

# Glycosaminoglycan Synthesis Is Depressed during Mitosis and Elevated during Early G1

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**ABSTRACT** [<sup>35</sup>S]Sulfate incorporation was measured in populations of Chinese hamster ovary cells enriched for mitotics, early G1 cells, and interphase monolayers or suspensions. Incorporation was determined by biochemical analysis of extracts and quantitative autoradiography of thick sections. 90% of [<sup>35</sup>S]sulfate was incorporated into glycosaminoglycan (GAG). Incorporation was depressed fourfold in mitotics and stimulated by from two- to three-fold in early G1 cells relative to mixed interphase cells. GAG synthesis was maintained into late G2. Thus, the rate of GAG biosynthesis was correlated temporally with the detachment and reattachment of cells to substrate.

Inhibitors of protein synthesis brought about the rapid arrest of GAG biosynthesis. However, xylosides, which bypass the requirement for core protein, did not bring oligosaccharide sulfation in mitotics to interphase levels. These observations indicate an inhibition of Golgi processing and are consistent with a generalized defect of membrane vesicle-mediated transport during mitosis.

In their pioneering work on glycosaminoglycan (GAG)<sup>1</sup> synthesis by cultured cells, Kraemer and Tobey (14) showed that a significant fraction of trypsin-releasable (i.e., surface) GAGs was shed during mitosis. Although the Chinese hamster ovary (CHO) cells analyzed were grown in suspension, viewed from the perspective of recent studies of cell adhesion (e.g., reference 32), it seemed likely that the shedding of GAGs was related to the familiar ease with which mitotic cells are detached from monolayers. We hypothesized that any effect of shedding would be reinforced by a coordinate reduction in GAG synthesis that would delay replacement of surface GAGs, and that a rapid resumption of GAG synthesis in early G1 would be required for cell reattachment.

A parallel motivation for our studies arose from the accumulating evidence that membrane vesicle-mediated transport is depressed during mitosis. This is shown clearly in work from our laboratory demonstrating depression of endocytosis (1, 2) and transferrin recycling (35) and from that of Warren and colleagues showing a decrease in surface transferrin receptors (41), and in depression of histamine secretion (9). These studies focus primarily on fusion and budding events at the plasma membrane. In addition, the insertion of the G

protein of vesicular stomatitis virus is also depressed during mitosis (42), which suggests a defect in its processing through the Golgi apparatus. Since carbohydrate chains of GAGs are largely assembled within the Golgi apparatus by multiple enzymes (31), a general defect in processing should affect GAG synthesis. Moreover, the apparent localization of sulfation to the *trans*-Golgi apparatus (7, 44) would serve to focus on later steps of the processing sequence.

We describe here the rates of labeled sulfate incorporation into CHO cells during mitosis, in their subsequent movement into G1, and in late G2. We followed incorporation using both biochemical and autoradiographic techniques. Our results indicate that the rate of GAG biosynthesis parallels the normal cycle of cell-substrate detachment associated with the rounding up of mitotic cells and their subsequent reattachment after cell division. In addition, the depressed incorporation of label during mitosis indicates an associated defect in Golgi apparatus processing that is consistent with a generalized defect in membrane-vesicle transport.

## MATERIALS AND METHODS

*Cell Culture and Collection of Mitotic Cells:* CHO cells were grown in a 5% CO<sub>2</sub> atmosphere in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco Laboratories, Inc., Grand Island, NY). Cells were plated

<sup>1</sup> *Abbreviations used in this paper:* CHO, Chinese hamster ovary; GAG, glycosaminoglycan.

into 100- $\mu$ m-diam plastic tissue culture dishes (Falcon Labware, Oxnard, CA). For some experiments, 1 mM  $\beta$ -umbelliferyl-D-xyloside (Sigma Chemical Co., St. Louis, MO) was added to the cultures 6–7 h before sulfate labeling.

Mitotic cells were collected by selective detachment (6). Cultures were partially synchronized by the addition of 5 mM thymidine overnight. 5 h after removal of the thymidine, mitotic cells were detached by rinsing the dish gently with the culture medium; the collected medium was diluted into ice-cold phosphate-buffered saline containing 1% bovine serum albumin (BSA). Fresh medium was added to the plates and rinsing was repeated every 30 min. The mitotic index for each collected sample was determined before pooling from an aliquot stained with Hoechst dye 33242 (Sigma Chemical Co.); samples with <70% mitotics were discarded. A precise mitotic index was determined after the pulse-labeling. Since the mitotic interval of CHO is  $\sim$ 25 min, and agents to arrest mitosis were purposely not employed, the percent mitotics decreased during the 15 min labeling period (described below); mitotic indices of 50–65% at the conclusion of the pulse interval were typical. This implies that the activity of extracts is dominated by mitotic cells but mixed with appreciable contributions from interphase cells.

**Radiolabeling Procedures:** For short-term radiolabeling, cells were rinsed in warm pulse medium (70 mM sodium chloride, 30 mM Tris, pH 7.2, 5 mM magnesium acetate, 0.5 mM EDTA, 50 mM potassium phosphate, 0.1% wt/vol BSA, and 75 mM glucose; 270–280 mOsmol (16); and incubated in pulse medium with 10  $\mu$ Ci/ml  $H_2^{35}SO_4$  (specific activity 4.2 mCi/mmol) at 37°C. Cells were routinely incubated for 15 min, or, to examine the kinetics of sulfate incorporation, up to 1 h. In this defined pulse medium mitotic cells incorporated enough sulfate to quantify before their progression into G1. Subsequent plating efficiency and growth of cells incubated with pulse medium for 1 h were equivalent to those of control cultures. Furthermore, mitotic cells in pulse medium progressed quantitatively into G1.

