Cell Surface Changes Correlated with Density-Dependent Growth Inhibition. Glycosaminoglycan Metabolism in 3T3, SV3T3, and Con A Selected Revertant Cells†

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ABSTRACT: A $^{35}SO_4$-labeling/chromatography technique has been developed which facilitates quantitation of sulfated glycosaminoglycan (GAG) synthesis in mammalian cell cultures. The technique has been used to compare sulfated GAG biosynthesis, degradation, and turnover in three related cell lines with differing degrees of density-dependent inhibition of growth *in vitro* (Balb/c 3T3, SV3T3, and SV3T3 revertant cells). Viral transformation of Balb 3T3 cells is accompanied by a 2–5-fold decrease in cell associated sulfated GAG. SV3T3 revertant cells, which show partial reversion to low saturation density *in vitro*, show a 2.5–8-fold increase in cell-associated sulfated GAG compared to the parental SV3T3 cells from which they were selected. In addition, the distribution of $^{35}SO_4$ and [^{3}H]glucosamine among the different GAG species produced by SV3T3 revertant cells reverts so that it is similar to the distribution characteristic of untransformed 3T3 cells rather than SV3T3 cells. Mild trypsin treatment of $^{35}SO_4$-labeled cells removed 68–84% of the cellular sulfated GAG, suggesting that at least this proportion of the total cellular sulfated GAG was located at the cell periphery. Removal of $^{35}SO_4$-labeled cells from the Petri dish with a Ca^{2+} selective chelating agent revealed a fraction of the sulfated GAG that remained tightly bound to the Petri dish. A higher proportion of the total cell-associated sulfated GAG remained attached to the Petri dish in cultures of untransformed and revertant cells compared to that present in cultures of transformed cells. A role for sulfated GAG in density-dependent growth inhibition of fibroblast cultures is proposed and discussed in the light of the data obtained.

Simian virus 40 (SV40) induced cell transformation of mouse 3T3 cell lines results in loss of density-dependent inhibition of cell growth *in vitro* (Todaro *et al.*, 1964). Since density-dependent inhibition of cell growth cannot be explained simply on the basis of visible cell-to-cell contacts (Martz and Steinberg, 1972), many investigators have studied the possibility that loss of such inhibition is due to biochemical changes at the cellular membrane surface of virus-transformed cells. Changes is sialic acid content (Ohya *et al.*, 1968; Culp *et al.*, 1971), membrane glycoproteins (Sakiyama and Burge, 1972), and glycolipids (Yogeeswaran *et al.*, 1972) have been shown to accompany SV40 virus-induced cell transformation, although proof that these changes are connected with loss of density-dependent inhibition of growth is still lacking.

Morphological variants of transformed cells, which showed lowered saturation densities *in vitro*, have been selected from populations of SV3T3 cells by exposing the transformed cells to the plant lectin, concanavalin A (Ozanne and Sambrook, 1971; Culp and Black, 1972a). These variant cell lines have been called "revertants" because their morphologies and *in vitro* saturation densities have reverted to resemble those of untransformed 3T3 cells. By comparative study of cell surface materials of 3T3, SV40 transformed 3T3 (SV3T3), and revertant (Rev) cells, it may be possible to correlate changes in cell surface components with changes in cell saturation density *in vitro*. Reversion of the 50–70 Å microfilament content (McNutt *et al.*, 1973) and sialic acid content (Culp and Black, 1972a) in concanavalin A selected revertants toward that found in untransformed cells has already been demonstrated.

Sulfated glycosaminoglycans (GAG)[1] are components of the intercellular matrix of connective tissue fibroblasts, and are secreted by fibroblast-like cells grown in tissue culture (Suzuki *et al.*, 1970). Highly sulfated GAG (dextran sulfate and heparin) have been shown to exert a growth inhibitory action on untransformed cells grown in agarose suspension cultures (Montagnier 1971). In addition, low concentrations (10–50 μg/ml) of exogenous dextran sulfate can reduce the saturation density (Goto *et al.*, 1973) as well as the fraction of cells which synthesize DNA in conventional liquid medium cultures (Clarke and Stoker, 1971). Previous studies have consistently demonstrated decreases in endogenous sulfated GAG synthesis in SV3T3 cells, compared with their untransformed 3T3 cell counterparts (Saito and Uzman, 1971; Goggins *et al.*, 1972, Terry and Culp, 1974). We have, therefore, compared the production, distribution, and turnover of sulfated GAG in untransformed Balb 3T3 cells, SV3T3 cells, and concanavalin A selected SV3T3 Rev cells in order to determine whether these aspects of the metabolism of sulfated GAG are correlated with density-de-

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[1] Abbreviations used are: GAG, glycosaminoglycan; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid, disodium salt; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; [^{3}H]TdR, thymidine-^3H; CPC, cetylpyridinium chloride; CTAB, cetyltrimethylammonium bromide; Con A, concanavalin A; SH, streptomyces hyaluronidase; TH, testicular hyaluronidase; $(but)_2$cAMP, N^6-$O^{2'}$-dibutyryl cyclic adenosine 3′,5′-monophosphate, C4S, chondroitin 4-sulfate; C6S, chondroitin 6-sulfate; DS, dermatan sulfate; HA, hyaluronic acid; Ch, chondroitin; Paps, 3′-phosphoadenosine 5′-phosphosulfate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

pendent inhibition of cell growth *in vitro*.

Materials and Methods

Origin and Preservation of Cell Lines. Balb/c 3T3 cells (clone A31) after 40 generations and Balb/c SV3T3 cells (clone SVT2) after 150 generations were obtained from Dr. Stuart Aaronson and preserved by storage in liquid nitrogen in the presence of 7.5% dimethyl sulfoxide. Two independently derived clones (80 and 84) of concanavalin A-selected Balb SV3T3 Rev cells (Culp and Black, 1972a) were obtained through the courtesy of Dr. Lloyd Culp and were similarly preserved. All experiments reported here were done with the cell lines between the 5th and 30th passage levels in our laboratory.

When used in these experiments, the SV3T3 Rev lines had saturation densities of 2.5×10^5 cells/cm^2 (Rev 84) and 3.1×10^5 cells/cm^2 (Rev 80), higher than those previously reported for these lines (Culp and Black, 1972a), but still substantially below that of SV3T3 cells. In addition, both Rev cell lines showed enhanced density-dependent inhibition of DNA synthesis (determined by autoradiography of [^{3}H]TdR-labeled confluent cultures) compared to the transformed SV3T3 parent cell line. Since it had the lower saturation density, Con A Rev line 84 was used in most of the experiments described below.

Mycoplasma Analyses. All cell lines have been repeatedly checked for mycoplasma contamination by two different assays: (a) autoradiography after 4-hr pulse of [^{3}H]thymidine (method in Culp and Black, 1972a); and (b) attempts to culture mycoplasma in the laboratories of Dr. Louis Dienes (Madoff, 1960) and/or Dr. Leonard Hayflick. After the cells had been used for a series of experiments, and reached the 30th passage level, they were checked for mycoplasma by these two assays. No mycoplasma contamination has been detected in any of these cell lines during their use in the experiments reported here.

Conditions of Cell Culture and $^{35}SO_4$ Labeling. All cell lines were passaged routinely in Eagle's minimal essential medium containing four times concentrated amino acids and vitamins (MEM X4), 10% fetal calf serum, 250 U/ml of penicillin, and 250 μg/ml of streptomycin sulfate. For $^{35}SO_4$ labeling experiments cells were plated in 100-mm Falcon plastic Petri dishes at $0.5\text{–}2 \times 10^6$ cells/dish. After 18–24 hr in unlabeled medium, 8 ml of fresh medium containing $Na^{35}SO_4$ (New England Nuclear, 660–946 Ci/mol) at 20 μCi/ml was added. The inorganic sulfate concentration in the medium was approximately 1.3 mM. GAG were labeled with [^{3}H]glucosamine by growing cells in medium containing 5 μCi/ml of [^{3}H]glucosamine (Amersham/Searle, 2.6 Ci/mmol).

