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Evidence for rapid metabolic turnover of hyaluronate synthetase in Swarm rat chondrosarcoma chondrocytes

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1. Synthesis of [^{3}H]hyaluronate from [6-^{3}H]glucosamine was investigated in cultures of Swarm rat chondrosarcoma chondrocytes treated with various concentrations (0.1 μM–0.1 mM) of cycloheximide for various times. Concentrations > 1 μM inhibited protein synthesis by > 90%. Hyaluronate synthesis was decreased, with a $t_{\frac{1}{2}}$ for 50% inhibition of 80–120 min, depending on the concentration of cycloheximide present. 2. Similar experiments using [1-^{3}H]glucose as a precursor label gave similar results. 3. Experiments using [6-^{3}H]glucosamine as a precursor label and 0.18 mM-puromycin to inhibit protein synthesis inhibited hyaluronate synthesis ($t_{\frac{1}{2}}$ = 82 min) with similar kinetics to cycloheximide-induced inhibition. 4. Cultures incubated with 3.6 μM-cycloheximide for up to 9 h and supplemented with *p*-nitrophenyl β-D-xyloside during the last 75 min of treatment showed increased synthesis of [^{3}H,^{35}S]chondroitin sulphate, demonstrating that UDP-hexose precursors for glycosaminoglycan synthesis are not rapidly depleted on blockage of protein synthesis. 5. Rapid metabolic turnover of hyaluronate synthetase is the most likely cause for decreased hyaluronate synthesis in chondrocytes in which protein synthesis is inhibited.

INTRODUCTION

The glycosaminoglycan hyaluronate is synthesized by chondrocytes and has an important role in forming proteoglycan aggregates in the extracellular matrix of cartilage and, possibly, a regulatory role in controlling the extent of proteoglycan synthesis by the cells (for review, see [1]). The regulation of hyaluronate synthesis may therefore be important in the maintenance of normal cartilage function.

Hyaluronate is synthesized by a different mechanism from other glycosaminoglycans. The chains grow at the reducing end by alternate addition of UDP-*N*-acetylglucosamine and UDP-glucuronate, with the release of UDP from the polymer at each addition [2,3]. Other glycosaminoglycans, e.g. chondroitin sulphate, are synthesized by transfer of hexoses from UDP-hexoses, first to an amino acid side chain of a core protein, and subsequently to the non-reducing end of the growing chain. The first hexose transfer is usually from UDP-xylose to the hydroxyl group of serine or threonine (for review, see [4]). The extent of chondroitin sulphate synthesis in chondrocytes is limited by the available core protein, since large increases occur if the cells are given *p*-nitrophenyl β-D-xyloside, or similar compounds, which act as artificial initiators for chain polymerization [5–7].

The mechanism proposed for hyaluronate synthesis would not require a core protein, and none was identified in newly synthesized hyaluronate in cultured chondrocytes [8]. Thus the extent of hyaluronate synthesis is likely to be regulated by mechanisms different from those affecting chondroitin sulphate–proteoglycan synthesis. We show here that hyaluronate synthesis decreases rapidly after inhibition of protein synthesis in Swarm rat chondrosarcoma chondrocytes. Since UDP-hexose concentrations in inhibited cells remain adequate for nitrophenyl xyloside–chondroitin sulphate synthesis, the decline in hyaluronate synthesis is most likely due to rapid turnover of the hyaluronate synthetase.

METHODS

Primary chondrocyte cultures were established from the Swarm rat chondrosarcoma [9]. Cultures were maintained at 37 °C in a humidified air/CO_2 (19:1) atmosphere in Dulbecco's modified Eagle's medium supplemented with 15% (v/v) foetal-calf serum (Flow Laboratories, batch no. U6004015), organic buffers, penicillin and streptomycin [9]. The medium was changed daily. The chondrocytes (2×10^6 cells per 35 mm dish) were used for experiments on day 2 in culture.

