Effects of [^{3}H]glucosamine concentration on [^{3}H]chondroitin sulphate formation by cultured chondrocytes

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GlcN (glucosamine) is now promoted over the counter for implied treatment of osteoarthritis, ostensibly by stimulating biosynthesis of cartilage chondroitin sulphate. In order to evaluate whether exogenous GlcN has any stimulatory effect, we have incubated mouse chondrocytes with [^{35}S]sulphate and various amounts of GlcN, to determine whether any increment in chondroitin [^{35}S]sulphate formation occurs. Similarly we have used varying concentrations of [^{3}H]GlcN to determine the dilution of incorporation into [^{3}H]chondroitin sulphate due to provision of endogenous GlcN by metabolism from glucose at two different glucose concentrations. The incorporation of both ^{35}S and ^{3}H was essentially linear over a 5 h time period. We found no stimulation of chondroitin [^{35}S]sulphate synthesis at lower concentrations of GlcN, and a significant reduction at higher concentrations. Even at concentrations of [^{3}H]GlcN that were greater than could be achieved with standard doses of oral GlcN, there was significant dilution of exogenous GlcN. Furthermore, an artificial acceptor for glycosaminoglycan synthesis in cell culture, 4-methylumbelliferyl β-D-xyloside, did not modify the provision of GlcN from endogenous sources, even though it stimulated chondroitin sulphate synthesis 4–5-fold at each GlcN concentration. We conclude that the cells have excess capacity to form maximal amounts of GlcN from glucose so that exogenous GlcN does not stimulate chondroitin sulphate synthesis.

Key words: cartilage, glycosaminoglycan, osteoarthritis, proteoglycan, xyloside.

INTRODUCTION

GlcN (glucosamine) is sold over the counter for oral use and widely advertised to 'build, support, lubricate and maintain cartilage' and thereby treat osteoarthritis, ostensibly by stimulating formation and/or stabilization of chondroitin sulphate. However, there has been no clear demonstration that stimulation of the production or maintenance of proteoglycans occurs when exogenous GlcN is utilized with cartilage or chondrocytes cultured under physiological culture conditions.

GlcN, which together with GalN (galactosamine) accounts for 30–50 % of the weight of most proteoglycans, is not ordinarily available in the circulation as a source for synthesis. However it is readily produced intracellularly from glucose by biological reactions that essentially all cells perform [1–3]. Cartilage, which contains the largest amount of proteoglycans (mainly proteochondroitin sulphate) of any tissue, is a major producer of GlcN and therefore utilizes large amounts of glucose for this purpose. Thus essentially the entire pathway (Scheme 1) in humans, as well as other vertebrates, for production of GlcN and GalN for glycosaminoglycans is ordinarily from glucose and not from exogenous GlcN.

If exogenous GlcN is made available to cultured cells or tissue slices, it can be incorporated directly into chondroitin sulphate by formation of GlcN 6-phosphate and to a lesser extent *N*-acetyl GlcN 6-phosphate, bypassing the formation from fructose 6-phosphate (Scheme 1). Nevertheless, high-specific-activity ^{14}C- or ^{3}H-labelled GlcN has been utilized by many investigators as an excellent tool to measure and characterize the formation of GlcN-containing and GalN-containing glycoconjugates by cell or tissue cultures. When presented to cultured cells in the absence of glucose, GlcN serves as the sole source for hexosamine in glycoconjugates, so that synthesis of glycosaminoglycans is directly proportional to GlcN concentration [4]. However, when glucose is present in the cell cultures the exogenous GlcN is diluted by endogenous GlcN formed from glucose. Because of this concomitant cellular production of undetermined amounts of non-radiolabelled GlcN from glucose, a technique has been developed and used regularly in our laboratory [5–7], as well as by others [8], to measure the degree of [^{3}H]GlcN dilution by the endogenously produced GlcN. This consists of incubating [^{35}S]sulphate of known specific activity together with [^{3}H]GlcN of known specific activity in the same or parallel cell cultures, followed by isolation and then use of chondroitin ABC lyase to degrade the resulting [^{3}H]chondroitin [^{35}S]sulphate, which is produced by essentially all cells. The resulting ^{3}H/^{35}S-labelled sulphated disaccharide products, which have equimolar amounts of sulphate and GalN (derived from the GlcN), are then counted to calculate how much the exogenous [^{3}H]GlcN has been diluted by endogenous GlcN produced from glucose. Using this technique we previously found that exogenous high-specific-activity [^{3}H]GlcN (final concentration, 0.00123 mM) was diluted as much as 635-fold by endogenous GlcN derived from glucose in human skin fibroblast cultures [6], and diluted as much as 1800-fold when we used mouse mast cell cultures [9] under conditions of high chondroitin sulphate formation.