Analysis of $^{35}SO_4$-Labeled Cell Cultures. $^{35}SO_4$-containing growth medium from labeled cultures was centrifuged at 10,000g for 30 min at 4°, and subsequently dialyzed against TD buffer (8.0 g of NaCl, 0.38 g of KCl, 0.1 g of Na_2HPO_4 and 3.0 g of Tris base per l. of distilled water, pH 7.4) containing 10^{-2} M sodium sulfate. The $^{35}SO_4$-labeled cultures were quickly washed three times with 5 ml of warm complete medium. Three such washes sufficed to bring the $^{35}SO_4$ radioactivity in the wash solutions to a constant value, less than 0.01% of the original radioactive medium. The entire $^{35}SO_4$-labeled cell monolayer was then solubilized by adding 2 ml of sodium dodecyl sulfate (SDS) buffer (1% SDS (w/v), 10^{-4} M ethylenediaminetetraacetic acid, disodium salt, 10^{-3} M dithiothreitol, 0.05 M phosphate buffer (pH 7.2) and 10^{-3} M sodium azide), and scraping the resulting viscous solution from the Petri dish with a rubber policeman. This solution was homogenized with 10–15 strokes in a small Dounce homogenizer to complete disruption of the sample and reduce its viscosity. Cell numbers were determined by counting cells removed by trypsin from replicate unlabeled plates, seeded, and maintained in parallel with those containing $^{35}SO_4$ medium. The protein content of cell monolayers was determined by careful removal of all growth medium, solubilization of the monolayer in 2 ml of 0.5 N NaOH, and protein assay by a slight modification of the method of Lowry *et al.* (1951). In this modification, omission of the 0.1 N NaOH in the initial alkaline solution (Na_2CO_3/NaOH) of the Lowry method was required to obtain reproducible results, and sodium citrate was used in place of potassium tartrate.

SDS/G-200 Sephadex Chromatography. Sephadex G-200, swollen in distilled water, was equilibrated with SDS buffer (above) by repeated washing and decantation. Columns (1 cm × 25 cm) were eluted with SDS buffer; 0.3-ml samples for chromatography were made 1% SDS and 10^{-2} M dithiothreitol and heated at 100° for 2 min to help ensure complete disaggregation. Approximately 0.6-ml column fractions were collected, combined with 10 ml of Aquasol, and counted in a Packard scintillation counter.

Trypsin Treatment of $^{35}SO_4$-Labeled Cell Monolayers. Samples of the different cell lines were labeled by growth in medium containing $^{35}SO_4$ for 48 hr. After removal of the labeled medium, the plates were washed three times with prewarmed, unlabeled medium and the cells were released from the plates by incubation at 37° with 3 ml of 0.02% trypsin/EDTA solution (NaCl, 8.0 g; KCl, 0.4 g; dextrose, 1.0 g; $NaHCO_3$, 0.58 g; Na_2EDTA, 0.2 g; distilled water to 1 l., final pH 7.3). The trypsin solution contained 40 mg of trypsin-TPCK (Worthington Biochem., specific activity = 190 U/mg) dissolved in 20 ml of EDTA buffer immediately before use. The cells were incubated with the prewarmed trypsin solution just long enough to release them from the plates (8–9 min), then the action of trypsin was stopped by adding 0.5 ml of fetal calf serum. Aliquots of the resulting cell suspension were removed and the percentage of viable cells was determined with the Trypan Blue dye exclusion technique. The percentages of viable cells after trypsin/EDTA detachment were 99.5, 99.3, and 98.0% for Balb 3T3, Balb SV3T3, and Balb SV3T3 Rev 84 cells, respectively. The remaining cell suspension was centrifuged at 10,000g for 20 min at 4°, the clear supernatant ("trypsinate") was withdrawn, and the cell pellet was dispersed by adding 2 ml of SDS buffer and homogenizing the resulting solution; 0.3-ml aliquots of the "trypsinate" and the SDS pellet extract were chromatographed on SDS/G-200 columns as described above.

EGTA Detachment of Cells, Dish-Attachment of Sulfated GAG. Samples of the different cell lines were labeled by growth in medium containing $^{35}SO_4$ (20 μCi/ml) and [^{3}H]TdR (0.25 μCi/ml) for 36 hr. After removal of the labeled medium, the plates were washed three times with prewarmed complete medium, then the cells were removed from the dishes by incubation with 4 ml of a 37° solution of EGTA (2×10^{-3} M in phosphate buffered saline (8.0 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, and 0.2 g of KH_2PO_4/l. of distilled water)) on a shaker at 37°. After the cells were completely removed from the dishes (20–40 min), the EGTA cell suspension was removed and the plates were rinsed three times with 5-ml portions of the EGTA solution. Radioactive material remaining attached to the dish

after removal of all the microscopically visible cells was extracted by adding 1 ml of SDS chromatography buffer and scraping the dish with a rubber policeman. The EGTA cell suspension was centrifuged at 10,000*g* for 20 min at 4°, the EGTA supernatant was removed, and the resulting cell pellet was dissolved by homogenization in 2 ml of SDS buffer. These SDS cell pellet extracts were dialyzed against three changes of a buffer containing 1% SDS, 0.05 M sodium phosphate (pH 7.2), 10^{-3} M sodium azide to remove $^{35}SO_4$, and other small molecular weight precursors. Aliquots of the SDS dish extract, and SDS cell pellet extract were counted in 10 ml of Aquasol. Appropriate standard solutions containing [^{3}H]TdR or $^{35}SO_4$ in SDS buffer were counted to permit accurate separation of ^{3}H from $^{35}SO_4$ cpm.

Turnover of Labeled Cellular Sulfated GAG. Cells were seeded into 60-mm Petri dishes at 2–4 × 10^5 cells/dish; 24 hrs after plating, the cells were labeled by growth in medium containing 20 μCi/ml of $^{35}SO_4$ until the cell layer became confluent (36–48 hr). The radioactive medium was removed and the plates were washed four times with warm medium and incubated in fresh complete medium at 37°. At various times after removal of the radioactive medium, the chase medium was removed and the entire contents of the cell layer was solubilized by addition of 2 ml of SDS buffer and homogenization. Aliquots of the cell extract were chromatographed on SDS/G-200 Sephadex columns as described above to determine the amount of high molecular weight sulfated GAG remaining. The chase medium was dialyzed against three changes of TD/SO_4 buffer and aliquots were counted to determine the amount of high molecular weight sulfated GAG released into the medium.

Characterization of $^{35}SO_4$-Labeled Materials. Aliquots of $^{35}SO_4$-labeled materials from cell culture media were dialyzed against either TD buffer containing 10^{-2} M sodium sulfate or 0.1 M NaCl–0.1 M sodium acetate buffer (pH 5.1) and portions were digested with chondroitinase ABC (Miles Laboratories, 0.25 unit) or testicular hyaluronidase (Worthington, HSEP 11,000 USPU/mg, 50 μg) respectively. Following overnight incubation at 37°, control and enzyme-treated samples were made 1% SDS and 10^{-2} M dithiothreitol, heated for 2 min at 100°, and chromatographed on SDS/G-200 Sephadex columns. Control samples, incubated in buffer alone, contained more than 93% of the $^{35}SO_4$ label in a high molecular weight form.