For analysis of glycopeptide and protein synthesis, cells were incubated at 37°C for from 10 min to 1 h with either 20  $\mu$ Ci/ml D-[6- $^3H$ ]glucosamine (specific activity, 31 Ci/mmol) in pulse medium in which glucose had been exchanged for sucrose in order not to inhibit glucosamine transport and supplemented with 0.1 mM inorganic sulfate, or with 20  $\mu$ Ci/ml L-[ $^3H$ ]tyrosine (specific activity, 52.1 Ci/mmol) in Ham's F-12 nutritive mixture (Gibco Laboratories, Inc.) supplemented with 10% fetal bovine serum.

After radiolabeling in monolayers or in suspension, cells were washed three times with ice-cold PBS. For biochemical characterization of labeled products, cells were sonicated into buffer A (50 mM sodium phosphate, pH 8.0, 1 M sodium chloride) and further subjected to SDS PAGE, enzyme studies, and molecular-sieve and ion-exchange chromatography. For light autoradiography, samples were fixed in 2% glutaraldehyde in 0.1 M cacodylate, pH 7.4, for 30 min at room temperature. Cells were washed three times with 0.1 M cacodylate buffer. To produce more adherent pellets, all cell samples were incubated with BSA (10% wt/vol) after fixation and rinsed again.

**Autoradiography of Thick Sections:** The protocol of Oliver (28) was generally followed for light microscopy autoradiography. 1- $\mu$ m-thick epoxy-embedded sections were mounted on subbed slides and dipped in NTB-2 (Eastman Kodak Co., Rochester, NY) nuclear track emulsion (1:1 vol/vol, 42°C, 60% relative humidity). After drying, the coated slides were stored refrigerated for 2 wk in bakelite boxes containing dessicant, then developed in full strength D-19 (Eastman Kodak Co.) at 13°C for 2.5 min and counterstained with methylene blue.

The cells were photographed using a Zeiss 40 $\times$  oil Planapo objective. Prints (8.5  $\times$  11 in) of the center portion of each negative were used for analysis. For mitotic populations, all cells identifiable as mitotics on each print were scored. Interphase cells, with a clear nuclear envelope, were also scored on the same prints; these were selected in order from one corner of the print to reduce bias in selection. In nonmitotic samples all cells with a defined nuclear envelope were scored. The area of each selected cell was determined using a sonic digitizer (Graf Pen, Science Accessories Corp., Southport, CT), and grains over cells were counted by eye.

Data are expressed as grains per area. These ratios were determined by dividing the sum of grains by the sum of areas for each population sample. Standard deviations were derived from the estimate of variance for ratios as published by Weibel (43). For distribution analyses, ratios of grains per area for individual cell profiles were determined and plotted.

**Characterization of Radiolabeled Products:** The extent of incorporated radiolabel was routinely assayed by gel filtration on Bio-Gel P-6DG (Bio-Rad Laboratories, Richmond, CA) for [ $^{35}S$ ]sulfate and Bio-Gel P-2 for [ $^3H$ ]glucosamine. Aliquots were applied to 0.6  $\times$  8.0 cm columns; excluded fractions were collected directly into scintillation vials. Counts were normalized to the protein content of the original cell suspension, determined by the method of Lowry et al. (22) using BSA as standard.

Enzymatic and chemical treatments for specific analyses were preceded by pronase digestion in 2 mg/ml *S. griseus* protease (Type VI, Sigma Chemical

Co.) 1.5 mM  $CaCl_2$  at 37°C for 18–24 h. Free label was then removed by column chromatography on Bio-Gel P-6DG or P-2. Excluded fractions were pooled for subsequent chondroitinase and nitrous acid digestions or chloroform/methanol extraction. Chondroitinase AC or ABC (Sigma Chemical Co.) was added to a final concentration of 2.5 U/mg and allowed to incubate for 18–24 h at room temperature. Nitrous acid deamination, initiated by adjustment to 1.8 M  $CH_3COOH$  and 0.24 M  $NaNO_3$ , was allowed to proceed for 90 min (20). The extent of degradation was estimated after fractionation on Bio-Gel P-6DG (for chondroitinases) or P-10 (for nitrous acid). Lipids were extracted according to the chloroform/methanol treatment of Heifetz and Snyder (8), and phases were counted directly.

Stepwise ion-exchange chromatography was adapted from the basic procedure of Kraemer (15). Free label was first removed by gel filtration on Bio-Gel P-6DG concomitant with buffer exchange into 10 mM ammonium acetate, pH 7.0. Excluded fractions were pooled and subjected to pronase digestion as outlined above. The samples were then applied to 1–2 ml DEAE-cellulose (Sigma Chemical Co.) columns equilibrated in the same buffer; elution proceeded with 1.5–2 times the column volume for each subsequent step of 0.5, 1.1, and 2.0 M ammonium acetate, pH 7.0.