Cultures were treated for various times with or without either cycloheximide (0.36 μM–0.36 mM) or puromycin (0.18 mM) in Dulbecco's modified Eagle's medium containing 15% foetal-calf serum and the other supplements. During the last 30 or 45 min of treatment, the cells were labelled with ^{3}H or ^{35}S-labelled precursors of macromolecules in medium of the same composition except for foetal-calf serum, which was omitted. The precursors were L-[5-^{3}H]proline (37 Ci/mmol), L-[3-^{3}H]-serine (10–30 Ci/mmol), D-[6-^{3}H]glucosamine (20–40 Ci/mmol), D-[1-^{3}H]glucose (3–10 Ci/mmol) and $Na_2{}^{35}SO_4$ (25–40 Ci/mg). All radionuclides were obtained from Amersham International, Amersham, Bucks., U.K. At the end of the labelling period the medium was removed and the cell layer solubilized with 4% (w/v) Zwittergent TM^{12}/4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, a procedure which extracts > 95% of the macromolecules present [10]. The extract was combined with the medium for further analysis.

In some experiments, chondrocyte cultures were

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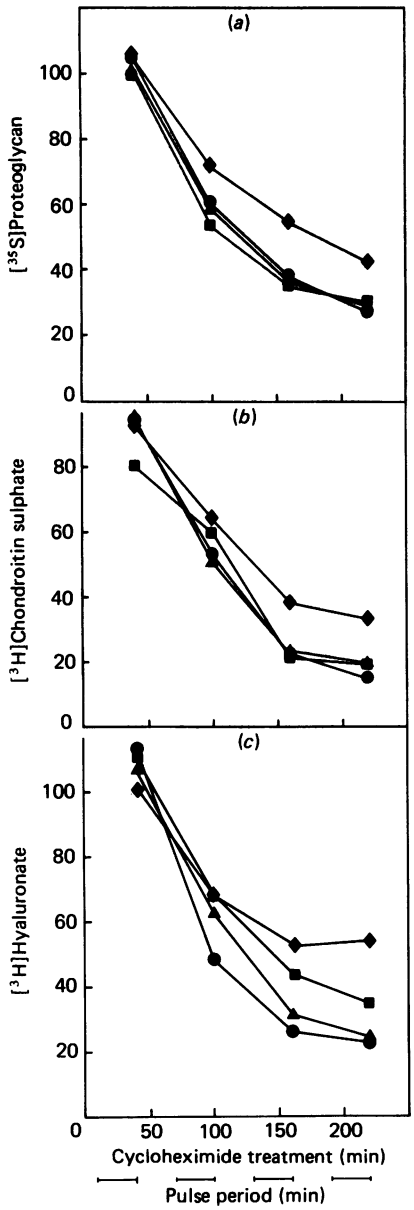

Fig. 1. Effect of cycloheximide on [^{3}H]hyaluronate and [^{3}H,^{35}S]chondroitin sulphate synthesis in chondrocyte cultures

Cultures were treated with various concentrations of cycloheximide (◆, 0.36 μM; ■, 3.6 μM; ▲, 36 μM; ●, 0.36 mM) for 45 to 220 min and pulsed with [^{3}H]glucosamine and [^{35}S]sulphate during the last 30 min of treatment. Incorporation of radioactivity into (*a*) [^{35}S]proteoglycan, (*b*) [^{3}H]chondroitin sulphate and (*c*) [^{3}H]hyaluronate was measured. Control cultures incorporated 7.7×10^5 d.p.m. for (*a*), 2.1×10^5 d.p.m. for (*b*) and 0.35×10^5 d.p.m. for (*c*). Incorporation of radioactivity by treated cultures is expressed as a percentage of the control. Three other experiments gave similar results.

treated with cycloheximide (3.6 μM) for various times up to 9.25 h. At 75 min before the end of each treatment, the medium was changed to fresh medium containing cycloheximide and 0.3 mM-*p*-nitrophenyl β-D-xyloside [10] dissolved in dimethyl sulphoxide (final concn. 0.3 mM). After 30 min incubation the medium was changed again to medium of the same composition except for the omission of foetal-calf serum and the inclusion of $Na_2{}^{35}SO_4$ and [^{3}H]glucosamine. Incubation was continued for 45 min. Control cultures containing dimethyl sulphoxide but lacking cycloheximide, or nitrophenyl xyloside, or both were set up for each time point and treated in the same way.

The combined medium and extract from treated and control cultures were analysed for [^{35}S]proteoglycans and [^{3}H]proteins by gel filtration on Sephadex G-25 (PD10 columns; Pharmacia) [11]. ^{3}H-labelled chondroitin sulphate isomers and hyaluronate were analysed after digestion with papain and chondroitinase ABC to liberate specific unsaturated disaccharides for each, which were separated by cellulose t.l.c. and counted for radioactivity [11].