For the current experiments, we utilized an immortalized line of mouse chondrocytes (MC615) as a source for controlled experiments to represent the metabolism of cartilage. These cells have been shown to make cartilage proteoglycans (decorin, biglycan,

Abbreviations used: GlcN, glucosamine; GalN, galactosamine; ΔDi-0S, 2-acetamido-2-deoxy-3-*O*-(β-D-Glc-4-enepyranosyluronic acid)-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-Glc-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-Glc-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactose.

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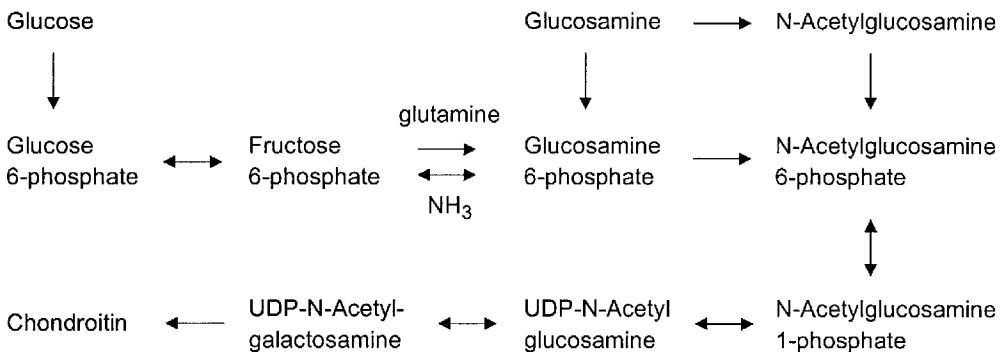

Scheme 1 Biosynthetic scheme for incorporation of GalN into chondroitin from glucose or GlcN precursors

aggrecan, link protein), and cartilage-specific type II, IX and XI collagen [10], but do not make any type I or III collagen, indicating that they have maintained their cartilage-specific characteristics during repeated monolayer subcultures. Monolayers of these cells were incubated for varying times with [^{35}S]sulphate and varying concentrations of GlcN to measure the formation of chondroitin [^{35}S]sulphate and incubated for varying times with varying concentrations of [^{3}H]GlcN and glucose to determine the dilution of [^{3}H]GlcN by endogenous GlcN formed by the cells. We found no stimulation of chondroitin [^{35}S]sulphate formation at lower concentrations of GlcN, while a significant reduction rather than stimulation was found at higher levels. This is consistent with an earlier report by others utilizing cultured chick embryo cartilage [11] and with a recent report using high GlcN concentrations with bovine cartilage explants [12]. Furthermore, dilution of [^{3}H]GlcN by endogenous GlcN was apparent, even in the presence of exogenous GlcN concentrations that were considerably higher than could be obtained by GlcN ingestion of amounts provided for oral consumption. We have also added an artificial acceptor for glycosaminoglycan formation, 4-methylumbelliferyl β-D-xyloside, to cultures in order to increase synthesis of [^{3}H]chondroitin [^{35}S]sulphate [9], thus increasing the demand for GlcN.

