Journal of Supramolecular Structure 13:281–294 (1980)

Cell Cycle Dependent Rate of Labelling of Cellular and Secreted Glycosaminoglycans in Mouse Embryonic Fibroblasts

Angela M. Otto and P.F. Mühlradt

Gesellschaft für Biotechnologische, Forschung mbH., Mascheroder Weg 1, D-3300 Braunschweig-Stöckheim, Federal Republic of Germany

Cultures of embryonic fibroblasts from Balb/c or CBA/J mice were given 12-h pulses of ^{14}C-galactose, or were double-labelled with ^{3}H-galactose and ^{35}H-sulfate. The time course of the rates of labelling of glycosaminoglycans – galactose label was found in the uronic acid moiety – was studied in synchronously and asynchronously growing cultures. Partial synchrony was achieved by trypsinising quiescent, confluent cells and subsequent transfer of cells to new cultures with fresh medium. Synchrony was monitored by measurement of thymidine uptake in parallel cultures. The distribution of label in the hyaluronic acid, chondroitin sulfate, and heparan sulfate fractions from cells and culture media was determined at each time point.

Peaks of DNA synthesis were accompanied by or followed 12 h later by a maximal rate of labelling with galactose of secreted glycosaminoglycans, and – with the exception of hyaluronic acid – also of cellular glycosaminoglycans. The rate of labelling with galactose of glycosphingolipids in parallel cultures followed a different time course. In double-label experiments the rates of labelling of glycosaminoglycan sulfates with ^{3}H-galactose and ^{35}S-sulfate did not go parallel. In older, quiescent cultures the labelling rate with galactose decreased while the sulfation rate increased.

It is discussed that the labelling rate with galactose is indicative of the biosynthetic rate of the glycosaminoglycans. The conclusion is reached that glycosaminoglycans are preferentially synthesized and secreted after the S phase of the cell cycle.

Key words: **glycosaminoglycans, cell cycle, biosynthesis, fibroblasts**

Glycosaminoglycans are strongly negatively charged polysaccharides conjugated to a protein core. They are found intracellularly, cell surface bound, and secreted into the culture media of cells from various origins [1].

Angela Otto is now at the Friedrich Miescher Institut, P.O. Box 273, CH-4002 Basel, Switzerland.

Received June 27, 1980; accepted July 14, 1980.

Hyaluronic acid, an unsulfated species, has been implicated in cellular adhesion [2], or attachment of fibroblasts to the substratum [3, 4]. Sulfated glycosaminoglycans have been discussed as controlling density inhibition of growth in fibroblasts [5], or as control elements in eucaryotic cells in general [6].

Glycosaminoglycans labelled by various precursors, usually glucosamine and ^{35}S-sulfate, have been compared by several investigators in "normal" and virus-transformed fibroblast cell lines [1, 5, 7, 8] or in fibroblasts seeded at high and low cell densities [7].

The possible influence of the cell cycle on the biosynthetic rate of the glycosaminoglycans has received relatively little attention. An early study of Onodera et al [9] suggested a cell cycle dependency of glucosamine incorporation into cell surface material of 3T3 fibroblasts, which at that time was not identified but very likely consisted primarily of glycosaminoglycans. A study by Kraemer et al [10] showed a preferential release of cell surface heparan sulfate from synchronised hamster CHO cells before mitosis.

We have, therefore, made a comprehensive study of the rate of biosynthesis of hyaluronic acid and of sulfated glycosaminoglycans in mouse embryonal fibroblasts as a function of the cell cycle. We have investigated both the rates of labelling of cellular glycosaminoglycans and of those released into the culture medium. It will be shown that the labelling of the carbohydrate backbone is largely cell cycle dependent, whereas the rate of sulfation follows a different time course.

MATERIALS AND METHODS

Primary Cultures

Balb/c or CBA/J mice (Zentralinstitut für Versuchstierzucht, Hannover, FRG) were sacrificed on day 14 of gestation. Head, limbs, and entrails of embryos were removed and the remaining material was minced with scissors. The minced tissue was treated for 20 min at room temperature with 50 ml 0.25% trypsin (Flow). An addition of 1 mg deoxyribonuclease (Boehringer) decreased clumping of cells. The suspension was centrifuged into a cushion of serum. Cells (1×10^6/ml) were seeded in 20 ml Dulbecco's modified Eagles medium with 20% fetal calf serum (Gibco), containing 100 mg/liter streptomycin and 65 mg/liter penicillin in 75-cm^2 tissue culture flasks (Falcon), and were cultured in humidified air with 5% CO_2 at 37°C. After the first day nonadherent cells were decanted and the medium was changed. After growing to confluency the cells were detached and a single cell suspension was prepared by a 10-min treatment of the cell layer with 0.013% EDTA, 0.08% trypsin (Flow) in phosphate-buffered saline containing 1.2 gm glucose · H_2O/liter and 0.2 gm KCl. The cells were then passaged – usually 5×10^6 cells in 30 ml medium per 75-cm^2 flask – at least five times before use in the experiments indicated below.

Synchronisation and Labelling of Cultures

Fibroblasts were grown to maximal density in 75-cm^2 tissue culture flasks (Balb/c: 9×10^4/cm^2; CBA/J: 18×10^4/cm^2) until they became quiescent (Balb/c: 8–10 days, CBA/J: 5 days). For obtaining synchronous growth the cells were then trypsinised as described above, washed, taken up in Dulbecco's modified Eagles medium with 5% heat-inactivated, non-dialysed fetal calf serum (Gibco), and seeded at a density of $4–7 \times 10^4$ cells/cm^2 into plastic petri dishes (Greiner). Five-ml cultures were grown in 60-mm dishes, 2-ml cultures in 35-mm dishes. Growth was monitored by counting trypsinised cells in a hemocytometer or by microscopical inspection of the cultures.