SDS PAGE was performed in 5–15% gradient slab gels according to the method of Laemmli (17). Proteins were fixed and stained with Coomassie Brilliant Blue R in acidic isopropanol. Radiolabeled materials were detected by fluorography of Enlightning (New England Nuclear, Boston, MA) impregnated gels by exposing Kodak SB-5 X-ray film to the dried gels at  $-70^\circ C$ .

## RESULTS

### [ $^{35}S$ ]Sulfate Incorporation into Mitotic and Interphase CHO Cells

The incorporation of [ $^{35}S$ ]sulfate into macromolecules was first determined in cell extracts. After a short lag, incorporation was linear for  $\sim$ 20 min (Fig. 1), and we adopted 15 min as the standard labeling period. This allowed characterization of sulfate incorporation into cells within small segments of the cell cycle and particularly in mitosis without the use of agents to arrest cell progression. The shake-off populations selected for biochemical analysis were >70% mitotic at the beginning of the pulse labeling period. The initial distribution of cells within mitosis was predominantly metaphase; 95–100% of the mitotics were able to progress into G1 within 20 min. Virtually 100% of the interphase cells were viable as assessed by their capacity to pinocytize fluorescein-labeled dextran (1).

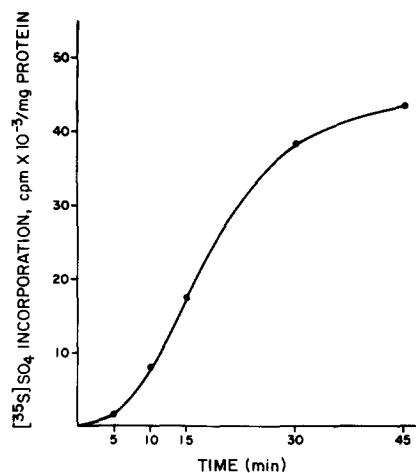


FIGURE 1 Time course of [ $^{35}S$ ]sulfate incorporation by CHO monolayers. Cells were incubated at 37°C in pulse medium for the times indicated and sonicated into cold buffer A, and aliquots were subjected to molecular-sieve chromatography. Incorporated [ $^{35}S$ ]sulfate that appeared in the excluded volume was normalized to the protein content in the original cell suspension.

Incorporation of [ $^{35}\text{S}$ ]sulfate into high molecular weight material was decreased fourfold in the shake-off (mitotic) population (Fig. 2). This was not related to altered transport of the inorganic sulfate precursor. Suspensions of interphase cells and shake-off population cells were incubated with [ $^{35}\text{S}$ ]sulfate for 15 min and sedimented through silicone oil to remove the bulk of the extracellular fluid (25). After correction for residual extracellular contamination and normalization to cell protein, no differences in uptake were seen (data not shown).

Since the shake-off population was a mixture of mitotic and interphase cells, the extent of the depression of sulfate incorporation during mitosis could not be assessed precisely from biochemical data alone. To measure the separate activities of mitotic and interphase cells, we employed quantitative analysis of light autoradiographs of thick sections in which the two cell types could be readily distinguished. The labeling was performed under conditions identical to those of the biochemical experiments described.

Table I A summarizes the data from three separate experiments. The incorporation was depressed almost 80% on the average, consistent with the biochemical analysis (Fig. 1). Note that the actual depression of incorporation must be even greater since the pulse interval was 15 min and the mitotic interval in CHO cells is only 25–30 min (45). (We have confirmed this figure under the conditions of our experiments using the pinocytosis-marker method [1]; data not shown).

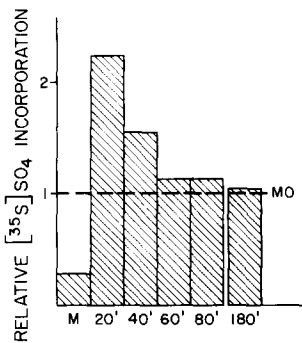


FIGURE 2 Incorporation of [ $^{35}\text{S}$ ]sulfate into mitotic cells and cells emerging from mitosis. Mitotic shake-off populations were collected and either pulse-labeled directly (M, mitotic population) or incubated for 20–180 min before labeling for 15 min. Sulfate incorporation is plotted relative to the incorporation of [ $^{35}\text{S}$ ]sulfate by cells in monolayer (MO).

Thus, the mitotic population enumerated would be enriched for cells that had been partially labeled in interphase. In contrast to the >80% inhibition of GAG synthesis during mitosis, under the same conditions protein synthesis is reduced ~30% (Table I B).

### Sulfate Incorporation into Late G2 Cells

Does the depression of sulfate incorporation commence with mitosis or does a shut-down of GAG synthesis occur in G2 or earlier in preparation for cell rounding and detachment? To address this question, we determined the activity of late G2 cells by first pulse-labeling monolayers for the usual 15 min. The monolayers were then rinsed and incubated in growth medium for an additional 20 min before cells were collected by shake-off. Mitotic cells present in the shake-off would thus have been derived from the G2 population that had been incubated with the label. Quantitative analysis shows that the mitotic cells present were labeled almost as intensely as the interphase population (2.27 vs 2.87 grains per unit area). Thus, although the 15-min pulse interval did not allow us to resolve the precise time at which sulfate incorporation was so markedly reduced, it could not have been more than a few minutes before mitosis.