EXPERIMENTAL

Materials

D-[1,6-^{3}H]GlcN (40.8 Ci/mmol), [^{35}S]sulphate (carrier free) and [^{35}S]methionine (1175 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Glucosamine chloride and 4-methylumbelliferyl β-D-xyloside were purchased from Sigma (St. Louis, MO, U.S.A.). Chondroitin ABC lyase and ΔDi-0S [2-acetamido-2-deoxy-3-*O*-(β-D-Glc-4-enepyranosyluronic acid)-D-galactose], ΔDi-4S [2-acetamido-2-deoxy-3-*O*-(β-D-Glc-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose] and ΔDi-6S [2-acetamido-2-deoxy-3-*O*-(β-D-Glc-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactose] disaccharide standards were purchased from Seikagaku America (Rockville, MD, U.S.A.). Sepharose CL-6B and Sephadex G-50 were purchased from LKB (Piscataway, NJ, U.S.A.).

Cell culture and labelling

MC615 mouse chondrocytes [10], generously provided by B. Olsen (Department of Cell Biology, Harvard Medical School, Boston, MA, U.S.A.), were first grown in six-well plates (3.5 cm wells) in high-glucose (4 g/l, equal to 22 mM) Dulbeccos's modified Eagle's medium/Ham's F12 medium (1:1) plus 10% (v/v) fetal bovine serum (Hyclone). The total sulphate concentration of this growth medium is 0.45 mM, and methionine is 0.1 mM. When the cells were nearly confluent (usually about 2 days) the medium was removed and replaced with new medium with glucose lowered to 10 mM. Incubations were initiated and continued for 5 h at 37 °C in a final volume of 2 ml with the addition of 25 μCi of [^{35}S]sulphate or 100 μCi of [^{3}H]GlcN with or without addition of varying concentrations of non-radioactive GlcN (0.01–10 mM), with or without 0.1 mM β-xyloside. The [^{3}H]GlcN without any added GlcN provided a GlcN concentration of 0.00123 mM. In other experiments, cells were incubated as above in a final volume of 1 ml with 4.5 mM glucose as well as 10 mM glucose together with 5 or 25 μCi of [^{35}S]sulphate and 100 μCi of [^{3}H]GlcN to provide doubly labelled [^{3}H]chondroitin [^{35}S]sulphate, and also grown with 100 μCi of [^{3}H]GlcN and 25 μCi of [^{35}S]methionine instead of [^{35}S]sulphate.

Chromatography

Following incubation, medium from each of the cell cultures was assayed for radioactivity and then eluted with 0.5 M ammonium bicarbonate from 0.5 cm × 40 cm columns of Sephadex G-50 to separate the large amount of free [^{35}S]sulphate or [^{35}S]methionine and [^{3}H]GlcN from the macromolecular material consisting of ^{35}S-, or ^{3}H-labelled glycosaminoglycans and proteoglycans, plus some [^{3}H]GlcN-labelled and [^{35}S]methionine-labelled glycoproteins. Fractions containing the macromolecular material were lyophilized to remove the ammonium bicarbonate, and then taken up in 1 ml or less of water. Some aliquots of the ^{35}S-labelled macromolecular material were chromatographed on 0.5 cm × 40 cm columns of Sepharose CL-6B with 0.5 M ammonium bicarbonate, and fractions assayed for radioactivity after lyophilization as above.

Disaccharide analysis

Other aliquots of the excluded macromolecular ^{3}H/^{35}S-labelled material were then incubated with chondroitin ABC lyase [13] and chromatographed on Whatman no. 1 paper with *n*-butanol/acetic acid/1 M NH_4OH (2:3:1, by vol.) [13] for 30 h with added ΔDi-0S, ΔDi-4S and ΔDi-6S disaccharide standards. After localization of the ΔDi-0S, ΔDi-4S and ΔDi-6S spots by UV light, 1 cm strips were eluted and counted in a Packard scintillation counter to determine the amounts of ^{35}S and ^{3}H. Most were in ΔDi-4S. Since a mono-sulphated disaccharide has equimolar amounts of sulphate and GalN (derived from the GlcN), dilution of the exogenous [^{3}H]GlcN by endogenous GlcN produced from glucose endogenously can be easily calculated as follows.