The cultures were labelled for 12 h prior to harvesting at the indicated times. At each time point, parallel cultures were labelled as follows. Experiment 1, Figure 1: 0.1 μCi [Methyl-^{3}H]-thymidine (18 Ci/mmole, Amersham) per 2-ml culture; 2.36 μCi D-[1-^{14}C]-galactose (60 mCi/mmole, Amersham) per 5-ml culture. The galactose was adjusted to a final concentration of 20 μM with unlabelled sugar. Experiment 2, Figure 2: 1 μCi [Methyl-^{3}H]-thymidine (2 Ci/mmole, Amersham) per 5-ml culture; double-labelling with 100 μCi [^{35}S]-sulfate and 5 μCi D-[^{3}H(G)]-galactose (247 mCi/mmole NEN) per 5-ml culture. The galactose was again adjusted to 20 μM as in Experiment 1. Experiment 3, Figures 3 and 4 (all 5-ml cultures): [^{3}H]-thymidine labelling as in Experiment 2, [^{14}C]-galactose labelling as in Experiment 1, double-labelling with [^{3}H]-galactose as in Experiment 2, but only 25 μCi [^{35}S]-sulfate per culture.

The extent but not the time course of galactose incorporation has been observed to vary with certain batches of serum, though thymidine uptake and sulfate incorporation were not affected.

Determination of Thymidine Incorporation

The medium from the thymidine-labelled cultures was decanted. The petri dish was rinsed twice with phosphate-buffered saline containing 5 mM Na_2SO_4. The cells were then detached with 1.0 ml trypsin EDTA mixture as above and pipetted into a test tube. The dish was rinsed once with 1.0 ml phosphate-buffered saline and the wash combined with the cells. Three 0.2-ml aliquots were pipetted into the wells of microtiter plates (Greiner, Nürtingen, FRG), and the cells were washed onto filters using a Skatron multiple cell culture harvester (Flow, Bonn, FRG). Perchloric acid-precipitable radioactivity was counted in emulsifier scintillator 299 in a (Packard) scintillation counter. The data presented are computed from the average of the three aliquots.

Lipid Extraction of the [^{14}C]-Galactose-Labelled Cells

The medium was quantitatively removed. The cells were rinsed and detached as above, transferred into a conical glass tube, pelleted by centrifugation, and washed once with a 1-ml rinse of the petri dish with phosphate-buffered saline. The cells were frozen until analysis and then suspended in 2-ml chloroform/methanol 2/1 (v/v) by brief sonication. Insoluble material was removed by centrifugation. The extract was partitioned 4 times with 0.2 M glycine · HCl pH 2.2, 0.1 M $CaCl_2$ to remove pool material [11]. The organic phase was dried, redissolved in scintillation fluid, and radioactivity was determined as above.

Analysis of Labelled Cells for Glycosaminoglycans

The medium was quantitatively removed and stored at –20°C until analysis. The petri dish was washed as above and the cells were detached by incubation with 1 ml 0.02% EDTA in phosphate-buffered saline containing 1.2 gm/liter glucose · H_2O and 0.2 gm KCl/liter. After 30 min at 37°C, the cells could be quantitatively removed and were transferred into a small conical plastic tube. They were washed as described above and frozen until use. The cells were treated with 50 μg pronase P (Serva, Heidelberg) in 0.5 ml 0.05 M Tris Cl, pH 7.5, for 24 h at 37°C. This treatment solubilised about 80% of the galactose radioactivity and all of the sulfate radioactivity. Pronase was inactivated by heating for 3 min at 100°C, and insoluble material was removed by centrifugation. Aliquots of the supernatants containing the glycosaminoglycans were subjected to fractionation as described by Blumenkrantz et al [12] either before or after further enzymatic treatment.