Other aliquots of $^{35}SO_4$ and [^{3}H]glucosamine labeled materials from dialyzed cell culture media were concentrated 10–20-fold by negative pressure dialysis and digested with Pronase (Calbiochem, B. grade, 1 mg/1.0–1.5 ml of concentrated medium) at 50° overnight. The digests were then dialyzed against 500 volumes of distilled water, frozen and thawed once, and centrifuged at 27,000*g* for 20 min at 4°. The clear supernatants containing 63–76% of the initial $^{35}SO_4$ radioactivity were withdrawn and duplicate 1-ml aliquots were treated with chondroitinase ABC (0.05 unit, in 0.01 M Tris (pH 8.0), 0.1 M NaCl, and 0.01% bovine serum albumin buffer), streptomyces hyaluronidase (Amano Chem. Co., 10 TRU, in 0.1 M acetate (pH 4.8)–0.1 M NaCl buffer), or testicular hyaluronidase (Sigma Type III, 0.5 mg in 0.1 M acetate (pH 4.8)–0.1 M NaCl buffer) overnight at 37°. The samples were then immersed in a boiling water bath for 3 min, cooled, combined with carrier GAG (250 μg of chondroitin sulfate and 250 μg of hyaluronic acid), and precipitated with 5 mg of cetyltrimethylammonium bromide (CTAB). After 2 hr at 37°, the precipitates were collected by centrifugation, washed twice by resuspension in 0.05% CTAB and 0.05 M NaCl, and dissolved in 1.5 ml of methanol. The samples were counted in 10 ml of Aquasol after waiting for several hours to eliminate spurious ^{3}H cpm due to autofluorescence. For nitrous acid degradation, other aliquots were first treated with chondroitinase ABC as described above, boiled to inactivate the enzyme, and subsequently treated at 25° with equal volumes of 33% (v/v) glacial acetic acid and 5% (w/v) sodium nitrite for 90 min (Kosher and Searls, 1973); 2 ml of distilled water and carrier GAG were then added and the samples were precipitated by addition of 0.25 ml of 2% CPC. After 2 hr at 37° to enhance flocculation of the precipitate, the samples were analyzed for CPC precipitable radioactivity as described above.

Results

Quantitative Assay of $^{35}SO_4$ Incorporation. Since inorganic $^{35}SO_4$ is apparently not transformed into sulfur-containing amino acids by mammalian cells (Gregory and Robbins, 1960), incorporation of $^{35}SO_4$ into high molecular weight compounds affords a sensitive and specific assay for the biosynthesis of sulfated GAG.[2] We wanted to develop a technique for examining the total cell-associated sulfated GAG that would eliminate the need for lengthy extraction techniques which lead to variable losses of sulfated GAG. Dissolving the $^{35}SO_4$-labeled cell monolayer in SDS-containing buffers, followed by SDS/G-200 Sephadex column chromatography, provided a rapid and convenient method for separation of the high molecular weight sulfated GAG products from low molecular weight precursors. As shown in Figure 1A, the sulfated GAG molecules found in the medium in contact with the cell cultures are all of high molecular weight and therefore are excluded from the column. Chromatography of an SDS buffer extract of a $^{35}SO_4$-labeled cell monolayer is shown in Figure 1B. In this case, in addition to the large excluded peak of sulfated GAG, a long trail of heterogeneous materials of intermediate molecular weight and a low molecular weight peak (fractions 35–40) of unincorporated precursors are observed.

Pulse-chase experiments (Figure 2) suggest that the intermediate molecular weight components seen in Figure 1B are degradation products of high molecular weight sulfated GAG. After 30 or 120 min of $^{35}SO_4$ labeling only the high molecular weight sulfated GAG peak and the low molecular weight precursor peak can be seen. If the intermediate molecular weight components were biosynthetic precursors of the high molecular weight sulfated GAG, then radioactivity should build up in the intermediate molecular weight region *before* its appearance in the high molecular weight components. Since this is not observed, and since the relative amounts of intermediate molecular weight components increase with extended labeling times (Figures 1B and 3) and do not "chase" into the high molecular weight peak, these components are more likely to be degradation products.

The results in Figure 2 also show a pronounced drop in

[2] $^{35}SO_4$ might also be possibly incorporated into sulfated lipids. However, in one experiment with $^{35}SO_4$-labeled Balb SV3T3 cells, we found that <1% of the macromolecular $^{35}SO_4$ label was incorporated into materials extractable into 2:1 chloroform–methanol (R. Roblin and S. Ruggieri, unpublished data). Thus, in this cell line at least, there does not appear to be appreciable incorporation of $^{35}SO_4$ into sulfated lipids.

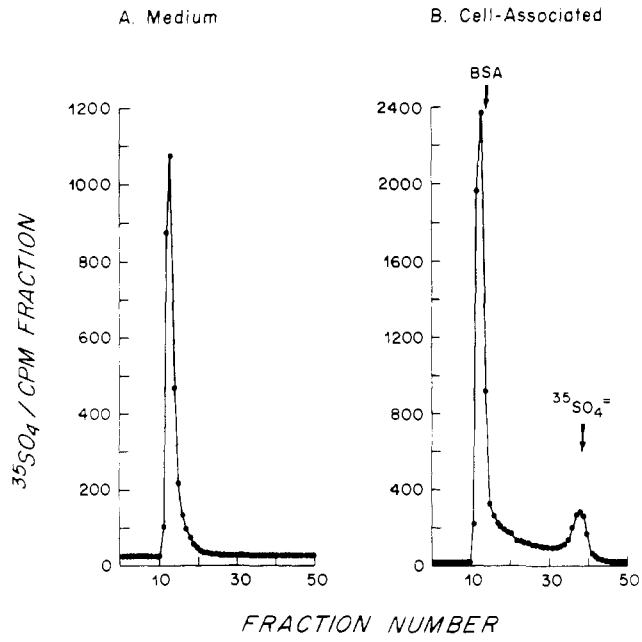

FIGURE 1: SDS/G200 Sephadex chromatography of sulfated GAG. (A) $^{35}SO_4$-labeled materials from the medium of a SV3T3 Rev cell culture, labeled by growth for 24 hr in medium containing 20 μCi/ml of $^{35}SO_4$; (B) $^{35}SO_4$-labeled material from an SDS cell monolayer extract of 2×10^6 SV3T3 Rev cells labeled for 24 hr as above. Arrows mark the elution positions of bovine serum albumin (BSA) and inorganic $^{35}SO_4$ chromatographed separately. Authentic $^{35}SO_4$-Paps (New England Nuclear) elutes at exactly the same position as does inorganic $^{35}SO_4$.

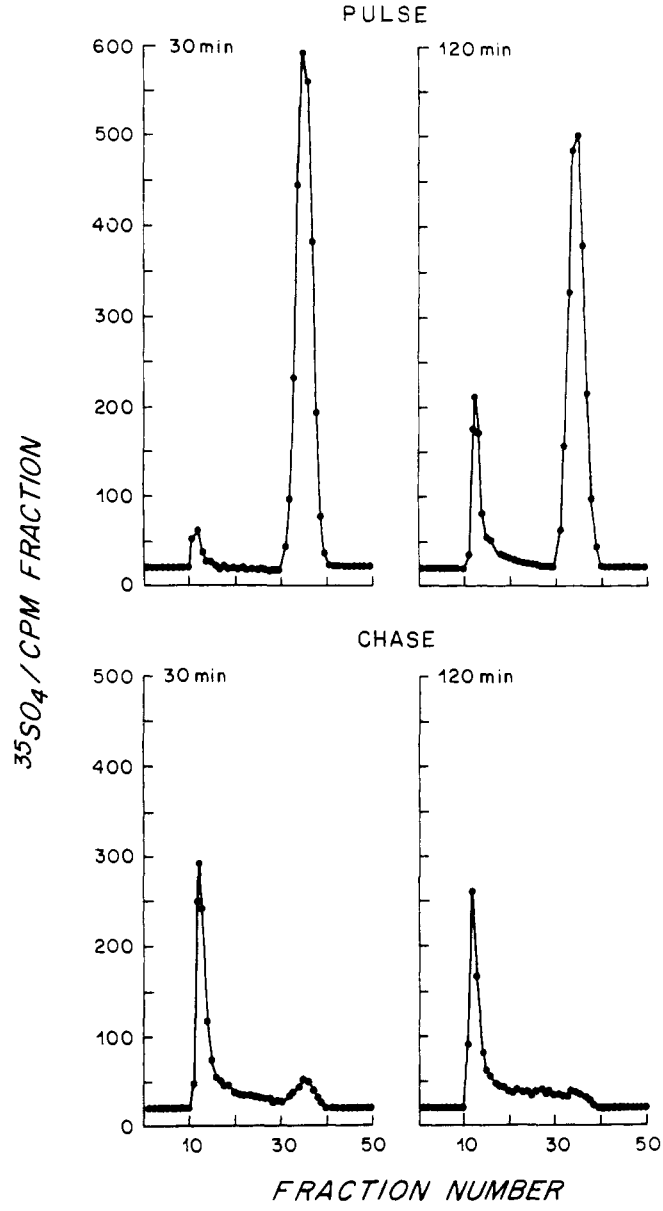

FIGURE 2: Pulse-chase analysis of cell associated sulfated GAG. Replicate plates of SV3T3 Rev cells were grown in the presence of 20 μCi/ml of $^{35}SO_4$ for 30 or 120 min, then the cell monolayers were washed, solubilized, and chromatographed as described under Materials and Methods. After 120 min of labeling, two additional plates were thoroughly washed with and reincubated in prewarmed complete medium. After 30 or 120 min in unlabeled medium, the chase monolayers were washed and solubilized in SDS buffer, and aliquots were chromatographed on SDS/G-200 columns.