The small decrease in labeling of these mitotics versus that of interphase cells is consistent with the nearly equal lengths of pulse and mitotic intervals. Due to cell-to-cell variation in mitotic interval there would have been some cells in late G2 or in mitosis during the labeling period that were still in mitosis after the 20-min chase. Such cells, having progressed to late stages of mitosis, should have been less active. An analysis of labeling as a function of the stage of mitosis supports this inference. Fig. 3 shows the frequency of cell profiles in different categories of labeling intensity (grains per area). We compared distributions of directly labeled interphase cells (Fig. 3B) with those of early and late mitotics labeled previously in G2. Such mitotics were divided into relatively early phases, prometaphase and metaphase (Fig. 3C), and late phases, anaphase and telophase (Fig. 3D). The distributions of early mitotics and the interphase cells are

TABLE I. Autoradiographic Comparison of Incorporation of [ $^{35}\text{S}$ ]Sulfate or [ $^3\text{H}$ ]Leucine into Mitotic and Interphase Cells in the Mixed Shake-off Population

| Experiment                                  |            | Grains/Area | $\pm$ SD | n   | Percent of interphase |
|---|------------|-------------|----------|-----|-----------------------|
| A. [ $^{35}\text{S}$ ]Sulfate incorporation |            |             |          |     |                       |
| 1   | Mitotic*   | 0.50        | 0.14     | 51  | 24                    |
|   | Interphase | 2.07        | 0.36     | 175 |                       |
| 2   | Mitotic    | 0.35        | 0.01     | 35  | 13                    |
|   | Interphase | 2.74        | 0.08     | 49  |                       |
| 3   | Mitotic    | 0.73        | 0.09     | 46  | 30                    |
|   | Interphase | 2.43        | 0.14     | 132 |                       |
|   |            |             |          |     | mean = 22             |
| B. [ $^3\text{H}$ ]Leucine incorporation    |            |             |          |     |                       |
| 1   | Mitotic    | 0.58        | 0.10     | 44  | 59                    |
|   | Interphase | 0.99        | 0.05     | 42  |                       |
| 2   | Mitotic    | 0.79        | 0.01     | 33  | 81                    |
|   | Interphase | 0.98        | 0.01     | 44  |                       |
| 3   | Mitotic    | 0.65        | 0.01     | 58  | 64                    |
|   | Interphase | 1.01        | 0.01     | 41  |                       |
|   |            |             |          |     | mean = 68             |

As described in Materials and Methods, a 15-min pulse, followed by fixation and processing, was used.

\* Mitotic and interphase cells were chosen at random from the same sections, as described. Only cell profiles with identifiable chromosomes or nuclei were quantified (see Materials and Methods).

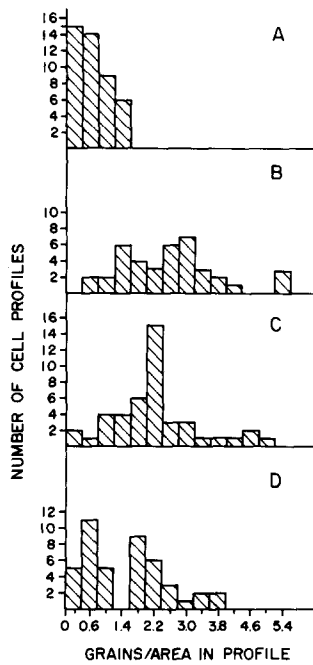


FIGURE 3 Frequency of cell profiles versus labeling intensity (grains per area) from cells labeled during mitosis, interphase, and late G2. Grain densities were determined for individual cell profiles for directly labeled mitotic cells (A), and interphase cells (B) within a mitotic shake-off population. Late G2 cells were labeled in monolayer and incubated for 20 min more; cells that progressed into mitosis were then collected by mitotic shake-off and classified as in early mitotic stages, prometaphase and metaphase (C), or late mitotic stages, anaphase and telophase (D). Whereas cells in the early mitotic stages (C) resembled interphase cells (B) in their intensity distribution, some later-stage mitotics (D) were poorly labeled and resembled directly labeled mitotic cells (A).

virtually equal. These mitotics were derived presumably from cells labeled entirely in G2. However, the anaphase–telophase population is mixed. The intensity over most profiles overlaps the interphase distribution; a subpopulation is more similar to the control poorly labeled, mitotic population (Fig. 3A) that was pulsed after shake-off.

### Sulfate Incorporation Into Early G1 Cells

To study early G1 cells, mitotic shake-off populations were incubated in complete medium for from 20 to 180 min and then pulse-labeled. After a 20-min incubation, mitotics were present at only 5–20%, after 60 min, at 1–5%. [<sup>35</sup>S]Sulfate incorporation was compared with that of growing cell monolayers. At 20 min, sulfate incorporation was increased 2.3-fold (Fig. 2). By 60 min, incorporation was still elevated but approached that of the monolayer. These results indicate that emergence from mitosis is accompanied by a burst of sulfate incorporation.