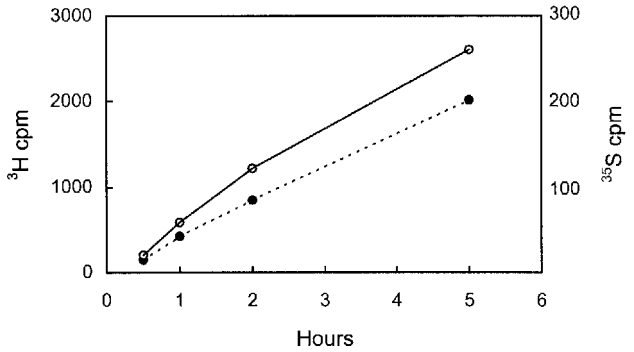

Figure 1 Timed incorporation of ^{35}S from [^{35}S]sulphate (●) and ^{3}H from [^{3}H]GlcN (○) into [^{3}H]chondroitin [^{35}S]sulphate

The specific activity of the [^{3}H]GalN in the disaccharide was calculated from the known specific activity of the [^{35}S]sulphate (see the Results section, which demonstrates that the cells do not produce any sulphate from methionine, so that the [^{35}S]sulphate concentration and specific activity remains constant). The known specific activities of the [^{3}H]GlcN added to the media at each concentration from 0.00123 to 10 mM was then compared with the specific activity of the [^{3}H]GalN calculated for the ΔDi-4S, providing a direct measurement of the dilution of GlcN by endogenous formation from glucose.

Total formation of chondroitin sulphate was calculated from the Sepharose columns and confirmed from the amounts of ^{35}S found in ΔDi-4S plus ΔDi-6S.

RESULTS

We have previously shown that essentially all [^{35}S]sulphate incorporated by the MC615 chondrocytes goes into chondroitin sulphate and dermatan/chondroitin sulphate, with little or no formation of heparan sulphate [14]. For the purposes of this investigation, we have not distinguished between chondroitin sulphate and dermatan/chondroitin sulphate, and refer to the total as chondroitin sulphate.

Incorporation of ^{35}S from [^{35}S]sulphate and ^{3}H from 1 mM [^{3}H]GlcN into chondroitin sulphate during a 5 h incubation is shown in Figure 1. Equilibrium of incorporation for both isotopes was seen after approx. 10–15 min, and similar results were seen with 0.1 and 0.01 mM [^{3}H]GlcN as well. When [^{35}S]methionine was incubated in sulphate-free media or media containing 0.2 mM non-radioactive sulphate, no ^{35}S-labelled ΔDi-4S was seen following degradation of chondroitin sulphate by chondroitin ABC lyase (Figure 2). Thus [^{35}S]methionine cannot be metabolized by the cells to provide [^{35}S]sulphate, demonstrating that the only source of sulphate is exogenous. This in turn demonstrates that the specific activity of the [^{35}S]sulphate and consequently the 3′-phosphoadenosine 5′-phospho[^{35}S]sulphate donor intermediate would remain constant for the entire 5 h incubation period following the first 10–15 min for equilibration.

Proteochondroitin [^{35}S]sulphate formation with and without added GlcN is shown by chromatography on Sepharose 4B (Figure 3). As has previously been demonstrated for these cells [15], the proteochondroitin [^{35}S]sulphate was found at or near the exclusion volume (fractions 8–18), indicating a mixture of large and smaller proteoglycans. A marked reduction in the total amount of [^{35}S]sulphate incorporated was found when 1 and 10 mM GlcN was added, in contrast to an increase that would be expected if the exogenous GlcN had stimulated synthesis. The presence of

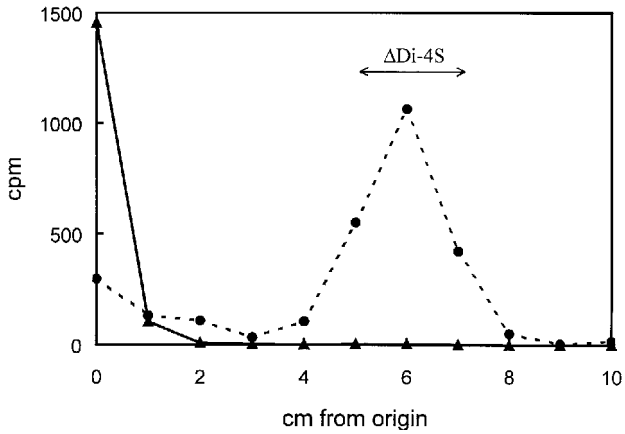

Figure 2 Paper chromatography of chondroitin ABC lyase degradation products of ^{35}S-labelled chondroitin sulphate

Disaccharides produced from chondroitin sulphate formed in media containing [^{35}S]sulphate (●) or [^{35}S]methionine (▲) were chromatographed as described in the Experimental section, and the radioactivity of the major product (ΔDi-4S) was determined.