the size of the cell-associated low molecular weight precursor peak after a 30-min chase in unlabeled medium. Thus, much of this peak is probably unincorporated $^{35}SO_4$ which is lost from the cells by rapid exchange with exogenous unlabeled inorganic SO_4. After a 120-min chase in unlabeled medium, incorporation of $^{35}SO_4$ into the high molecular weight peak has ceased, the low molecular weight precursor peak has almost completely disappeared, and intermediate molecular weight components (fractions 20–30) continue to accumulate.

Comparison of Incorporation by Different Cell Lines. Comparative data on the synthesis of sulfated GAG by untransformed 3T3, SV3T3, and Con A selected SV3T3 revertant cell lines are given in Table I. Since the cells of the different cell lines have different sizes and contain different amounts of protein/cell, we have expressed the results both in terms of cpm/10^6 cells and cpm/mg of cell protein. Regardless of which of these methods is used to express the results, (1) the amount of *cell-associated* sulfated GAG is decreased in the transformed cells relative to their untransformed counterparts, in agreement with previous data (Saito and Uzman, 1971; Goggins *et al.*, 1972; Terry and Culp, 1974); (2) the amount of cell-associated sulfated GAG increases 2.5–8.0-fold in two independently derived clones of Con A revertant SV3T3 cells compared to that in the transformed parental cells; and (3) the amount of sulfated GAG in the medium of SV3T3 cultures is also decreased when compared to the amount detected in the media of cultures of untransformed and revertant cells (Table I).

In the experiments reported in Table I, cells were grown in the presence of $^{35}SO_4$ for 24–48 hr before analysis, to allow sufficient time for maximum labeling of precursor pools and to enable newly synthesized sulfated GAG to replace preexisting unlabeled molecules. To determine whether 3T3, SV3T3, and Rev cells differed in the rate of sulfated GAG biosynthesis, and whether this rate was affected by the growth state of the cells, we measured the incorporation of a 6-hr "pulse" of $^{35}SO_4$ into cell-associated sulfated GAG in sparse and dense cultures of all three cell lines. The data presented in Table II show that the growth state of the cells has at most about a twofold effect on $^{35}SO_4$ incorporation into sulfated GAG, regardless of whether the results are expressed as $^{35}SO_4$ incorporation per 10^6 cells (column A) or $^{35}SO_4$ incorporation/mg of protein (column B). Thus, the labeling time (Tables I and II) and growth state of the cells have only minor effects upon the level of $^{35}SO_4$ incorporation into cell-associated sulfated GAG and SV3T3 cells

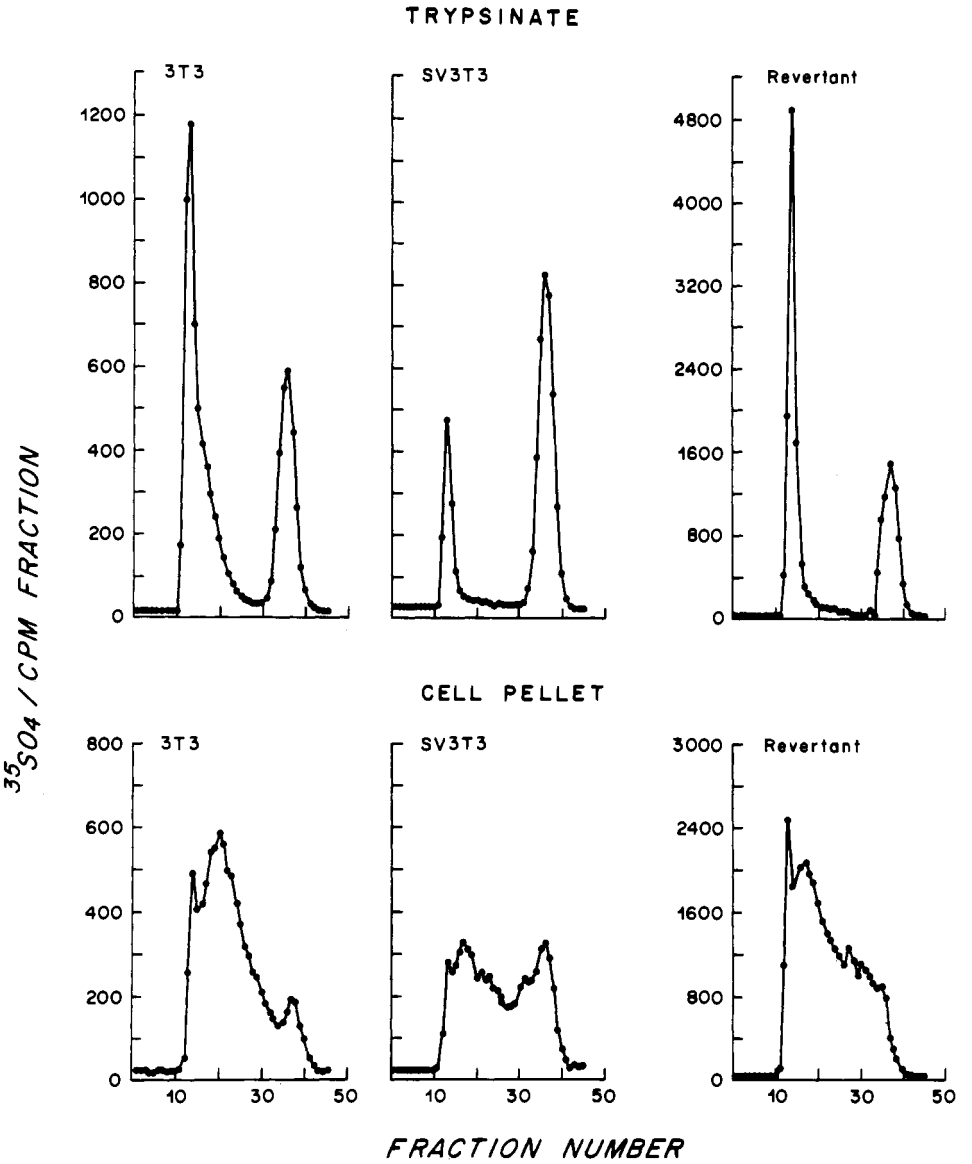

FIGURE 3: Trypsin release of sulfated GAG from the cell periphery. $^{35}SO_4$-labeled monolayers of 3T3, SV3T3, or SV3T3 Rev cells were converted to single cell suspensions by mild trypsin/EDTA treatment as described under Materials and Methods; 0.3 ml aliquots of the "trypsinate" (3.0 ml) and the cell pellet extract (2.0 ml) were chromatographed on SDS/G-200 Sephadex columns to separate high molecular weight sulfated GAG. The initial 3T3, SV3T3, and SV3T3 Rev monolayers contained 4.0×10^6, 8.6×10^6, and 9.0×10^6 cells per 100-mm Petri dish, respectively.

are relatively deficient in the synthesis of cell-associated sulfated GAG.