Table II summarizes the data gathered from quantitative autoradiography on comparable cell populations. At 20 min, the activity of a suspension containing ~60% early G1 cells was 2.5-fold greater than that of a suspension containing mixed interphase cells, and 7–8-fold greater than one containing their mitotic progenitors (see Table II A). By 60 min, the stimulation had declined, but was on the average 1.7-fold greater than that of mixed interphase populations. There was clearly a marked but short-lived enhancement of sulfate incorporation during early G1, a period associated with the reestablishment of substrate attachment and shape change. Since the populations examined contained a significant fraction of interphase cells, some of which would not have been in early G1 (but could not be identified separately), the grain-per-area ratios obtained yielded minimal estimates of the degree of stimulation. In general, somewhat larger ratios were obtained by autoradiographic than by chromatographic analysis. This may have been due in part to elimination in the

TABLE II. Autoradiographic Analysis of [<sup>35</sup>S]Sulfate Incorporation into Populations Enriched for Early G1 Cells

| Experiment |                        | Grains/<br>Area | ± SD | n   | Percent of<br>interphase |
|------------|------------------------|-----------------|------|-----|--------------------------|
| 1          | Interphase             | 2.07            | 0.36 | 175 |                          |
|            | Shake-off*<br>+ 60 min | 3.56            | 0.14 | 220 | 172                      |
| 2          | Interphase             | 2.74            | 0.08 | 49  |                          |
|            | Shake-off<br>+ 60 min  | 3.30            | 0.10 | 100 | 120                      |
| 3          | Interphase             | 2.43            | 0.14 | 132 |                          |
|            | Shake-off<br>+ 20 min  | 6.36            | 0.10 | 138 | 262                      |
|            | Shake-off<br>+ 60 min  | 5.03            | 0.37 | 138 | 207                      |
|            |                        |                 |      |     | mean in 60 min =<br>166  |

As described in Materials and Methods, a 15-min pulse, followed by fixation and processing, was used.

Cells in early G1 were obtained by incubating the mitotic shake-off population for 60 or 20 min in complete medium. The shake-off was 70% mitotic; after incubation, it was <5%. Therefore, ~60% of cells were in G1 after incubation.

former of cell profiles from damaged or vacuolated cells that would have been included in sonicated extracts.

### Comparison of Interphase Cells in the Shake-off and Monolayer Populations

In the autoradiographic experiments of pulse-labeled mitotic cells described in Table I, mitotics were compared with interphase cells in the shake-off suspension. It was thus important to determine whether these interphase cells were comparable to interphase cells in monolayer. If depressed relative to monolayer values, the apparent hyperactivity in G1 could be simply a recovery period unrelated to the potential for reattachment associated with G1 populations. Cells in monolayers were labeled and their activities were compared with those of interphase cells obtained by shake-off from the same monolayers. Quantitative autoradiography indicated that interphase cells in the shake-off population were actually slightly stimulated as compared with those in monolayer (from  $2.32 \pm 0.03$  to  $2.74 \pm 0.08$  mean  $\pm$  SD grain/area). This increase may reflect the presence of early G1 cells derived from mitotics during the labeling period.

### Characterization of the [<sup>35</sup>S]Sulfate-labeled Macromolecule

90% of the sulfate label could be accounted for as GAG. No radioactivity was extractable into chloroform/methanol (8). This finding eliminated sulfatides as significant constituents after short labeling periods. Cell extracts were routinely desalted on polyacrylamide (P-6DG) columns to eliminate unincorporated [<sup>35</sup>S]sulfate. By SDS PAGE (data not shown), the label appeared in a diffuse band and at the stacking–running gel interface. After exhaustive pronase digestion, the number of counts recoverable in the high molecular weight fractions was >95%, ruling out appreciable formation of tyrosine-O-SO<sub>4</sub> label (18, 21). SDS PAGE of the pronase-treated material showed a still more diffuse band that had moved further into the gel. However, the radioactivity was retained under conditions in which virtually all of the Coomassie Blue–staining material was absent (data not shown).

60–65% of the pronase-digested material was sensitive to nitrous acid, 30–35% to chondroitinase ABC or AC, consistent with a corresponding composition of heparan and chondroitin sulfates, respectively. This agrees with earlier analyses by Kraemer (15), although he used longer pulse intervals. Extracts of interphase and mitotic cell preparations behaved similarly. However, we emphasize that because of the decreased incorporation into mitotic cells, the bulk of labeled material in all preparations was derived from interphase cells.

A small fraction of labeled material was probably sulfated glycopeptide. Fig. 4 shows the elution profile of pronase-digested, sulfate-labeled cell extract from DEAE-cellulose columns according to the basic scheme of Kraemer (15). The major peaks eluted with 1.1 and 2.0 M ammonium acetate ( $\text{NH}_4\text{Ac}$ ) and correspond to heparan and chondroitin sulfates, respectively. The relative counts in these two peaks agreed with the values obtained by degradation (above). The 2.0 M eluted fraction was completely digested by chondroitinase ABC. 80% of the 1.1 M fraction was degraded by nitrous acid and the balance by chondroitinase ABC. In addition, 8–11% of the radioactivity eluted with 0.5 M  $\text{NH}_4\text{Ac}$ . Heifetz and Synder (8) found this material in endothelial cells to be charged glycopeptide of low sulfate content. They found that its formation was inhibited by tunicamycin, indicating N-linkage to protein (27, 38).