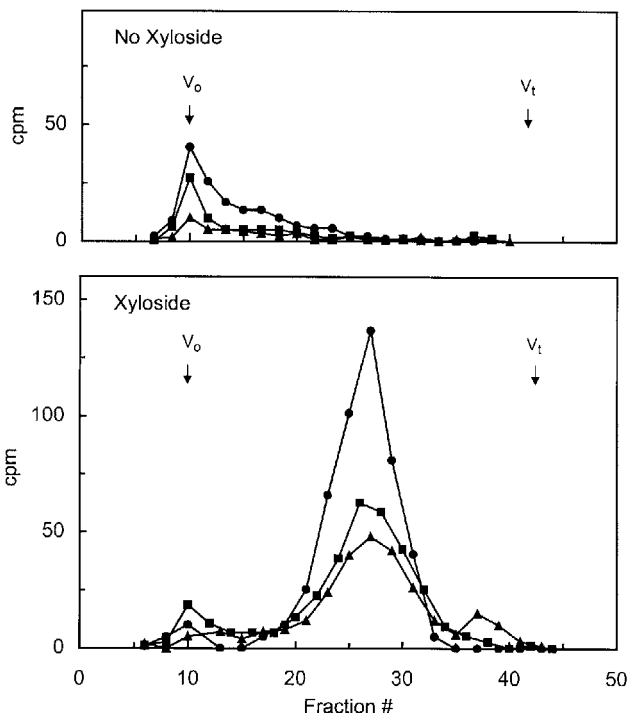

Figure 3 Sepharose CL-6B gel chromatography of proteochondroitin [^{35}S]sulphate/chondroitin [^{35}S]sulphate formed with various concentrations of GlcN in the absence or presence of β-xyloside

Equal aliquots of proteochondroitin [^{35}S]sulphate/chondroitin [^{35}S]sulphate formed by chondrocyte cultures with no added GlcN (●), 1 mM GlcN (■) and 10 mM GlcN (▲) without (upper panel) or with (lower panel) 0.1 mM β-xyloside were chromatographed on 0.5 cm × 40 cm Sepharose CL-6B columns and fractions assayed for radioactivity.

β-xyloside at each GlcN concentration in the cell cultures (Figure 3) resulted in a 4–5-fold increase in [^{35}S]sulphate incorporation over the amounts incorporated when β-xyloside was not present. The patterns indicated glycosaminoglycan (fractions 20–32) rather than proteoglycan, as has been reported by us [9] and many others for various cell types following incubation with β-xyloside.

Table 1 Contribution of exogenous [^{3}H]GlcN to total GalN in [^{3}H]chondroitin sulphate

The percentage of GalN derived from [^{3}H]GlcN was calculated by dividing the ^{3}H/^{35}S ratios of the ΔDi-4S values by the ^{3}H/^{35}S ratios of the specific activities and multiplying by 100.

GlcN (μM)	Specific activity [(cpm × 10^6)/μmol]			ΔDi-4S (cpm)			% of GalN derived from [^{3}H]GlcN
	^{3}H	^{35}S	^{3}H/^{35}S	^{3}H	^{35}S	^{3}H/^{35}S	
10 000	6.0	53	0.113	60	570	0.105	93
1000	60	53	1.13	629	770	0.817	73
101	595	53	11.2	1290	975	1.32	12
11.2	5350	53	101	4380	940	4.66	5
1.23*	48 600	53	917	3720	908	4.1	0.4

* The concentration of the [^{3}H]GlcN itself without added non-radioactive GlcN.