Localization of Sulfated GAG at the Cell Periphery. Although biochemical (Suzuki *et al.*, 1970) and electrokinetic (Kojima and Yamagata, 1971) observations have indicated the presence of sulfated GAG at the cell surface, it seemed desirable to determine how much of the $^{35}SO_4$-labeled high molecular weight sulfated GAG was located at the cell periphery of our cell lines. Accordingly, we exposed $^{35}SO_4$-labeled cell monolayers to mild trypsinization conditions, centrifuged to separate cells and the trypsin/EDTA supernatant ("trypsinate"), and determined the high molecular weight sulfated GAG content of each fraction by SCS/G-200 Sephadex chromatography. Results of this analysis (Figure 3) showed that 84, 71, and 68% of the high molecular weight sulfated GAG were released from the periphery of 3T3, SV3T3, and SV3T3 Rev cells, respectively, by this trypsin/EDTA treatment. It is unlikely that high molecular weight sulfated GAG in the "trypsinate" come from the inside of cells disrupted by trypsin/EDTA treatment since (a) essentially all the intermediate molecular weight materials remain associated with the cell pellet and are not seen in the "trypsinates" and (b) more than 98% of the cells remained viable by the Trypan Blue dye exclusion test after trypsinization. The appearance of a low molecular weight precursor $^{35}SO_4$ peak in the "trypsinates" suggests either that there is a fraction of the $^{35}SO_4$ which is loosely cell associated after washing and which can be released by trypsin, and/or (b) that the rate of leakage of $^{35}SO_4$ across the cell membrane is rapid enough so that an appreciable amount leaks out of the cell during the trypsinization procedures. Thus, at least 70–80% of the high molecular weight sulfated GAG is associated with the cell periphery, and viral transformation and reversion did not markedly affect the fraction of the sulfated GAG located at the cell periphery which was released by this trypsin/EDTA treatment.

The SDS/G-200 chromatograms of the cell pellet fractions (Figure 3) reveal considerable amounts of intermediate molecular weight materials which are probably degradation products. The amount of $^{35}SO_4$ cpm in these inter-

Table I: Synthesis of Sulfated GAG by 3T3, SV3T3, and SV3T3 Rev Cell Lines.[a]

Cell Line	Labeling time (hr)	Cell-Associated Sulfated GAG cpm/10^6 Cells	cpm/mg of Protein	In Medium cpm/10^6 Cells
3T3				
Expt 1	24	6,940	14,450	20,800
Expt 2[b]	48	11,740	23,600	
SV3T3				
Expt 1	24	1,320	8,050	7,200
Expt 2[a]	48	1,850	11,500	
SV3T3 Rev #84				
Expt 1	24	8,440	21,000	32,000
Expt 2[b]	48	16,430	42,420	
SV3T3 Rev #80				
Expt 1	24	8,650	22,800	18,500

[a] Cells were grown in the presence of $^{35}SO_4$, and the amount of radioactivity incorporated into cell-associated and secreted sulfated GAG was determined as described under Materials and Methods. Medium radioactivity measurements, cell counts, and protein contents are the averages of duplicate determinations. [b] The data for experiment 2 come from the trypsinization experiment illustrated in Figure 3. In this case, high molecular weight sulfated GAG from both "trypsinate" and cell pellet fractions were determined from the areas under the SDS/G-200 chromatogram peaks and the sum was taken as the "cell-associated sulfated GAG."

Table II: Effect of Growth State on Rate of $^{35}SO_4$ Incorporation into Cell-Associated Sulfated GAG.[a]

Cell Line	Cells/Dish	A: HMW $^{35}SO_4$ cpm/10^6 Cells	B: HMW $^{35}SO_4$ cpm/mg of Protein
3T3			
Sparse	2.8×10^5	6,516; 8,143	15,025; 18,996
Dense	3.0×10^6	7,912; 8,052	41,460; 42,702
SV3T3			
Sparse	1.37×10^6	1,608; 1,143	8,607; 6,115
Dense	7.62×10^6	1,835; 1,512	14,492; 11,942
SV3T3 Rev #84			
Sparse	6.09×10^5	22,167; 20,623	113,445; 101,290
Dense	2.64×10^6	11,129; 10,816	93,269; 83,739

[a] $^{35}SO_4$ incorporation into cell-associated sulfated GAG was measured as described in the legend to Table I.

Table III: Characterization of $^{35}SO_4$-Labeled GAG from Cell Culture Medium.

Cell Line	Expt	% Low Molecular Weight cpm[a] after: Buffer Control	Chondroitinase ABC	Buffer Control	Testicular Hyaluronidase	Average Net % Hydrolysis[c] by: ABC	TH
3T3	1	6.9	63.0		43.1		
						56.2	39.7[b]
	2		63.2				
SV3T3	1	4.4	29.0	6.3	25.5		
	2	1.4	29.9	2.4	22.7	26.6	21.3
	3			3.0	27.5		
Rev	1		64.7		50.2		
						55.5	47.1
	2	6.3	58.8	2.0	48.1		

[a] As determined by SDS/G200 Sephadex chromatography following enzyme digestion. [b] Calculated assuming control = 3.4. [c] After subtraction of appropriate buffer control.

mediate molecular weight products was estimated from the areas under the curves (Figure 3), and, when expressed as a percentage of the total $^{35}SO_4$ cpm in the intermediate and excluded regions of both "trypsinate" and cell pellet fractions, was shown to be 54.2% (3T3), 54.8% (SV3T3 Rev), and 63.7% (SV3T3).

Characterization of GAG. Glycosaminoglycans elaborated into the medium by the different cell lines were characterized by degradation with chondroitinase ABC, testicular hyaluronidase, Streptomyces hyaluronidase (Ohya and Kaneko, 1970), and by nitrous acid treatment. In some experiments, $^{35}SO_4$-labeled medium GAG were characterized by direct enzyme digestion followed by SDS/G-200 Sephadex chromatography (Table III). In other experiments, the GAG were double labeled with [^{3}H]glucosamine and $^{35}SO_4$, isolated from cell culture medium following Pronase digestion, and then subjected to enzyme treatment (Table IV). The distribution of incorporated $^{35}SO_4$ label among the different sulfated GAG types was calculated using the general method of Toole and Gross (1971) and is shown in Table V. Note that there is good agreement in the calculated sulfated GAG composition between the two methods.

All three cell lines elaborate chondroitin 4-sulfate (C4S) and/or chondroitin 6-sulfate (C6S) but dermatan sulfate is low or not detectable in medium from SV3T3 cultures. In addition, GAG from SV3T3 cultures contains a higher percentage of chondroitinase ABC resistant $^{35}SO_4$ label than medium from either 3T3 or revertant cultures. Since 99% of the nondialyzable $^{35}SO_4$ label in Pronase digested medium is degraded by treatment with chrondroitinase ABC and nitrous acid (Table VI) most of the chondroitinase ABC resistant $^{35}SO_4$ label is probably contained in N-sulfated GAG (*e.g.*, heparin and heparan sulfate). Since 66–76% of the incorporated $^{35}SO_4$ radioactivity in the starting culture fluid material is recovered following Pronase treatment and dialysis, it is apparent that most, if not all, of the $^{35}SO_4$ in the culture medium resides in sulfated GAG. Significantly,

Table IV: Characterization of [^{3}H]Glucosamine, $^{35}SO_4$-Labeled GAG from Pronase Digested Cell Culture Media.

Cell Line	CPC-precipitable $^{35}SO_4$ cpm[a] after					CPC-precipitable ^{3}H cpm[a] after					Calcd[d] HA + "resistant"
	Control[b]	SH	TH	Control[c]	ABC	Control[b]	SH	TH	Control[c]	ABC	
3T3	2915	2945	1420	2846	874	78,668	36,716	16,432	79,006	51,139	50,801
SV3T3	1525	1665	995	1421	1138	42,503	34,628	18,862	41,533	32,689	26,737
Rev	854	898	437	924	422	50,155	14,782	7,990	52,272	39,733	37,616

[a] Average of duplicate samples. [b] Control sample incubated in 0.1 M sodium chloride–0.1 M sodium acetate (pH 4.8) buffer. [c] Control sample incubated in 0.05 M Tris (pH 8.0), 0.1 M sodium chloride, and 0.01% bovine serum albumin buffer. [d] Sum of calculated hyaluronic acid cpm and ^{3}H cpm not contained in C4S, C6S, chondroitin, and DS subfractions.