When CHO cells were labeled for 30 min with  $^3\text{H}$ glucosamine, 95% of the label in pronase-digested extracts was eluted in a single peak by 0.5 M  $\text{NH}_4\text{Ac}$  (Fig. 4). The peak fractions were rechromatographed on a polyacrylamide gel (P-10) and co-eluted in a broad peak with the vitamin B12 marker (1,335 mol wt), consistent with their identity as short protein-linked oligosaccharides.  $^3\text{H}$ Glucosamine-labeled extracts run on SDS PAGE showed multiple discrete bands, in contrast to the diffuse sulfate-labeled GAGs. No label was seen after electrophoresis of pronase-digested  $^3\text{H}$ -labeled material (data not shown). Tunicamycin, even at doses (2  $\mu\text{g}/\text{ml}$ , 6 h) high enough to depress protein synthesis by 20% and to block N-linked glycosylation of vesicular stomatitis virus G protein in CHO cells (27), did not suppress this incorporation. Thus, the predominant material labeled by short glucosamine exposure was protein-linked oligosaccharide, probably in O-linkage. Its character is under study.

In any event, when mitotic shake-off populations (> 70% mitotics) were incubated with  $^3\text{H}$ glucosamine for 15 min, incorporation into pronase-resistant material was depressed about fourfold. Thus, we suggest that the synthesis of the oligosaccharide backbone, as well as sulfation, is depressed during mitosis.

### Protein Synthesis and Sulfation

One possible explanation for decreased sulfation of GAGs during mitosis is a deficiency of the core protein to which the glycopolymers are linked and elongated. We first determined the effects of a general inhibition of protein synthesis on sulfation in interphase cells. Cells were incubated with cycloheximide and then pulse-labeled in the continued presence of cycloheximide for 15 min. After only 5 min of preincubation, sulfate incorporation was reduced by 80%. Essentially identical effects were observed with puromycin (data not shown). Under these conditions, protein synthesis, determined by incorporation of  $^3\text{H}$ tyrosine, was decreased by ~90%. These findings are, of course, consistent with the hypothesis that the

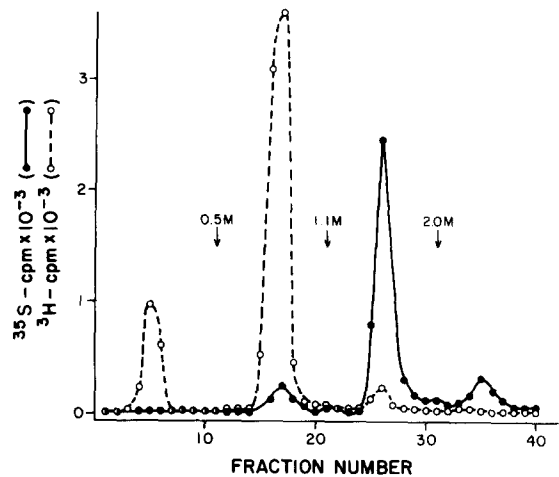


FIGURE 4 Ion-exchange chromatography of radiolabeled products. CHO monolayers were pulse-labeled for 15 or 30 min with  $^{35}\text{S}$ sulfate or  $^3\text{H}$ glucosamine, respectively, desalted, and exchanged into 10 mM  $\text{NH}_4$  acetate, and excluded fractions were subjected to pronase digestion. Digested samples applied to 1 ml DEAE-cellulose columns were eluted stepwise with 2 ml each of starting buffer, 0.5, 1.1, and 2.0 M  $\text{NH}_4$  acetate. More than 95% of the  $^3\text{H}$ glucosamine label is eluted before the GAG fractions that elute with 1.1 and 2.0 M  $\text{NH}_4$  acetate.

decrease in sulfation in mitotics is secondary to decreased availability of core protein. Even though protein synthesis is reduced only 30% during mitosis (Table 1B), it is possible that inhibition was selective for core protein.

To examine this question further we studied sulfation after incubation with xylosides. The linkage of the oligosaccharide side chains to the core protein of chondroitin sulfate is through an O-glycoside bond between xylose and the hydroxyl group of serine (19). Incubation of cells with membrane-permeable xylosides stimulates GAG production; the core protein is bypassed (36). The xylosides provide increased numbers of nuclei for oligosaccharide chain elongation (5).

Incorporation of  $^{35}\text{S}$ sulfate into monolayer suspensions was stimulated by from two to threefold by the xyloside (see below). Cycloheximide, as expected, did not affect sulfate incorporation into the xyloside-based oligosaccharides (data not shown). Extracts of xyloside-treated and untreated cells were subjected to chondroitinase or nitrous acid digestion and chromatographed to identify undegraded products. The elution profiles are illustrated in Fig. 5, A (nitrous acid) and B (chondroitinase). The character of the labeled GAG was clearly shifted by xyloside from predominantly heparan sulfate to largely chondroitin sulfate.

If sulfation in mitotics was reduced entirely because of the limited availability of core protein, xyloside might normalize sulfation. To test this, growth medium was supplemented with xyloside 6–7 h before shake-off, and the activities of mitotic and interphase cells present in the shake-off cell population were assessed by quantitative autoradiography. Table III summarizes the data. Mitotic cells were clearly stimulated by xyloside. In fact, the percentage increment was greater than that in the interphase populations. More important, however, the absolute increment of activity was smaller. The mitotic population, even when treated with xyloside, was not as intensively labeled as the untreated interphase cells. Thus, bypassing the requirement for core protein was not

sufficient to bring the level of sulfate incorporation by mitotic cells to that of interphase.