It is of note that the proportional effects of 1 and 10 mM GlcN on chondroitin sulphate formation were similar to the effects on chondroitin sulphate formation in the absence of β-xyloside. The disaccharide products of digestion by chondroitin ABC lyase demonstrated that the high degree of sulphation remained the same at all GlcN concentrations with or without β-xyloside, confirming that sufficient formation of 3′-phosphoadenosine 5′-phospho[^{35}S]sulphate for full sulphation was available at all concentrations of GlcN.

Dilution of [^{3}H]GlcN by endogenous GlcN produced by metabolism from glucose (calculated from the isolated ^{3}H/^{35}S-labelled ΔDi-4S as described in the Experimental section) is shown in Table 1. At exogenous [^{3}H]GlcN concentrations of 10 mM (equal to the glucose concentration) and 1 mM (one-tenth that of the glucose concentration), most of the incorporation was directly from the added exogenous [^{3}H]GlcN. However, when concentrations were reduced further, only a minor to tiny proportion was from the exogenous [^{3}H]GlcN, indicating dilution factors as high as 220:1 at the lowest concentration of exogenous [^{3}H]GlcN.

In another experiment, 1.0, 0.1 or 0.01 mM [^{3}H]GlcN was incubated together with 0.45 mM [^{35}S]sulphate in media containing 4.5 mM glucose and in media containing 10 mM glucose to examine the effects of glucose concentrations equal to the physiological range of 80–180 mg%. The contribution of exogenous [^{3}H]GlcN to total chondroitin sulphate GalNAc in media containing 10 mM glucose (calculated as described in Table 1) was 50, 8 and 1.2 % for 1.0, 0.1 and 0.01 mM GlcN respectively, while in media containing 4.5 mM glucose it was 99, 18 and 3 % for 1.0, 0.1 and 0.01 mM GlcN respectively, These results are consistent with what might be expected for competitive inhibition of [^{3}H]GlcN by glucose.

Although similar magnitudes of dilution were observed with repeat cultures at the various GlcN concentrations, the degree of dilution varied somewhat because of uncontrolled variations such as cell density and growth rate. The least degree of dilution that we found for 0.00123 mM [^{3}H]GlcN was 95:1. We have previously found such variations with cell cultures from human skin fibroblast [6] and many other cell types. In repeat experiments there were also similar reductions (see Table 1) in [^{35}S]sulphate incorporation when 1 and 10 mM GlcN was present in the incubations.

The presence of β-xyloside in cultures with [^{3}H]GlcN did not have a significant effect on the amounts of GlcN obtained endogenously at each exogenous [^{3}H]GlcN concentration, confirming that the cells had at least a 4–5-fold excess capacity to provide for the endogenous production of GlcN. Thus the cells were capable of producing sufficient GlcN to increase their production of chondroitin sulphate manyfold without relatively increasing their use of exogenous GlcN.

DISCUSSION

Diet is not the primary source of GlcN for humans since substances with large amounts of GlcN or GalN, such as proteoglycans, are not degraded in the stomach or intestines to any great extent. Only a small portion of orally administered chondroitin sulphate was reported by others [15] to be digested and bioavailable, with 90 % being recovered in faeces and urine, mainly as glycosaminoglycan. Similarly, oligosaccharides from glycoproteins, which contain small amounts of GlcN or GalN, may be digested, but free GlcN or GalN from either glycosaminoglycan or glycoprotein sources can only be made available by lysosomal degradation of some or all of the relatively small amount of oligosaccharides or polysaccharides that might be taken up by cells.

Commercial GlcN preparations for oral use, as GlcN sulphate or GlcN chloride, are usually 500–1000 mg, which is equal to as much as 850 mg of GlcN, slightly less than 5 mmol. If this were to be immediately quantitatively absorbed into the general blood stream without any of it reaching interstitial or intracellular fluid or being metabolized or secreted, a maximum plasma GlcN concentration of approx. 1.25 mM would be reached for a 90 kg person and 2.5 mM for a 45 kg person. If the GlcN were equally distributed rapidly in interstitial fluid as well, then the concentrations would be approx. 0.3 and 0.6 mM respectively. If distributed rapidly and quantitatively in intracellular fluid, the concentration would be approx. 0.1 and 0.2 mM. However, this molarity would be reduced depending upon the time over which the absorption took place and would be reduced even more rapidly through metabolism and renal clearance. Thus it would be highly unlikely for concentrations in cartilage ever to be as high as 0.1 mM, a level at which we found that only 12–23 % of the GalN in chondroitin sulphate was derived from exogenous [^{3}H]GlcN.