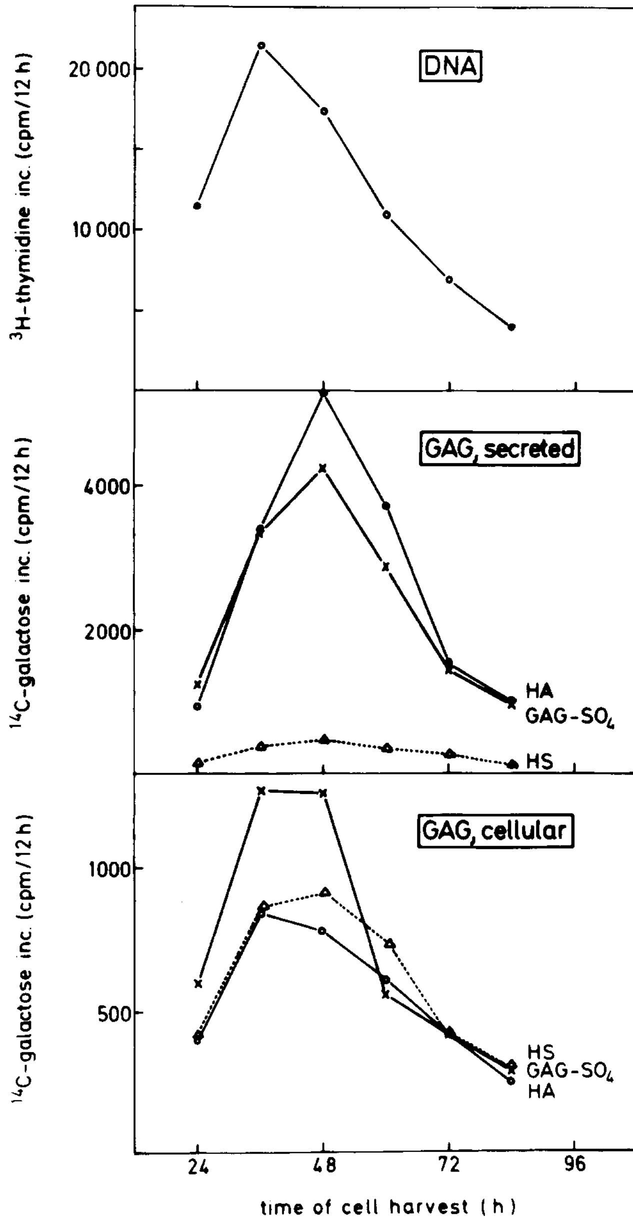

Fig. 1. Rate of labelling with ^{14}C-galactose of glycosaminoglycans in Balb/c fibroblast cultures undergoing one synchronous division. Cells derived from a confluent monolayer were seeded at 4.3×10^4 cells/cm^2 in 60-mm petri dishes. Parallel cultures were pulsed for 12 h with thymidine and galactose and harvested at the indicated times. A) thymidine incorporation; B) ^{14}C-galactose incorporation into glycosaminoglycans in the medium, (o) hyaluronic acid, (X) sulfated glycosaminoglycans, (△) heparan sulfate; C) ^{14}C-galactose incorporation into cellular glycosaminoglycans, same symbols as in B.

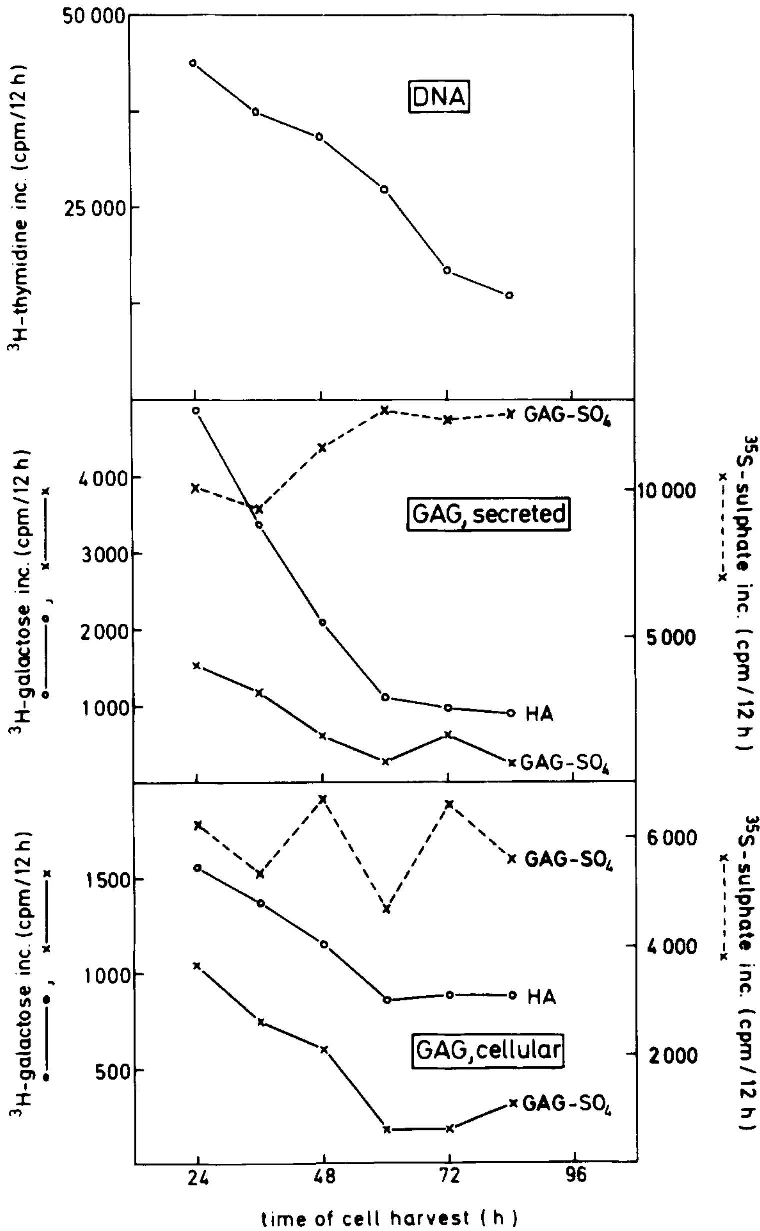

Fig. 2. Rate of labelling with ^{3}H-galactose and ^{35}S-sulfate of glycosaminoglycans in Balb/c fibroblast cultures undergoing nonsynchronous divisions. Cells derived from an actively dividing culture were seeded at 5 × 10^4 cells/cm^2 into 60-mm petri dishes. Parallel cultures were pulsed for 12 h with thymidine or double-labelled with ^{3}H-galactose and ^{35}S-sulfate, and harvested at the indicated times. A) thymidine incorporation; B) ^{3}H-galactose and ^{35}S-sulfate incorporation into glycosaminoglycans of the medium; (○—○) hyaluronic acid – tritium label, (X—X) sulfated glycosaminoglycans – tritium label, (X– – –X) sulfated glycosaminoglycans – ^{35}S-label, C) ^{3}H-galactose and ^{35}S-sulfate incorporation into cellular glycosaminoglycans, same symbols as in B.