## DISCUSSION

These studies establish that GAG biosynthesis is markedly depressed during mitosis and stimulated during early G1. A short labeling period made possible the examination of [<sup>35</sup>S]sulfate incorporation into macromolecules during the normal mitotic interval; drugs were not required to arrest cells in mitosis, and virtually all mitotic cells progressed into interphase under the experimental incubation conditions. Light level autoradiography allowed discrimination between mitotic and interphase constituents of the heterogeneous cell population obtained by shake-off, but the biosynthetic changes were so great in the shake-off and early G1 populations that they were readily observed in extracts that could be characterized biochemically.

The only other analysis of the cell-cycle dependence of GAG synthesis of which we are aware is the recent study of Blair et al. (3) in which a growing culture of melanoma cells was labeled and the cells were separated by flow cytometry and then analyzed. The result shows a gradual increase in labeling from G1 through G2-M. However, the cell sorting did not allow discrimination of G2 from M, or early from late G1. As we have shown here, dramatic changes in GAG synthesis occur at precisely these transitions. Of course GAG synthesis is not the only synthetic activity affected during mitosis. RNA synthesis (except for 4S and 5S RNA [46]) decreases rapidly in prophase and resumes in late telophase (29), and, as we confirmed under our experimental conditions, there is a decline in protein synthesis. There is great variability in this decline, from little or none (13) to nearly complete (10), the latter associated with the use of antimicrotubule agents such as vinblastine. The overall stimulation of GAG synthesis as cells emerge from mitosis shown here is not paralleled by RNA or protein synthesis.

Although GAG synthesis during mitosis was depressed and subsequently underwent transient hyperstimulation, it was difficult to set precisely the interval during which GAG sulfation was inhibited. A pulse interval of 15 min obviously limited resolution. However, allowing for this uncertainty, it was clear that G2 cells were not inhibited during the major part of G2 and that G1 cells became rapidly hyperactive and remained so for ~1 h. This time course parallels the rounding up and partial detachment of mitotic cells from their substrate and the subsequent spreading and reattachment that occurs

upon emergence from mitosis (into early G1).

Despite this striking parallel, it is known that in CHO cells only a fraction of cellular GAGs are present on the surface and thus are directly relevant for attachment (33). The precise

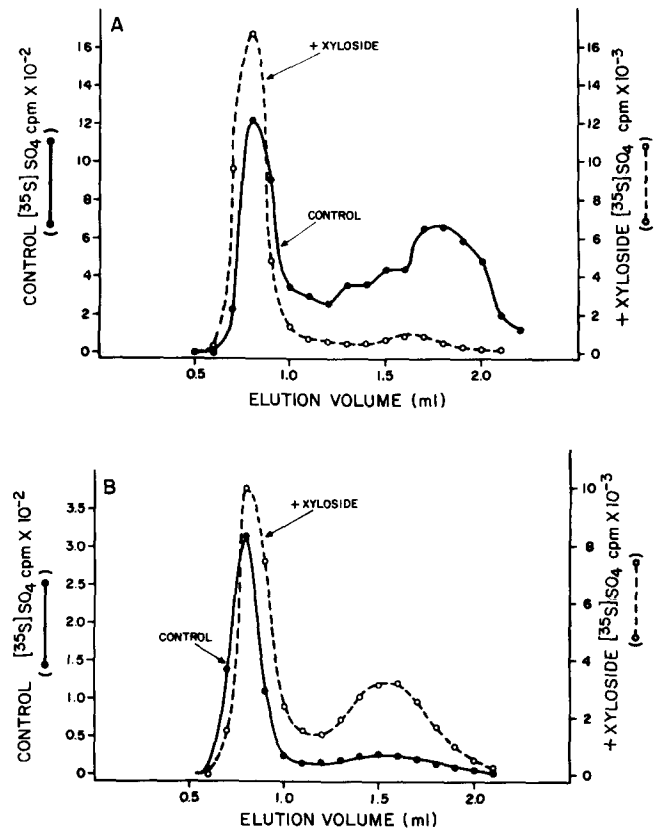


FIGURE 5 Effect of incubation with  $\beta$ -D-xyloside on the species of GAG synthesized. Xyloside was added to monolayers for 7 h before standard pulse-labeling conditions for [<sup>35</sup>S]sulfate. Extracts were digested with pronase and desalted on P-6DG columns. Excluded fractions were pooled and subjected to further degradative assays. (A) Nitrous acid deamination. Molecular-sieve chromatography on P-10 shows a typical elution profile for the control extracts in which 61% of GAG label exhibits sensitivity and is thus identified as heparan sulfate. 85% of the xyloside-derived sample remains intact after nitrous acid treatment and further P-6DG chromatography. (P-6DG was employed because of the short-chained oligosaccharides produced with xylosides.) (B) Chondroitinase AC degradation. Gel filtration on P-6DG columns indicates a shift in chondroitinase sensitivity from 20% in the control to 55% in the xyloside-derived sample.