Table V: Calculated Percentage Distribution of [^{3}H]Glucosamine and $^{35}SO_4$ Radioactivity among Different GAG Types from Cell Culture Media.

Cell Line		$^{35}SO_4$ Label		
		C4S + C6S	DS	Resistant
3T3	1[a]	39.7	16.5	43.8
	2[b]	51.3	16.4	32.3
SV3T3	1[a]	21.3	5.3	73.4
	2[b]	33.0	0	67.0
Rev	1[a]	47.1	8.4	44.5
	2[b]	48.8	10.0	41.2

Cell Line	[^{3}H]Glucosamine Label[c]			
	C4S + C6S + Ch	DS	HA	Resistant
3T3	25.8	9.6	53.3	11.2
SV3T3	37.1	0	18.5	44.4
Rev	13.5	11.5	70.5	4.5

[a] Calculated from the data in Table III, assuming that material degraded by testicular hyaluronidase is C4S and/or C6S, and that material degraded by chondroitinase ABC is C4S, C6S, and DS. Resistant fraction is that resistant to degradation by chondroitinase ABC. [b] Calculated from the data in Table IV, using the assumptions in (*a*) above. [c] Calculated from the data in Table IV, assuming, in addition to the assumption in (*a*) above that radioactivity degraded by Streptomyces hyaluronidase is exclusively hyaluronate (Ohya and Kaneko, 1970). Resistant fraction is calculated as the total radioactivity minus that in hyaluronate, minus that digested by chondroitinase ABC.

Table VI: Degradation of $^{35}SO_4$-Labeled GAG[a] by Combined Chondroitinase ABC and Nitrous Acid Treatment.

Cell Line	CPC Precipitable $^{35}SO_4$ cpm[b] after			% $^{35}SO_4$ cpm Resistant[c] to	
	Control	Chondroitinase ABC	ABC + nitrous acid	Chondroitinase ABC	ABC + Nitrous Acid
3T3	1065	356	9	33.3	0.85
SV3T3	1322	1065	14	80.6	1.06
Rev	867	503	10	58.0	1.15

[a] Samples from cell culture medium after Pronase digestion as described under Materials and Methods. [b] Average of duplicate samples. [c] % cpm resistant = (cpm remaining after treatment/cpm in control sample) × 100.

the qualitative composition of the sulfated GAG produced by revertant cells (Table V) has shifted so that it now resembles that of 3T3 cells, rather than the SV3T3 cells from which the revertants were selected.

Labeling the acid mucopolysaccharides with [^{3}H]glucosamine and $^{35}SO_4$ produced information about hyaluronate production by the three different cell lines as well as providing some useful checks on the specificity of some of the enzyme degradation reactions. For example, treatment of ^{3}H and $^{35}SO_4$ double-labeled GAG with Streptomyces hyaluronidase produced appreciable loss of CPC-precipitable ^{3}H radioactivity without any loss of $^{35}SO_4$ (Table IV) as expected if the degradative action of Streptomyces hyaluronidase is confined to hyaluronic acid (Ohya and Kaneko, 1970).

The distribution of ^{3}H radioactivity among the different types of GAG is also summarized in Table V. A greater fraction of the non-hyaluronate ^{3}H label in SV3T3 GAG is resistant to chondroitinase ABC and is presumably N-sulfated GAG, in agreement with the data on $^{35}SO_4$ label in Table V. In addition, the distribution of incorporated [^{3}H]glucosamine for revertant cells resembles that of untransformed 3T3 cells (high hyaluronate, low "resistant" GAG) and is very different from that of SV3T3 cells (low hyaluronate, high "resistant" GAG). Since chondroitinase ABC degrades hyaluronic acid at a slow rate compared to other GAG (Yamagata *et al.*, 1968), comparison of the sum of the CTAB-precipitable radioactivity in hyaluronic acid and "resistant" (*e.g.*, N-sulfated GAG) with the amount of chondroitinase ABC restant CPC-precipitable radioactivity affords a check on the internal consistency of the technique. As is seen in comparing Table IV, columns 10 and 11, these numbers are in excellent agreement for 3T3 and revertant GAG and in reasonable agreement for SV3T3 GAG.

Turnover of Sulfated GAG. To determine how long high molecular weight sulfated GAG remain associated with the cells we measured the kinetics of loss of high molecular weight $^{35}SO_4$ labeled GAG from confluent monolayers of Balb 3T3 and Balb SV3T3 Rev cells and dense cultures of Balb SV3T3 cells. We reasoned that if sulfated GAG were components of an intracellular-matrix involved in cell-to-

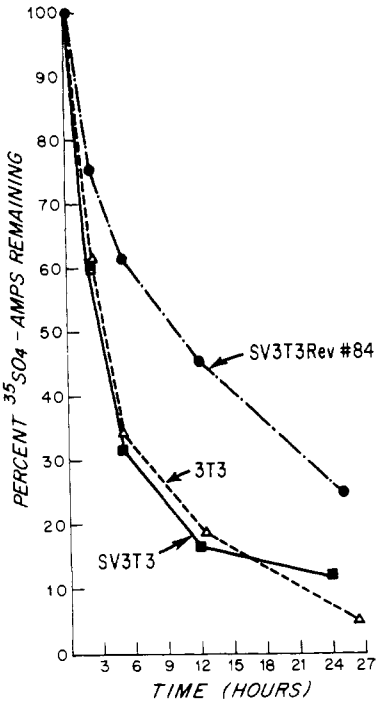

FIGURE 4: Rate of loss of sulfated GAG from prelabeled monolayers. $^{35}SO_4$-labeled confluent monolayers of 3T3, SV3T3, and SV3T3 Rev cells were washed and incubated in unlabeled medium, and the contents of the plates were solubilized and chromatographed to determine the amount of remaining sulfated GAG. The area under the high molecular weight portion of curves like those for Figures 1B and 2 was calculated, and the amount of sulfated GAG remaining at various times was expressed as a percentage of that present at the beginning of the chase period (zero hours).

cell or cell-to-dish adhesion which contributed to density-dependent inhibition of growth, then a decreased rate of turnover might be observed in untransformed and revertant cells compared to transformed cells. When we measured the amount of $^{35}SO_4$-labeled GAG in labeled confluent monolayers as a function of time after careful washing and chase in unlabeled medium, we found that the rate of loss of $^{35}SO_4$-labeled sulfated GAG was essentially the same for monolayers of 3T3 and SV3T3 cells (Figure 4). However, for the Con A SV3T3 Rev cells, the rate of loss was appreciably slower (Figure 4). For all three cell lines, most of the $^{35}SO_4$-labeled sulfated GAG appeared to be lost from the monolayer into the medium, rather than by intracellular degradation, since essentially all the lost sulfated GAG could be recovered in the dialyzed medium.

For all three cell lines there was a progressive slowing of the rate of loss of sulfated GAG with time. This may indicate the existence of more than one population of sulfated GAG molecules, one fraction of which is rapidly lost from the cell periphery and another fraction or fractions which are retained by the cell monolayer for longer periods of time. It is unlikely that the slowing of the rate of loss is due to continued incorporation of $^{35}SO_4$ during the chase period, since the washing procedure and chase in the presence of 1.3 mM unlabeled SO_4 reduce the cell-associated inorganic $^{35}SO_4$ to a very low level within 30 min of initiating the chase (Figure 2).