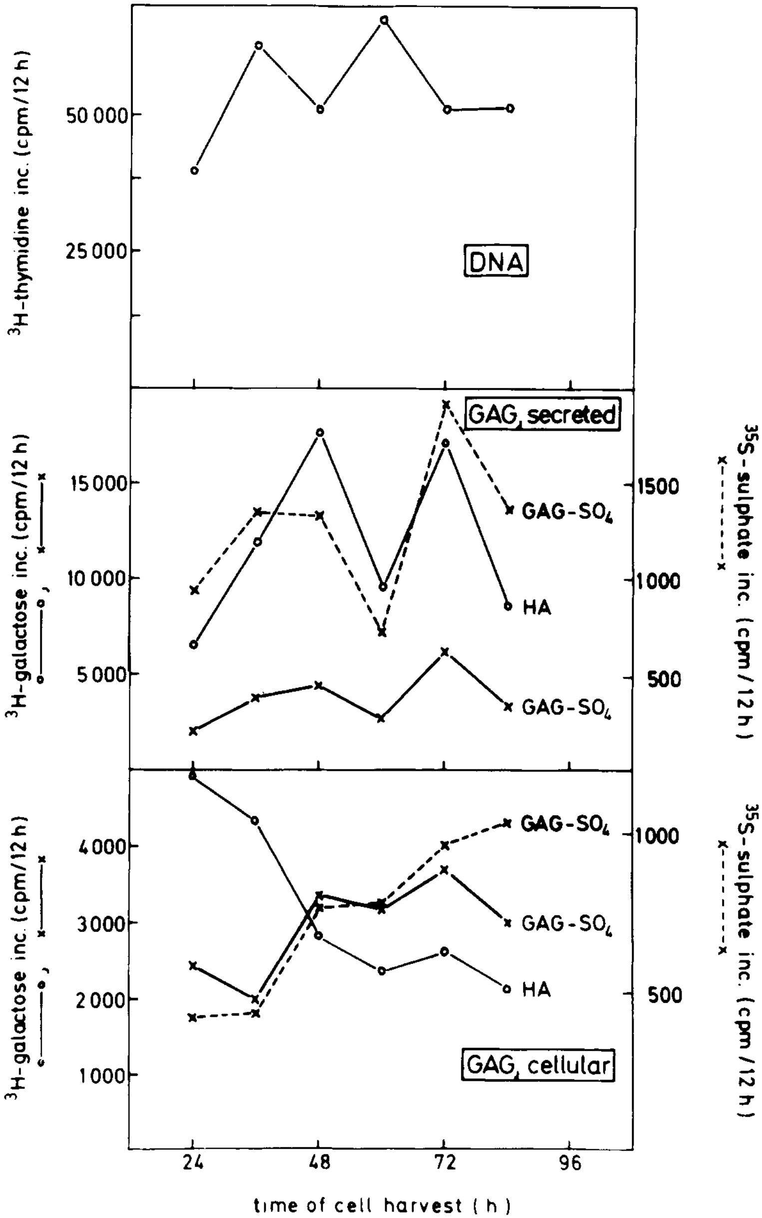

Fig. 3. Rate of labelling with ^{3}H-galactose and ^{35}S-sulfate of glycosaminoglycans in Balb/c fibroblast cultures undergoing two divisions. Cells derived from a resting confluent culture were seeded at 5×10^4 cells/cm^2 into 60-mm petri dishes. Three parallel cultures were pulsed for 12 h with (a) thymidine, (b) double-labelled with ^{3}H-galactose and ^{35}S-sulfate, or (c) with ^{14}C-galactose, and harvested at the indicated times. The ^{14}C-labelled cells were extracted for lipids (see Fig. 4). A) Thymidine incorporation; B) ^{3}H-galactose and ^{35}S-sulfate incorporation into glycosaminoglycans of the medium, (o—o) hyaluronic acid – tritium label, (X—X) sulfated glycosaminoglycans – tritium label, (X – – – X) sulfated glycosaminoglycans – ^{35}S-label, C) ^{3}H-galactose and ^{35}S-sulfate incorporation into cellular glycosaminoglycans, same symbols as in B.