However, it is questionable whether much, if any, free GlcN gets into the systemic blood stream after ingestion. This is strongly indicated by a report describing oral administration of GlcN to humans [16] followed by repeat determinations of blood levels over a period of many hours. Recently experiments with dogs [17] and rats [18] have shown that less than 15 and 20 % respectively appeared in plasma as free GlcN after weight-adjusted oral dosages of GlcN that were 20 times and 6 times, respectively, that of human dosages. These results indicate that most of the orally administered GlcN, being first transported to the liver by the portal system, was metabolized or incorporated into glycoprotein before reaching the systemic circulation. Thus parenteral administration apparently would be necessary to achieve any significant free GlcN blood levels.

Nevertheless, we investigated the effects on chondroitin sulphate formation by GlcN concentrations that were up to 100 times higher than 0.1 mM. In contrast to any enhancement of chondroitin sulphate formation by provision of GlcN, we found that addition of GlcN resulted in a concentration-dependent decrease of proteochondroitin sulphate production (Figure 3, Table 1). This inhibition by added GlcN was also seen when cultures were incubated with 0.1 mM β-xyloside, which provided a 4–5-fold increase in chondroitin sulphate formation over that produced in the absence of the β-xyloside (Figure 3). Thus the cells were capable of providing more than sufficient GlcN from endogenous sources. At the lowest GlcN concentration, the production of ^{3}H- and ^{35}S-labelled ΔDi-4S indicated that less than 0.5 % of the GalN in the chondroitin sulphate was derived from the exogenous [^{3}H]GlcN with more than 99.5 % being derived by metabolism

from glucose (Table 1). Only with concentrations of GlcN of 1 and 10 mM, much higher than could be achieved *in vivo*, was most of the GalN in chondroitin sulphate derived from the exogenous GlcN rather than by metabolism from glucose. In previous work with other types of cells, including human skin fibroblasts, the dilution factors were even higher [6,9].

Our results are compatible with previous information concerning the metabolism of GlcN. When GlcN is presented to cells along with glucose, the formation of glucose 6-phosphate is more efficient than the formation of GlcN 6-phosphate [19], which is catalysed by the same hexokinase. Thus the presence of glucose will act as a strong competitive inhibitor to the utilization of any exogenous GlcN, and one would expect cells to dilute exogenous GlcN by endogenous GlcN formed from glucose in direct proportion to the glucose concentration, as described in the Results section. GlcN can undergo many other reactions in molecular mimicry of glucose, but each of these is at a lower efficiency than that of glucose. In addition to the formation of GlcN 6-phosphate with hexokinase, these include phosphoglucomutase acting on GlcN 6-phosphate to produce GlcN 1-phosphate [19], utilization with UTP and UDP-glucose pyrophosphorylase to form UDP-GlcN [19], and formation of UDP-GalN from UDP-GlcN by UDP-glucose epimerase, as well as formation of UDP-glucosaminuronate catalysed by UDP-glucose dehydrogenase [20].

The results with this chondrocyte cell system indicate that excess exogenous GlcN does not stimulate synthesis of chondroitin sulphate, and that concentrations higher than can be achieved by oral or even intravenous administration still do not constitute the major provision of GlcN for formation of chondroitin. Thus it would be unlikely that any slight increment in blood levels of GlcN provided by oral or even parenteral administration would have an effect on increasing cartilage chondroitin sulphate formation or maintenance. Indeed, the results suggest that high levels of GlcN have an inhibitory effect on chondroitin sulphate synthesis, perhaps due to reversal of the formation of GlcN 6-phosphate from fructose 6-phosphate resulting in some decrease of the formation of fructose 6-phosphate from glucose 6-phosphate.

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