Sulfated GAG in Cell-to-Dish Adhesion. Since much of the cellular sulfated GAG appears to be located at the cell periphery, these molecules could play a role in adhesion of the cells to the substrate on which they grow. To determine whether cellular sulfated GAG become attached to the Petri dish, we removed $^{35}SO_4$ labeled cells by a mild treatment with Ca^{2+} ion-selective chelating agent EGTA, and extracted the acellular material remaining on the dish with SDS buffer. The loss of surface material from the cells has been shown to be less if EGTA rather than EDTA is used for detachment of the cells from their substrate (Culp and Black, 1972b). In order to monitor the completeness of cell removal, the cells were also labeled with [^{3}H]TdR, and the ratio of [^{3}H]TdR to $^{35}SO_4$ was determined for both the pellet of removed cells and for the material extracted from the dish. A decrease in the [^{3}H]TdR/$^{35}SO_4$ ratio of the dish extracted material compared with that of the cell pellet would indicate selective retention of sulfated GAG on the dish. The results of these cell removal experiments, summarized in Table VII, show that for all three cell lines, the ^{3}H/$^{35}SO_4$ ratio is decreased for the material extracted from the dish compared to the ratio of the cell pellet material from the same cell culture. Thus, the $^{35}SO_4$ radioactivity which remains dish associated after cell removal cannot be solely due to contamination with a few whole cells which remained undetected when the plates were examined with a microscope.

Both the untransformed 3T3 and SV3T3 Rev cells show a higher percentage of their sulfated GAG bound to the Petri dish compared with the virus transformed cells. Chro-

Table VII: Dish Attachment of Sulfated GAG.[a]

Cell Line	[^{3}H]DNA/$^{35}SO_4$ GAG Ratio		Total Dish-Associated $^{35}SO_4$ GAG	Dish-Associated % of Total[b]	
	Cell Pellet	Dish Extract		$^{35}SO_4$ GAG	[^{3}H]DNA
3T3 (3.7×10^6 cells)	7.15, 7.35	1.00, 0.74	6820; 5790	13.8, 12.7	2.2, 1.4
SV3T3 (6.3×10^6 cells)	15.7, 15.6	5.54, 9.90	410, 500	2.1, 2.6	1.7, 0.76
SV3T3 Rev #84 (5.0×10^6 cells)	6.47, 5.40, 5.78	0.80, 0.56, 0.82	3130, 3530; 3540	5.5, 4.5, 4.9	0.73, 0.50, 0.73

[a] Cells were grown in the presence of [^{3}H]TdR and $^{35}SO_4$ and released from the Petri dish with EGTA solution, and the cell pellet and dish-extract fractions were prepared as described under Materials and Methods. Results are given for two or three replicate plates, which were seeded, labeled, and processed in a single experiment. [b] Total $^{35}SO_4$ GAG = dish associated $^{35}SO_4$ GAG plus cell-associated $^{35}SO_4$ GAG.

matography of the SDS plate extract on SDS/G-200 Sephadex columns shows all of the [^{3}H]TdR and $^{35}SO_4$ to be high molecular weight and therefore [^{3}H]DNA and sulfated GAG, respectively. Similarly, chromatography of the SDS cell pellet extract showed that all the ^{3}H cpm were DNA. For the cell pellet extract about 70% of the $^{35}SO_4$ cpm were excluded from the column, while the remainder were intermediate molecular weight materials. No $^{35}SO_4$ cpm were observed in the low molecular weight region, indicating that dialysis effectively removed all cell-associated inorganic $^{35}SO_4$.

The fact that there is generally very good agreement among the results for replicate plates indicates that the EGTA removal technique results in a reproducible cleavage of the cells from their Petri dish substrate. The observation that some [^{3}H]DNA is found in SDS plate extracts indicates either that removal of the cells with EGTA breaks open a small fraction of the cells, or that a few cells remained on the plates after EGTA treatment, even though careful microscopic examination failed to reveal them. However, since a greater proportion of the $^{35}SO_4$-labeled GAG than [^{3}H]DNA remains attached to the dish (Table VII, columns 4 and 5), EGTA treatment must disrupt cell-to-dish attachments in such a way that sulfated GAG remain selectively attached to the Petri dish. Alternatively, the $^{35}SO_4$-labeled GAG may be part of a cellular "microexudate" secreted onto the Petri dish by the cells.

Discussion

Utilizing the SDS/G-200 Sephadex chromatography technique for measuring $^{35}SO_4$ incorporation into sulfated GAG, we have shown (1) that SV40-virus transformed 3T3 cells incorporate 2–5-fold less $^{35}SO_4$ than their untransformed 3T3 cell counterparts, (2) that two independently selected SV3T3 Rev cell lines incorporated 2.5–8.0-fold more $^{35}SO_4$ into sulfated GAG than the SV3T3 cells from which they were selected, (3) that a portion of this sulfated GAG remains adherent to the Petri dish when the cells are removed with EGTA solutions, (4) that the fraction of the total cell associated sulfated GAG which remains adherent to the Petri dish correlates well with the saturation density of the cell lines, (5) that the rate of turnover of cell-associated sulfated GAG is essentially the same for 3T3 and SV3T3 cells, while it is appreciably slower for SV3T3 Rev cells, and (6) that 68–84% of the cell-associated sulfated GAG can be released from the cells by mild trypsin treatment and is thus presumably located at the cell periphery. These data establish a correlation between a relatively high level of sulfated GAG synthesis and low saturation density in cell culture.

The relatively high levels of sulfated GAG synthesis in 3T3 and SV3T3 Rev cells may be, in part, the cause of the low saturation densities of these cell lines based on the following reasoning. Since both the cellular membrane surface and the surface of the Petri dish are negatively charged, counterion binding may play an important role in neutralizing these charges and in overcoming the mutual repulsion expected from the close apposition of two negatively charged surfaces. Calcium ions are known to play a role in attachment of fibroblast-like cells to glass or plastic surfaces (Yang and Morton, 1971), and cells detach from such surfaces when treated with EGTA solutions which selectively chelate calcium ions. Since sulfated GAG have been shown to bind calcium ions selectively in preference to other cations (Dunstone, 1962), cell-associated sulfated GAG and their bound calcium ions may bind the cellular membrane surface to the Petri dish. Increased amounts of cell-associated sulfated GAG in 3T3 and Rev cells could, therefore, enhance cell-to-dish adhesion, help prevent these cells from crawling over one another, and thus contribute to the lower saturation densities observed for 3T3 and SV3T3 Rev cells compared to SV3T3 cells.

Results consistent with this hypothesis have recently been published by several laboratories. Goto *et al.* (1973) showed that treatment of several transformed hamster cell lines and malignant mouse 3T6 cells with 2–10 μg/ml of high molecular weight dextran sulfate produced a pronounced reduction in their saturation densities but not in their growth rates. This reduction in saturation density was reversible upon removal of dextran sulfate from the medium, indicating that dextran sulfate was not simply toxic to the cells. In addition, Goto *et al.* (1973) showed that dextran sulfate treated 3T6 cells were more flattened and polygonal in shape than untreated cells, consistent with a role for sulfated GAG in cell-to-substrate attachment. In addition, Goggins *et al.* (1972) showed that $(but)_2$cAMP and theophylline treatment of SV3T3 cells increased the $^{35}SO_4$ incorporation into sulfated GAG about 3.5-fold compared to untreated control SV3T3 cells. Other investigators have shown that $(but)_2$cAMP and theophylline treatment of other transformed cells decreases their saturation density and agglutinability by Con A (Sheppard, 1971), and increases their adhesiveness to the Petri dish (Johnson and Pastan, 1972). Thus, increased synthesis of cell-associated sulfated GAG is again generally correlated with lowered saturation density, decreased Con A agglutinability, and increased cellular adhesiveness to the substratum, although all these parameters have not yet been compared in the same cell line. Finally, specific desquamation of cell surface heparan sulfate has been observed as cells pass through mitosis (Kraemer and Tobey, 1972), a time when cells generally round up and lose most of their adherence to the substrate.