Table 1. Times for 50% inhibition ($t_{\frac{1}{2}}$) of synthesis of [^{35}S]proteoglycan, [^{3}H]chondroitin sulphate and [^{3}H]hyaluronate in chondrocyte cultures treated with cycloheximide

Incorporation of radioactivity into [^{35}S]proteoglycan and [^{3}H]hyaluronate in cycloheximide-treated cultures slightly exceeded that in controls during the first pulse period (Figs. 1*a* and 1*c*) and was taken as maximum synthesis for these molecules. Maximum synthesis for [^{3}H]chondroitin sulphate (Fig. 1*b*) was taken as 100% of the control culture. The time (t min) taken for a 50% decrease from maximum synthesis was found for each labelled molecule in the treated cultures. $t_{\frac{1}{2}}$ was defined as $(t-30)/2$ min, since incorporation of labelled precursors into macromolecules within the pulse period (30 min) represents the mean synthesis during the pulse and not that at the end of it.

Cycloheximide concn....	$t_{\frac{1}{2}}$ (min)		
	3.6 μM	36 μM	0.36 mM
[^{35}S]Proteoglycan	95	105	105
[^{3}H]Chondroitin sulphate	90	85	100
[^{3}H]Hyaluronate	117	103	78

RESULTS AND DISCUSSION

Treatment of chondrocytes with 3.6 μM or higher concentrations of cycloheximide resulted in an immediate 90%, or greater, inhibition of protein synthesis, as measured by incorporation of [^{3}H]proline or [^{3}H]serine into macromolecules (results not shown). The results agree well with those reported previously [10]. Cycloheximide at 0.36 μM (0.1 μg/ml) inhibited [^{3}H]protein synthesis by only 65–70%.

The effect of cycloheximide on [^{3}H]hyaluronate synthesis was similar to its effect on [^{3}H]chondroitin sulphate and [^{35}S]proteoglycan synthesis (Fig. 1). The time taken for 50% inhibition ($t_{\frac{1}{2}}$) of the latter was about 100 min (Table 1), in agreement with previous reports [10,12], and consistent with chondroitin sulphate polymerization and sulphation following one another rapidly during the last few minutes of intracellular core-protein dwell time in these chondrocytes [13,14]. The kinetics of inhibition of [^{3}H]hyaluronate synthesis in chondrocyte cultures treated with 3.6 μM–0.36 mM-cycloheximide showed more variation with the concentration of the inhibitor than was found for the [^{3}H,^{35}S]chondroitin sulphate proteoglycan (Fig. 1, Table 1). This may reflect

Table 2. Ratio of ^3H:^{35}S radioactivity in unsaturated chondroitin 4-sulphate disaccharides isolated from cycloheximide-treated and control chondrocyte cultures

Cultures were dual-labelled with $Na_2{}^{35}SO_4$ and [^{3}H]glucosamine as described in the Methods section.

Cycloheximide concn.	Treatment time (min)...	40	100	160	220
0.36 mM		1.10	1.01	0.81	0.87
36 μM		1.05	1.01	0.82	0.78
3.6 μM		1.10	1.07	0.92	0.89
0.36 μM		1.10	0.96	0.99	1.13
0 (control)		1.19	1.30	1.40	1.30

changes in the specific radioactivity of the UDP-*N*-acetyl[^{3}H]hexosamine precursor pool. Decreases in the ratio of $^3H/^{35}S$ radioactivity in chondroitin 4-sulphate occurred with increasing cycloheximide concentration and treatment time (Table 2), consistent with a small expansion in the UDP-*N*-acetylhexosamine pool in inhibited cells [15], assuming that the intracellular concentration of sulphate which diffuses into chondrocytes [16] remains unchanged. Cycloheximide at 3.6 μM (1 μg/ml) causes the smallest changes in $^3H/^{35}S$ ratio while still inhibiting protein synthesis completely. By analogy, the $t_{\frac{1}{2}}$ value for inhibition of hyaluronate synthesis under these conditions (117 min) is probably the most reliable.