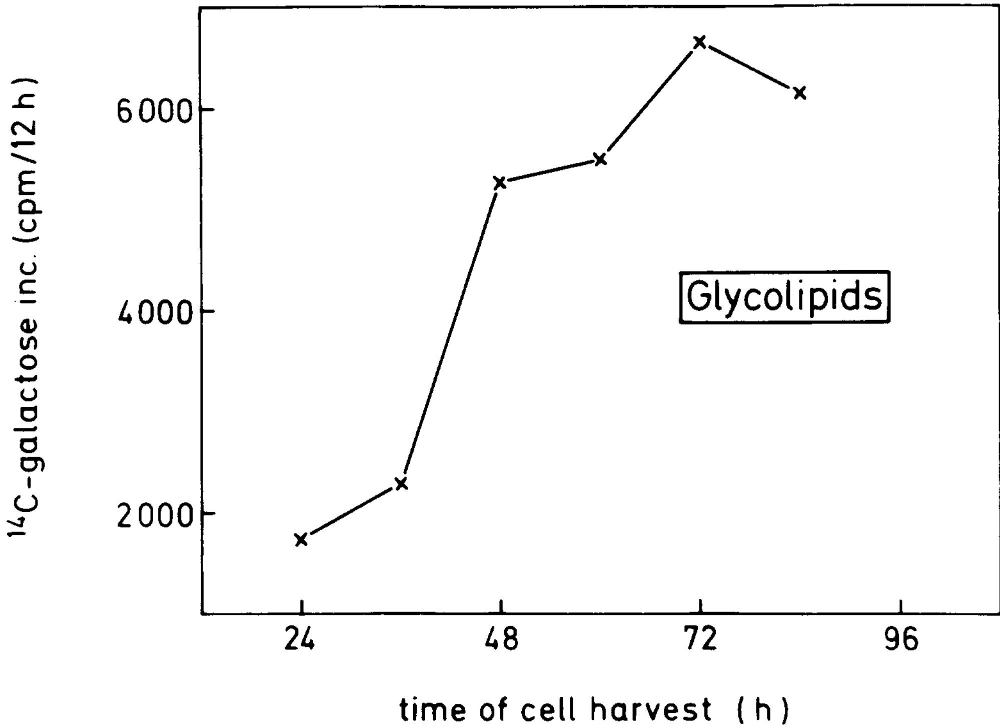

Fig. 4. Rate of labelling with ^{14}C-galactose of glycolipids in Balb/c fibroblast cultures undergoing two divisions. Cells from the third, ^{14}C-galactose-labelled parallel culture from the experiment depicted in Figure 3 were extracted with chloroform/methanol 2/1 (v/v). Nonlipid pool material was removed. The data represent ^{14}C-galactose radioactivity incorporated into lipid during a 12-h pulse.

Briefly, glycosaminoglycans are precipitated with 10% cetyl pyridinium chloride on a celite column, and are subsequently fractionated by increasing concentrations of HCl. Such a fractionation is shown in Table I. Radioactivity was determined in 0.5-ml aliquots. In double-labelled samples the percent spillover of [^{35}S]radioactivity into the tritium channel and the counting efficiency of tritium depend on the HCl content of these fractions. Both parameters were therefore determined with standards for each fraction, and the data corrected accordingly.

Analysis of Culture Media for Glycosaminoglycans

The medium from one culture (5 ml) was digested with 500 μg pronase P for 24 h at 37°C. After this time, 500 μg fresh pronase was added and the incubation was prolonged for another 24 hours. The digests were exhaustively dialysed against 0.05 M Tris-SO_4, pH 7.5, and were lyophilised. The residue was taken up in 1.0 ml water, was heated for 3 min at 100°C to inactivate pronase, and insoluble material was centrifuged down. Over 90% of the galactose radioactivity and all of the sulfate radioactivity remained in solution. The samples were analysed as described above.

TABLE I. Fractionation of Glycosaminoglycans From Culture Medium From CBA/J Fibroblasts Before and After Treatment With Chondroitin Sulfate ABC Lyase

			No enzyme treatment			After treatment with chondroitinase ABC		
Fraction number	Eluant	Volume (ml)	^{3}H-galactose label (counts/min)	^{35}S-sulfate label (counts/min)	Eluted components	^{3}H-galactose label (counts/min)	^{35}S-sulfate label (counts/min)	Eluted components
1	10% cetyl-pyridinium chloride	1.47	922	3,602	Glycopeptides	1,618	8,676	Glycopeptides and depolymerised hyaluronic acid and chondroitin sulfates (fractions 1–5)
2	0.005 N HCl	1.0	96	278	Glycopeptides	371	1,498	
3	0.005 N HCl	0.5	–	7	–	–	49	
4	0.5 N HCl	2.0	3,836	164	Hyaluronic acid (fractions 4–5)	3,169	380	
5	1.0 N HCl	1.0	1,282	74		1,679	122	
6	2.0 N HCl	1.0	850	498	Chondroitin sulfates and heparan sulfate (fractions 6–8)	993	554	Heparan sulfate (fractions 6–8)
7	3.0 N HCl	2.5	2,649	6,215		1,316	1,065	
8	4.0 N HCl	2.0	181	132		452	72	

The medium (5 ml) from a ^{3}H-galactose, ^{35}S-sulfate double-labelled culture was digested with pronase, dialysed, and freeze dried. The sample was redissolved, divided in two equal portions – one of which was treated with chondroitinase ABC and the other left untreated. Both samples were then applied to a 0.5-ml celite column and the glycosaminoglycans were eluted according to Blumenkrantz et al [12].

Enzyme Treatments of Glycosaminoglycans

Pilot experiments established that the following conditions were adequate for complete enzymatic breakdown of the respective glycosaminoglycans derived from cells or medium of 5-ml cultures: One-tenth units chondroitinase ABC from Proteus vulgaris (Sigma), 0.05 M Tris·Cl, pH 8.0, 30 min 37°C in a total volume of 500 μl; 0.025 units chondroitinase AC from Arthrobacter aurescens (Sigma), 0.05 M Tris·Cl, pH 7.5, 30 min 37°C, total volume 50 μl; 80 μg hyaluronidase from sheep testes (Boehringer), 0.05 M ammonium acetate, pH 5.5, 60 min 37°C, total volume 90 μl.