We have also presented here data which show that sulfated GAG are part of the dish-associated material which remains after removal of the cells with EGTA solutions, and that a higher proportion of the total cell-associated sulfated GAG remained attached to the Petri dish in cultures of 3T3 and Rev cells than in cultures of SV3T3 cells (Table VII). We suggest that this adherent fraction may be part of an intercellular "matrix" laid down in the immediate vicinity of the cells. Since chrondroitin sulfate protein can form insoluble complexes with collagen (Toole, 1969) and since all three of these cell lines have been shown to produce collagen (Culp and Black, 1972a), formation of insoluble collagen-sulfated GAG complexes may be responsible for some or all of the sulfated GAG which remains tightly adherent to the Petri dish. Alternatively, some of the cell-associated sulfated GAG may by itself be part of an adhesive layer which holds the cell surface to the Petri dish. It is of interest that the untransformed 3T3 cells, which exhibit the lowest saturation density *in vitro* show the highest percentage of retention of sulfated GAG on the dish, while the transformed SV3T3 cells exhibit little selective retention of sulfated GAG on the dish. SV3T3 Rev cells, which have reverted toward decreased saturation density *in vitro,* show an intermediate level of retention of sulfated GAG on the Petri dish.

Comparison of the spectrum of sulfated GAG elaborated into the culture medium by the three cell lines showed that

SV3T3 cells produce relatively little dermatan sulfate and relatively more chondroitinase ABC resistant sulfated GAG than do 3T3 and SV3T3 Rev cells (Table V). A similar shift toward enhanced elaboration of chondroitinase ABC resistant material in cultures of fibroblasts transformed by SV40 virus compared to untransformed cells has previously been noted by Goggins *et al.* (1972) and by Satoh *et al.* (1973). The specific desquamation of high molecular weight heparan sulfate (Kraemer and Smith, 1974) from the surface of mitotic CHO cells (Kraemer and Tobey, 1972) suggests an explanation for the relatively high percentage of chondroitinase ABC resistant material in the medium of SV3T3 cultures. Since SV3T3 cells continue to undergo frequent mitosis in dense cultures while 3T3 and Rev cells do not, desquamation of heparan sulfate from the surfaces of mitotic SV3T3 cells could be responsible for the relative enrichment of N-sulfated GAG in SV3T3 cell medium (Tables V and VI).

The results in Table V also reveal a pronounced difference in the flow of exogenous glucosamine into the glycosaminoglycans elaborated by these three cell lines. 3T3 and SV3T3 Rev cells both incorporate a major fraction of the [^{3}H]glucosamine into hyaluronate and relatively little glucosamine into N-sulfated GAG compared with SV3T3 cells. Further work will be required to determine whether these high levels of incorporation of glucosamine into hyaluronate actually means that 3T3 and Rev cells synthesize more hyaluronate than SV3T3 cells. However, it is of interest that the distribution of incorporated glucosamine in SV3T3 Rev cells (like the distribution of incorporated $^{35}SO_4$) has reverted to closely resemble that of untransformed 3T3 cells. Because of the greater amount of materials available we have reported here the characterization of the GAG species elaborated into the medium by the three different cell lines. Characterization of the GAG species at the cell periphery and those which remain dish-associated would be of considerable interest and such experiments are currently in progress. Along these lines, Terry and Culp (1974) have already presented evidence suggesting that hyaluronic acid is one component of the dish-associated material of Balb 3T3 and SV3T3 cells.

Our results show that the decrease in cell-associated sulfated GAG content of SV3T3 is particularly marked (Table I). Although we have provided evidence that the rate of sulfated GAG synthesis is decreased in SV3T3 cells relative to 3T3 and Rev cells (Table II), other processes such as proteolysis might also contribute to the paucity of cell-associated sulfated GAG in SV3T3 cells. In particular, Ossowski *et al.* (1973) have shown that SV3T3 cell cultures contain enhanced levels of "Fibrinolysin T" (plasmin) activity compared to those of 3T3 cells. It is also known that plasmin can liberate GAG from cartilage *in vitro* (Lack and Rogers, 1958) and *in vivo* (Lack *et al.*, 1961). Whether the enhanced plasmin activity of SV3T3 cultures plays a role in decreasing their cell-associated sulfated GAG content is currently under investigation.

The relatively high levels of sulfated GAG synthesis in Rev cells as compared with SV3T3 cells are probably a consequence of the fact that these Rev cells were originally selected by exposing cultures of SV3T3 cells to concanavalin A (Culp and Black, 1972a). Previous work has shown that treatment of cells with exogenous dextran sulfate makes them less agglutinable with Con A (Goto *et al.*, 1973), and that treatment of Rous sarcoma virus transformed cells with hyaluronidase makes them more agglutinable by Con A (Burger and Martin, 1972). Thus, it is perhaps not surprising that Con A exposure selects variant SV3T3 revertant cells which produce relatively higher levels of sulfated GAG. To further investigate this correlation between sulfated GAG synthesis and saturation density, it would be of interest to compare synthesis of GAG in other low density revertant SV3T3 cell lines such as those selected by the FUdR technique (Pollack *et al.*, 1968; Culp *et al.*, 1971).

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Hydrogen Exchange in Nucleosides and Nucleotides. Measurement of Hydrogen Exchange by Stopped-Flow and Ultraviolet Difference Spectroscopy†

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ABSTRACT: Time-dependent changes in the ultraviolet absorbance of the adenine chromophore are observed in the stopped-flow spectrophotometer when adenosine and its analogs are rapidly transferred from protium oxide to deuterium oxide. These absorbance changes are shown to result from hydrogen exchange in the exocyclic amino groups of the purine ribonucleosides by using derivatives of adenosine in which methyl groups replace exchangeable hydrogens and by showing that the general characteristics of hydrogen exchange in adenosine analogs agree with those found here. A study of the dependence of hydrogen-exchange rate constants on adenosine, AMP, and phosphate concentration showed there is a second-order dependence on AMP concentration which is primarily due to intermolecular catalysis by the phosphate group of the nucleotide. The deuterium oxide perturbation difference spectrum, obtained at equilibrium, was found to contain two components that result from blue shifts of the adenine chromophore absorbance: (1) a shift cause by the substitution of deuterium for protium in the ring (N^1) nitrogen and exocyclic nitrogens, and (2) a shift associated with a change in the polarizability of the medium. Since the theory of solvent perturbation, which is used to measure the relative "exposure" of chromophores in macromolecules, assumes that the spectral shifts observed are solely due to (2) above, the use of deuterium oxide as a measure of chromophore exposure to perturbants the size of water must be reexamined.

The use of hydrogen exchange methodology to study both the chemistry of the constituent groups of proteins and polynucleotides and the structural properties of macromolecules has been reviewed by Englander *et al.* (1972). Hydrogen exchange data have been obtained using a number of instrumental methods that measure total and specific exchange over a variety of time ranges. Near-infrared absorption properties of amides have been used by Miller and Klotz (1973) to measure hydrogen exchange rate constants which are on the order of 0.02 sec^{-1}. Overall hydrogen exchange in nucleic acids has been measured with Sephadex column chromotography using tritium (Printz and von Hippel, 1968; McConnell and von Hippel, 1970; Hanson, 1971; Englander, 1972). In these experiments the earliest data were obtained at approximately 6 sec after initiation of exchange. Marshall and Grunwald (1969) and McConnell and Seawell (1972) using nuclear magnetic resonance (nmr) and Lang *et al.* (1974) using ultrasonic absorption techniques have measured much faster rates of hydrogen exchange to specific chemical groups in analogs of adenosine.

I show that there are changes in the ultraviolet absorption spectra of adenosine and other purine and pyrimidine analogs which reflect the number of hydrogens that exchange with deuterium at the ring nitrogen and amino nitrogens. The ultraviolet difference spectra associated with these exchanges can be used to identify the exchanging chromophoric moiety. I use these ultraviolet absorption changes to study the general properties of hydrogen exchange including the effect of adenosine and AMP concentration on the apparent first-order rate constant of exchange. Furthermore, I determine the spectral contributions from deuterium exchange to the total deuterium oxide perturbation difference spectrum and reexamine the use of deuterium oxide to measure the accessibility of water to chromophores in macromolecules.

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