A similar inhibition of [^{3}H]hyaluronate and [^{3}H]chondroitin sulphate synthesis occurred in cultures treated with various concentrations of cycloheximide but labelled with [1-^{3}H]glucose (results not shown). Glucose is a precursor of both the hexuronate and the hexosamine moiety of glycosaminoglycans. Thus the results confirm that failure to incorporate [^{3}H]glucosamine into glycosaminoglycans (Fig. 1) in the presence of cycloheximide is not due solely to changes in the specific radioactivity of the UDP-*N*-acetylhexosamine pool.

Chondrocyte cultures were treated with 0.18 mM-puromycin and labelled with [^{35}S]sulphate and [^{3}H]glucosamine during the least 45 min of treatment. This concentration of puromycin inhibits protein synthesis by ~ 85% in the chondrocyte cultures [17], but by a mechanism different [18–20] from that of cycloheximide. [^{3}H]Hyaluronate synthesis was inhibited in the same way in puromycin-treated cultures (Fig. 2) as in those treated with cycloheximide. The time ($t_{\frac{1}{2}}$) taken for 50% inhibition of [^{3}H]hyaluronate and [^{35}S]proteoglycan synthesis by puromycin was 83 and 96 min respectively, within the range of values obtained with cycloheximide inhibition (Table 1).

In the absence of a requirement for a core protein for hyaluronate synthesis, the results suggest that either hyaluronate synthetase turns over quite rapidly in the Swarm chondrocytes, with consequent loss of activity after inhibition of protein synthesis, or that the latter leads to a depletion of the UPD-hexose precursors of glycosaminoglycan chains. Attempts to isolate hyaluronate synthetase for cell-free assays were unsuccessful, but the availability of UDP-hexoses during cycloheximide treatment was tested.

Nitrophenyl β-D-xyloside and similar β-xylosides initiate the synthesis of chondrotin sulphate chains *in vitro* [5–7]. The biosynthetic system reaches maximum

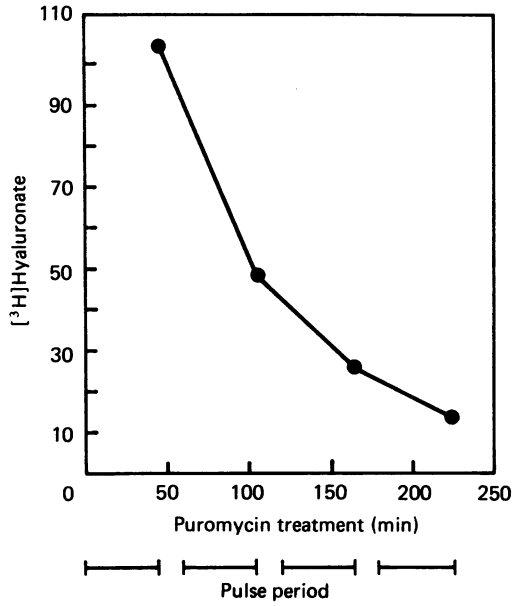

Fig. 2. Effect of puromycin on [^{3}H]hyaluronate synthesis in chondrocyte cultures

Cultures were treated with 0.18 mM-puromycin for 45 to 225 min and pulsed with [^{3}H]glucosamine during the last 45 min of treatment. Incorporation of radioactivity into [^{3}H]hyaluronate was measured and expressed as a percentage of the control culture.

capacity with 0.1 mM-β-D-xyloside in Swarm chondrocytes [10], a value determined by the amount of UDP-glycosyltransferases present and the availability of UDP-hexose precursors. The same UDP-hexoses are required for synthesis of both chondroitin sulphate and hyaluronate [4]. Chondrosarcoma chondrocyte cultures were treated with 3.6 μM-cycloheximide for various times up to 9 h. The cells were viable throughout the treatment period, as assessed by exclusion of Trypan Blue. Nitrophenyl β-D-xyloside (0.3 mM) was introduced during the last 75 min of treatment, and cultures were labelled with [^{3}H]glucosamine and [^{35}S]sulphate during the last 45 min. Replicate cultures were treated with cycloheximide and labelled without β-D-xyloside (Fig. 3). In these cultures the time taken for 50% inhibition of [^{35}S]proteoglycan, [^{3}H]chondroitin sulphate and [^{3}H]hyaluronate synthesis were 120, 135 and 150 min respectively. These times are rather longer than those shown in Table 1 and Fig. 1 and should be regarded as