TABLE III. Xyloside Stimulation of GAG Formation Determined by Autoradiography

| Stage of experiment 2 | Grains/Area | SD   | n   | Percent stimulation | Absolute increment<br>grains/area |
|-----------------------|-------------|------|-----|---------------------|-----------------------------------|
| Mitotics              |             |      |     |                     |                                   |
| Control               | 0.35        | 0.01 | 35  |                     |                                   |
| + Xyloside*           | 1.52        | 0.02 | 29  | 434                 | 1.17                              |
| Interphase            |             |      |     |                     |                                   |
| Control               | 2.74        | 0.08 | 49  |                     |                                   |
| + Xyloside            | 4.79        | 0.01 | 49  | 175                 | 2.05                              |
| 60-min incubation†    |             |      |     |                     |                                   |
| Control               | 3.30        | 0.10 | 100 |                     |                                   |
| + Xyloside            | 5.34        | 0.09 | 100 | 162                 | 2.04                              |

\* Incubation with xyloside 6 h before shake-off.

† Pulse-label after a 60-min incubation in complete medium.

relationship of the GAGs synthesized in early G1 to the early attachment phase is under study. Rollins et al. have carefully analyzed the evolution of GAG composition during reattachment of fibroblasts after trypsinization and found an ordered process of GAG association at sites of attachment (32). Nevertheless, the physiological mechanism by which cells become reassociated with the substratum after mitosis may proceed quite differently than the reattachment of trypsinized cells.

Since only a part of synthesized GAGs is incorporated into substrate attachments, the global inhibition of GAG synthesis may signify inhibition of a diverse group of intracellular functions that require GAGs. Little is known of such functions, in fact, but it is clear that GAGs are present within a variety of secretory organelles and perhaps Golgi apparatus (23, 24), whose activities are affected during mitosis. In cells not obviously involved in secreting massive quantities of GAGs into the extracellular milieu, these intracellular pools may regulate GAG synthesis and turnover. Moreover, these requirements may be highly dynamic, and the biosynthetic pathway must respond quickly. Consistent with this view, we note that in chondrocytes, inhibition of protein synthesis leads to a gradual reduction in chondroitin sulfate formation ( $t_{1/2} = 80\text{--}100$  min), which suggests the existence of a significant intracellular pool of core protein (12, 26). By contrast, inhibition of protein synthesis in CHO cells leads to a rapid decline ( $t_{1/2} < 5$  min) in GAG formation.

Whereas the biological significance of the cell-cycle-related changes is a matter of speculation, our data provide clear evidence on the specific questions of molecular processing and synthesis during mitosis. The Golgi apparatus is clearly implicated in GAG synthesis (39). The precise site at which the initial xylose-to-core-protein linkage is established is uncertain. However, by autoradiography, sulfation has been consistently localized to the *trans*-Golgi apparatus (e.g., reference 44). Golgi fractions of mastocytoma cells are enriched for UDP glucuronic acid, transferase activity (37), and studies of the synthesis of a melanoma cell line glycoprotein-proteoglycan complex noted above also strongly suggest that the glycosaminoglycan is synthesized in the Golgi apparatus (4). Although sulfation is coordinated with the activities of other enzymes that catalyze deacetylation of *N*-acetylglucosamine and isomerization of iduronic acid (30), it follows completion of chain elongation. In addition, consistent with autoradiographic studies, pulse-chase experiments show sulfation immediately before release of GAG from chondrocytes (12, 26). Thus, GAG synthesis almost certainly involves oligosaccharide processing in *cis*- and/or intermediate-Golgi apparatus, with the bulk of sulfation occurring late in the *trans*-Golgi apparatus.

The depression of GAG sulfation in mitotics is consistent with a defect in Golgi apparatus processing. However, since inhibition of protein synthesis leads also to decreased sulfation, the possibility that there is specific inhibition of core protein synthesis or its transfer to the Golgi apparatus must be considered. Without additional evidence, the question of processing through Golgi apparatus during mitosis would remain unanswered. Fortunately, the steps involving synthesis and transfer of core protein can be bypassed by the administration of xyloside. Since xyloside-nucleated oligosaccharide sulfation is still depressed in mitotics, a defect in Golgi apparatus processing is specifically implicated. The shift of GAG formation from heparan to chondroitin sulfates indirectly supports this conclusion. This shift with xylosides, although

not universal (11), has been observed previously (40). Although heparan and chondroitin sulfates share the same trisaccharide linkage to core protein (19), subsequent elongation depends on different specific enzymes. Inhibition of both of their syntheses during mitosis indicates inhibition of numerous enzymes of diverse properties, an effect virtually incompatible with control at a specific step of biosynthesis. Multiple steps in the transfer of the growing GAG chains through the Golgi apparatus are probably affected. Such transfers are believed to occur by membrane vesicles, and a defect in their capacity to fuse with Golgi membrane, as Warren points out (42), would represent the functional equivalent of the failure to form endocytic vesicles (1, 2), to discharge secretory vesicles (9), or recycle receptors (35, 41). All vesicle-mediated transport would appear to be inhibited during mitosis. However, it is also possible that the fragmentation of the Golgi apparatus that occurs in mitosis represents unproductive fusion events. Rothman et al. (34) have shown that productive transfer from the Golgi apparatus of one cell to that of another can occur after fusion, so the simple physical separation of Golgi elements that occurs during mitosis need not block transfer. However, the organization of protein and other elements within a Golgi stack or saccule presumably was preserved in the cell fusion experiments, whereas the fragmentation during mitosis could create vesicles that are structurally disorganized or restricted in enzymatic function, thus further reducing the efficiency of productive transfer, or perhaps affecting recognition signals that optimize productive fusions. A parallel reorganization of plasma membrane during mitosis could also inhibit endocytosis and exocytosis.

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