Identification of the Labelled Carbohydrate Moiety From 1-^{14}C-Galactose-Labelled Glycosaminoglycans

Five-ml culture medium from Balb/c fibroblasts was digested with pronase as described above. The digest was dialysed and heated to inactivate the pronase. An aliquot was subjected to gel filtration on a 0.9 X 60-cm Biogel P-100 (Biorad) column in 0.2 M pyridine, 0.1 M acetic acid buffer. Eighty percent of the radioactivity was excluded from the gel. This material was further treated for 26 h with 750 μg hyaluronidase from sheep testes. Filtration of an aliquot on the Biogel P-100 column showed complete disappearance of any high molecular weight material. The main portion was subjected to gel filtration on a 0.9 X 30 cm Biogel P-2 column in the same pyridine-acetic acid buffer. Acid hydrolysis of the radioactive oligosaccharides included in the gel (1 N H_2SO_4, 4 h, 100°C) yielded an apparently neutral substance as the major radioactive product that did not migrate on paper electrophoresis in pyridine/acetic acid/water (10/4/86) buffer, pH 5.4. Prolonged acid hydrolysis in 4 N HCl for 3, 6, or 12 h at 100°C led to eventual destruction of this material without release of radioactive glucosamine or any other well-defined radioactive product. Such properties can be expected from the zwitterionic disaccharide hyalobiuronic acid [13] when labelled in the uronic acid moiety. A 30-h treatment of the pronase- and hyaluronidase-digested material with β-glucuronidase from E coli (Boehringer) gave a poor yield of a substance eluting with monosaccharides from the P2 column and co-migrating with glucorunic on paper electrophoresis.

RESULTS

Fate of the Galactose Label Incorporated Into Glycosaminoglycans

Galactose was chosen as a common radiolabel for the carbohydrate moiety of several glycoconjugate species. Galactose was given preference over glucosamine, because pilot experiments showed that the incorporation of galactose radioactivity into glycosaminoglycans was about four times that of the aminosugar when both carbohydrates were offered at the same concentration and specific radioactivity.

In order to identify the radioactive carbohydrate moiety in glycosaminoglycans from galactose-labelled cultures, the medium, containing the bulk of radioactivity as hyaluronic acid (see below), was successively pronase-digested, dialysed, and hyaluronidase-treated. The depolymerised glycosaminoglycans were separated from glycopeptides by gel chromatography on biogel P12. The behavior of the included material upon acid hydrolysis and treatment with glucuronidase suggests strongly that the label was primarily in the glucuronic acid moiety.

Synchronisation of Growth

Partial synchronisation of growth was achieved by letting the fibroblasts grow to saturation density and subsequently transferring them into new cultures. We found that the cell density in these new cultures was not very critical for subsequent synchronous growth. Within certain limits of $3-7 \times 10^4$ cells/cm^2, synchronous thymidine uptake simply increased proportionately to the cell numbers. It proved, however, essential to let the cells become fully quiescent before transfer. The transfer of quiescent cells leads to one (Fig. 1, panel A) or two (Fig. 3, panel A) maxima of DNA synthesis, indicating a certain degree of synchrony. Two synchronous divisions were also observed by others using the same method of synchronisation of fibroblasts [9]. The first peak of thymidine incorporation occurred usually between 36 and 48 h with Balb/c cells, occasionally earlier with CBA/J cells. About 12 to 24 h after the maximal DNA synthesis, the cell number usually increased by 50% or more. When cells which had not yet reached confluency were transferred, no maxima of thymidine incorporation indicative of synchrony were observed (Fig. 2, panel A).

Effect of Synchronous Growth on the Rate of Labelling of Glycosaminoglycans With Galactose and ^{35}S-Sulfate

Two or more cultures were labelled in parallel at the same time-points with (a) thymidine, to follow synchrony and determine the S phase, (b) [^{14}C]-galactose to measure the rate of labelling of glycosaminoglycans and/or glycolipids (see below), or (c) [^{3}H]-galactose and [^{35}S]-sulfate for double-labelling glycosaminoglycans. The cultures were each labelled for 12 hours. Such long pulses were chosen to avoid a possible influence of transport rates on the labelling and also to obtain enough labelled material for subsequent analysis. Cells and culture supernatants were then analysed at each time-point as described in Materials and Methods. The chondroitinase ABC-resistant material eluting with 2–3 N HCl from the celite column (see Table I) was taken to be heparan sulfate [12, 8, 14]. The results of one such experiment in which good synchrony was obtained are shown in Figure 1. The rate of labelling with galactose of cellular as well as secreted glycosaminoglycans follows a similar time course as that of DNA synthesis, showing a pronounced maximum at or 12 hours after maximal DNA synthesis.

In a second experiment, cells were used which had not yet reached confluency prior to transfer. These cells did not grow synchronously (Fig. 2). In this case, glycosaminoglycans were double-labelled with [^{3}H]-galactose and [^{35}S]-sulfate. The rate of labelling of all glycosaminoglycan species with galactose decreased gradually with time as did the rate of DNA synthesis. The rate of sulfation of glycosaminoglycans proceeded independently and increased, particularly for the secreted glycosaminoglycans as the cultures became older.