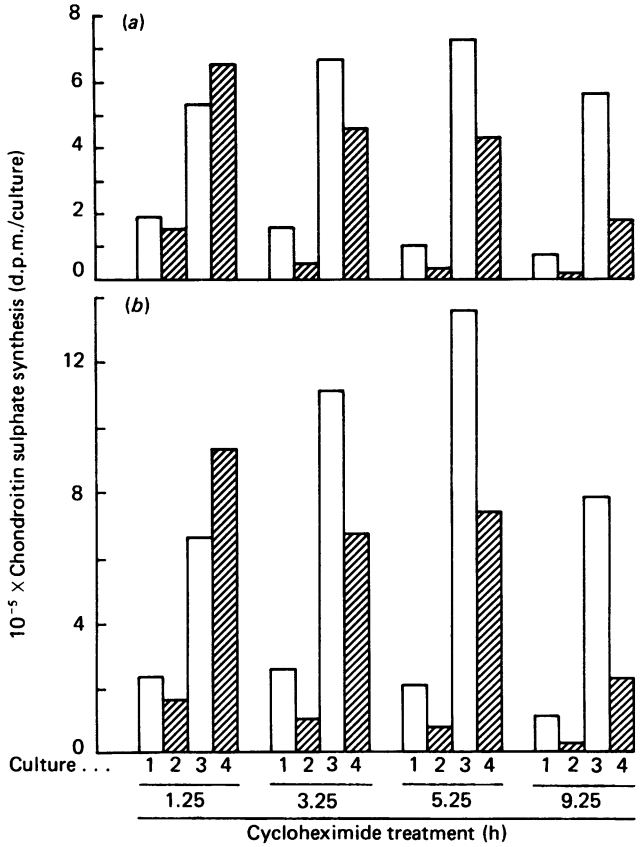

Fig. 3. Effect of nitrophenyl β-D-xyloside on [^{3}H,^{35}S]chondroitin sulphate synthesis in chondrocyte cultures treated with cycloheximide

Control cultures (1,3; □) and cycloheximide (3.6 μM)-treated cultures (2,4; ▨) were labelled with [^{3}H]glucosamine and [^{35}S]sulphate during the last 45 min of the times shown. The medium of some cultures (3, 4) was supplemented with 0.3 mM-nitrophenyl β-D-xyloside during the last 75 min of the experiment. Synthesis of [^{35}S]proteoglycan (*a*) and [^{3}H]chondroitin sulphate (*b*) was measured for each culture. Two other experiments gave very similar results.

less accurate, since the intervals between the pulses and the length of the pulses in this experiment were longer than for the experiment in Table 1.

[^{3}H]Hyaluronate synthesis was not altered significantly in cultures supplemented with nitrophenyl β-D-xyloside (results not shown), but incorporation of ^{35}S and ^{3}H radioactivity into chondroitin sulphate was stimulated in both control and cycloheximide-treated cultures (Fig. 3). The chondrocytes incorporated over twice as much radioactivity into chondroitin sulphate, even after 9 h of cycloheximide treatment, when supplemented with nitrophenyl β-D-xyloside (culture 4, Fig. 3) as they did in the non-supplemented cultures (culture 1, Fig. 3). Since both precursors gave similar results, and as [^{35}S]sulphate incorporation is a reasonably quantitative measure of chondroitin sulphate synthesis, the experiment shows that cycloheximide treatment does not lead to rapid depletion of the UDP-glucuronate and UDP-*N*-acetylhexosamine pools in the chondrocytes. Inhibition of [^{3}H]hyaluronate synthesis during cycloheximide treatment over the period shown in Fig. 1 is therefore unlikely to be due to non-availability of the UDP precursors.

