with resting 3T3 cells. However, other investigations indicate that virus transformation of 3T3 cells is not consistently accompanied by such a change in cell-surface-associated hyaluronic acid or heparan sulphate (Underhill & Keller, 1975; Cohn *et al.*, 1976).

The definition of chondroitin sulphate and dermatan sulphate used in the present investigation was based on the observed susceptibility of glycosaminoglycans to treatment with chondroitinases, and hence related to the relative content of glucuronic acid and iduronic acid (Yamagata *et al.*, 1968). Since glucuronic acid-containing units are known to form co-polymeric chains with iduronic acid-containing units (Fransson, 1970) the estimated amounts of dermatan sulphate represent minimum values. The underestimation of dermatan sulphate tends to be greater by the cetylpyridinium chloride procedure than by the gel-chromatography analysis, since the former procedure would not detect small iduronic acid-containing oligosaccharides among the chondroitinase-digestion products. The glioma-derived dermatan sulphate showed a higher proportion of short iduronic acid-containing sequences; these components were formed from co-polymeric chains with an unknown ratio of glucuronic acid to iduronic acid. However, irrespective of the co-polymeric composition, the glioma cells formed less iduronic acid-containing repeating units than did the glial cells. It is thus possible that the glioma cells are deficient in the enzymic complex dealing with the conversion of glucuronic acid into iduronic acid in the polysaccharide chain.

The malignant glioma cells produced far more hyaluronic acid, but less sulphated glycosaminoglycans, than did the normal glial cells (Tables 2 and 5). The significance of this finding is not clear. If the observed differences are representative of conditions *in vivo*, it is reasonable to conclude that the tumour cells surround themselves with and grow in an intercellular matrix that is quite different from that of normal glial cells. In some systems of virus-transformed cells, the transformed state has similarly been shown to be associated with an increase in hyaluronic acid production (Ishimoto *et al.*, 1966; Satoh *et al.*, 1973) or a decrease in the production of sulphated glycosaminoglycans (Underhill & Keller, 1975), as compared with the non-transformed state. However, these phenomena are probably not universal (Hamerman *et al.*, 1965; Cohn *et al.*, 1976). Preliminary results indicate that different lines of normal glial cells have a similar glycosaminoglycan pattern, whereas different glioma lines show a great variability (B. Glimelius, B. Norling, B. Westermark & Å. Wasteson, unpublished work); the variability in the production of

hyaluronic acid by different glioma lines (Westermark & Wasteson, 1973) is one illustration of that point.

Comparison between glial and glioma cultures indicated several differences with respect to amounts and proportions of various types of glycosaminoglycans in the different culture compartments. Since cell density had little influence on the parameters under study (Tables 2 and 4), it may be concluded that the observed differences between glial and glioma cultures were due to the malignant transformation itself rather than to differences in cell density or growth state. With the 3T3 cell system, other authors (Cohn *et al.*, 1976; Vannucchi & Chiarugi, 1977) reached an opposite conclusion, i.e. that cell density and possibly density-dependent inhibition of growth, but not viral transformation, are major factors controlling glycosaminoglycan metabolism.

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