In Figure 3 an experiment is shown in which cultures underwent two waves of DNA synthesis. Glycosaminoglycan sulfates were double-labelled with [^{3}H]-galactose and [^{35}S]-sulfate as in the previous experiment. The rate of labelling with galactose of secreted glycosaminoglycans shows two maxima, again 12 hours later than the maximal DNA synthesis. The cellular sulfated glycosaminoglycans show a similar time course, while the rate of labelling of cellular hyaluronic acid follows a quite different pattern.* Again, the sulfa-

*The more typical time course of labelling of hyaluronic acid is the one shown in Figure 1, which was also observed with CBA/J fibroblasts (data not shown).

TABLE II. Percentage Distribution of Galactose-Labelled Glycosaminoglycans Among Cells and Media in Balb/c and CBA/J Fibroblast Cultures

	Total glycosaminoglycans (%)	Hyaluronic acid (%)	Total sulfated glycosaminoglycans (%)	Chondroitin sulfate ABC (%)	Heparan sulfate (%)
Balb/c					
Cells	18	13	23	8	67
Medium	82	87	77	92	37
CBA/J					
Cells	23	24	21	39	12
Medium	77	76	79	61	88

Synchronous cultures were pulse labelled with ^{14}C-galactose (Balb/c) or ^{3}H-galactose (CBA/J) at the time of maximal labelling from 36 to 48 hours. Media and cells were harvested at 48 hours and analysed as in Table I.

tion rate increases in older cultures with increasing cell numbers. In similar experiments with CBA/J cells, this tendency of stepwise increase of sulfation rate with stepwise increasing cell numbers was even more pronounced (data not shown).

We conclude from these data that there is a cell cycle dependency of the rate of labelling with galactose of secreted hyaluronic acid and of sulfated glycosaminoglycans, be they cell-bound or secreted. Maximal rate of labelling with Balb/c cells occurs about 12 hours after maximal DNA synthesis. The sulfation rate of glycosaminoglycans appears less cell-cycle dependent but increases in older cultures, probably as a result of higher cell numbers.

The Rate of Labelling of Glycolipids During the Cell Cycle

In parallel cultures from the experiment shown in Figure 3, the incorporation rate of galactose into glycolipids was also determined. That galactose is a suitable precursor for the labelling of glycolipids has been shown for several systems [15, 16]. The purpose of this experiment was mainly to assess whether rates of labelling of different glycoconjugate species with carbohydrate follow the same time course. According to the results presented in Figures 3 and 4, this is not the case.

Distribution of Labelled Glycosaminoglycans Among Cells and Medium

When the distribution of carbohydrate label among cells and medium was compared at the time of maximal labelling, significant strain specific differences were noted between Balb/c and CBA/J fibroblast cultures (Table II). About 75%–80% of the total label in the sulfated glycosaminoglycans were found secreted in the media from both strains. However, in Balb/c cultures most of the chondroitin sulfates were secreted, the bulk of the heparan sulfate label being cell-associated, while in CBA/J cultures there was an opposite trend: a higher percentage of the labelled chondroitin sulfate was cellular, whereas most of the heparan sulfate was secreted.

Besides cell-cycle-dependent variations, other factors can influence the distribution of label among cells and medium. For example, in the older CBA/J cultures a significantly higher proportion of particularly hyaluronic acid was cell associated than in younger cultures (67% versus 24%). This effect is more likely due to increased cell density than to a cell cycle event.

TABLE III. Percentage Distribution of Galactose Label Among Cellular and Secreted Glycosaminoglycan Fractions in Balb/c and CBA/J Fibroblasts

	Balb/c		CBA/J	
	Cells (%)	Medium (%)	Cells (%)	Medium (%)
Hyaluronic acid	38	55	63	59
Chondroitin sulfate ABC	17	40	22	10
Heparan sulfate	45	5	15	32

The data are derived from the analysis of the culture described in Table II.

The Composition of the Labelled Cellular and Secreted Glycosaminoglycans

Table III shows the distribution of carbohydrate label in the various cellular and secreted glycosaminoglycans of Balb/c and CBA/J cultures at the time of maximal labelling rate. The bulk of the glycosaminoglycan label in the media is found in both cases in the hyaluronic acid fraction. In other experiments, up to 80% of the label was found in this fraction. The main labelled species of sulfated glycosaminoglycans are chondroitin sulfates in Balb/c and haparan sulfate in CBA/J culture media. The chondroitin sulfates from Balb/c media were analysed in more detail by digestion with hyaluronidase from sheep testes, chondroitinase AC, and chondroitinase ABC. The first two enzymes mentioned do not degrade chondroitin sulfate B (dermatan sulfate) and heparan sulfate; the latter enzyme also degrades chondroitin sulfate B. From the differential susceptibility of the chondroitin sulfates toward these enzyme treatments it could be estimated that the labelled chondroitin sulfates contain about 10%–20% chondroitin sulfate B.

The composition of cellular glycosaminoglycans also differs in Balb/c and CBA/J fibroblasts. In Balb/c cells a comparatively higher proportion of label is associated with sulfated glycosaminoglycans, in particular with heparan sulfate, than in CBA/J cells. In addition, the position in the cell cycle can distinctly influence the proportions of the labelled glycosaminoglycan species (see Figs. 1 and 3, bottom).

DISCUSSION

Our results indicate that the rate of labelling with galactose of all secreted glycosaminoglycan species goes through a maximum about 12 hours after maximal DNA synthesis. The observed variation of the galactose incorporation rate during the cell cycle could be caused by several effects. However, for the following reasons we consider the rate of labelling to be an indicator of the biosynthetic rate.