The $t_{\frac{1}{2}}$ for the xyloside–chondroitin sulphate polymerizing system after inhibition of protein synthesis could not be measured, for several reasons. First, increased incorporation of both ^{35}S radioactivity (1.23-fold) and ^{3}H radioactivity (1.40-fold) into xyloside–chondroitin sulphate occurred after 1.25 h of cycloheximide treatment (culture 4, Fig. 3) compared with controls (culture 3), showing a quantitative increase in its synthesis in blocked cells at this stage. This suggests that normally the size of the UDP-hexose precursor pools, rather than the amount of polymerizing enzymes present, is the limiting factor for maximum xyloside–chondroitin sulphate synthesis. After blockage of protein synthesis, decreased demands on UDP-hexose precursors for glycoprotein synthesis would increase their availability for xyloside–chondroitin sulphate. Later during the block, xyloside–chondroitin sulphate synthesis decreases progressively, but it is not known whether this is due to decreasing amounts of UDP-glycosyltransferases for chondroitin sulphate synthesis, or to decreases in UDP-hexoses below the values required for maximal xyloside–glycosaminoglycan synthesis. A further complication is that, for reasons unknown, incorporation of both ^{35}S and ^{3}H radioactivity into xyloside–chondroitin sulphate in untreated cultures increases progressively during the first 5.25 h of the experiment.

General discussion

Inhibitors of protein synthesis bring about a rapid decrease in [^{3}H]hyaluronate synthesis in cultures of Swarm rat chondrosarcoma chondrocytes. The results also confirm that chondroitin sulphate–proteoglycan synthesis is inhibited by only 50% about 100 min after cessation of protein synthesis [10,12], consistent with the existence of a fairly large pool of nascent core protein in the cells awaiting glycosylation [21].

Inhibitors of protein synthesis can have inhibitory effects on cytidylate and guanylate synthesis [22,23] and on sugar transport [24] in some cells. The high *p*-nitrophenylxylosyl–[^{3}H,^{35}S]chondroitin sulphate synthesis in chondrocytes treated with cycloheximide expands previous findings [10] by showing that [^{3}H]glucosamine must be taken up, converted into UDP-*N*-acetyl[^{3}H]glucosamine, and translocated to the Golgi apparatus, where the glycosaminoglycan is synthesized [25–27]. Hyaluronate synthesis may draw UDP-hexoses from pools different from those utilized for chondroitin sulphate [28] and other glycosaminoglycans [29], since it is probably located in a different cell compartment, i.e. the cytoplasmic face of the plasma membrane [30,31]. Nevertheless, since the xyloside experiments demonstrate an abundant supply of UDP-hexosamines in one compartment during cycloheximide treatment, it seems unlikely that there should be either a rapid depletion or a gross dilution of the same precursors in the pool supplying hyaluronate synthesis.

Since almost identical results were obtained in experiments using cycloheximide or puromycin and labelling with either [^{3}H]glucosamine or [^{3}H]glucose, the data, overall, support the conclusion that the rapid inhibition of [^{3}H]hyaluronate synthesis is directly related to inhibition of protein synthesis and not to secondary effects of the inhibitors on the chondrocytes. In the absence of a requirement for core-protein synthesis [8], and with the ability to synthesize UDP-hexoses maintained in cycloheximide-treated chondrocytes, it is likely that

inhibition of hyaluronate synthesis is due to loss of hyaluronate synthetase activity from continued metabolic turnover of the enzyme.

Cycloheximide and puromycin have variable effects on hyaluronate synthesis in other cells. In virally transformed chondrocytes it was inhibited by 80% after 6 h treatment with puromycin (55 μM) [32]. However, when calf rib cartilage slices were incubated for 110 min with either cycloheximide (0.35 mM) or puromycin (0.5 μM) in the presence of [^{3}H]glucosamine or [^{3}H]glucose, the specific radioactivity of [^{3}H]hyaluronate in the cartilage increased [28]. Currently there is no satisfactory explanation for this observation. Hyaluronate synthesis in 3T6 cells was inhibited by 85% after 6 h treatment with cycloheximide (0.1 mM), but was decreased by only 20–50% in synovial cells after 24 h [33]. With a similar concentration of cycloheximide, synthesis by skin fibroblasts from normal persons and Hurler-syndrome patients was decreased by 50% in 2 h [34], but with 36 μM only marginal inhibition (18% or less) occurred with normal and Marfan-syndrome fibroblasts over 18 h treatment [35]. In another report, no decrease in hyaluronate synthesis was observed in normal human skin fibroblasts after 48 h treatment with 0.36 mM-cycloheximide [36]. Although some of these variations are due, no doubt, to different experimental conditions, and especially to the inhibitor concentration used, others may reflect differences in the turnover rate of hyaluronate synthetase, or of enzymes generating UDP-hexoses, in different cell types.

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