A) Maximal galactose incorporation was measured in our experiments many hours after cell transfer. This effect was therefore unrelated to the increase in the carbohydrate transport rate which happened within minutes after release from contact inhibition of growth by serum or trypsinisation [17–19].

B) In one and the same experiment (see Figs. 3, 4) the rates of labelling of different glycoconjugates with galactose as common precursor followed different time courses. This finding makes it unlikely that the rate of labelling is primarily determined by the rate of transport or phosphorylation of galactose.

C) The rate of labelling of hyaluronic acid and sulfated glycosaminoglycans in cells from identical cultures (see Fig. 3, bottom) differed considerably. A cell-cycle-dependent variation of the rate of epimerisation or of conversion of the label to uronic acid is therefore equally unlikely to be the cause of the observed cell-cycle-dependent changes in galactose incorporation into glycoconjugates. We conclude that the rate of labelling of glycosaminoglycans reflects primarily the rate of biosynthesis of these compounds.

It is obvious from the experiments shown in Figures 1–3 that whenever there is active DNA synthesis it is closely followed by biosynthesis of glycosaminoglycans, in particular of those which are secreted. Our results confirm and extend those of Kraemer et al [10] in that we found that not only heparan sulfate but glycosaminoglycans in general are secreted during the time span between S phase and cell division, ie, during the G_2 or M phase of the growth cycle. Moreover, biosynthesis of cell-bound glycosaminoglycans, with the possible exception of hyaluronic acid, also appears to be enhanced at this time.

Observations of other investigators who found that (a) primarily growing, in contrast to nongrowing, cells secrete glycosaminoglycans [20, 21], (b) that serum (because it stimulates growth) stimulates glycosaminoglycan synthesis [22], or (c) that cortisol simultaneously inhibits glycosaminoglycan and DNA synthesis [23] can be understood on the basis of our findings that the biosynthesis of glycosaminoglycans appears to be closely linked to the cell cycle. In drawing conclusions from comparative studies of glycosaminoglycan biosynthesis in normal versus virus-transformed or sparsely versus densely growing cells, it may sometimes be difficult to separate the effects of the growth cycle from those caused by transformation or by cell density per se.

Surprisingly, the rate of sulfation of glycosaminoglycan sulfates does not generally parallel that of the formation of the polysaccharide backbone but tends to increase in older cultures containing nondividing cells (see Fig. 2). Trivial explanations, such as differential losses during analysis for example, can be ruled out because of the double-labelling technique. One reason for this apparent discrepancy may be the preferential synthesis of a highly sulfated species of glycosaminoglycan sulfate in older cultures. Indeed, cellular glycosaminoglycan sulfate synthesised by Balb/c fibroblasts towards the end of the culture period appears to consist primarily of the highly sulfated heparan sulfate (see Fig. 1, bottom panel), while actively dividing cells synthesize chondroitin sulfates (see Table III). It is additionally possible that the degree of sulfation of any glycosaminoglycan sulfate species can increase during cultivation. Both processes should lead to glycosaminoglycans in old cultures with a comparatively high degree of sulfation. Whether or not this phenomenon is causally related with a hypothetical role of sulfated glycosaminoglycans as growth control elements [5, 6] remains to be elucidated.

ACKNOWLEDGMENTS

We thank Mrs. Toni Mühlradt for patiently analysing all the samples, and Dr. D. Monner for critically reading the manuscript.

REFERENCES

1. Saito H, Uzman BG: Biochem Biophys Res Commun 43:723, 1971.
2. Underhill C, Dorfman A: Exp Cell Res 117:155, 1978.
3. Terry AH, Culp LA: Biochemistry 13:414, 1974.
4. Culp LA: Biochemistry 15:4094, 1976.

5. Roblin R, Albert SO, Gelb NA, Black PH: Biochemistry 14:347, 1975.
6. Chiarugi VP, Vannucchi S: J Theor Biol 61:459, 1976.
7. Cohn RH, Cassiman JJ, Bernfield MR: J Cell Biol 71:280, 1976.
8. Winterbourne DJ, Mora PT: J Biol Chem 253:5109, 1978.
9. Onodera K, Sheinin R: J Cell Sci 7:337, 1970.
10. Kraemer PM, Tobey RA: J Cell Biol 55:713, 1972.
11. Carter TP, Kanfer JN: Methods Enzymol 35:549, 1975.
12. Blumenkrantz N, Asboe-Hansen G: Methods Biochem Anal 24:78, 1977.
13. Rapport MM, Weissmann B, Linker A, Meyer K: Nature 168:996, 1951.
14. Keller KL, Underhill CB, Keller JM: Biochim Biophys Acta 540:431, 1978.
15. Chatterjee S, Sweeley CC, Velicer LF: Biochem Biophys Res Commun 54:585, 1973.
16. Rosenfelder G, van Eijk RVW, Monner DA, Mühlradt PF: Eur J Biochem 83:571, 1978.
17. Trash CR, Cunningham DD: Nature 252:45, 1974.
18. Vaheri A, Ruoslahti E, Nordling S: Nature 238:211, 1972.
19. Sefton BM, Rubin H: Proc Natl Acad Sci USA 68:3154, 1971.
20. Koyama H, Ono T: Biochim Biophys Acta 217:477, 1970.
21. Moscatelli D, Rubin H: Nature 254:65, 1975.
22. Kurtz MJ, Stidworthy GH: Biochim Biophys Acta 399:90, 1974.
23. Saarni H, Tammi M: Biochim Biophys Acta 540:117, 1978.