Post-translational alterations in newly synthesized cartilage proteoglycans induced by the glutamine analogue 6-diazo-5-oxo-L-norleucine

Time course of inhibition and recovery

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Incorporation of [³⁵S]sulphate by cultures of matrix-free cells from chick embryo sterna in the presence of the glutamine analogue 6-diazo-5-oxo-L-norleucine (0.58 mM) was inhibited in a time-dependent manner to < 15 % of that in control cultures after 2 h. Characterization of the major cartilage proteoglycan synthesized under these conditions showed that it contained few, if any, normal-sized chondroitin sulphate chains, and only about half of the normal complement of substituted serine residues. Subsequent addition of D-glucosamine hydrochloride (final concn. 2 mM) resulted in a time-dependent recovery of [³⁵S]sulphate incorporation to 90 % of control cultures after 2 h, but restored the chondroitin sulphate chains to normal size within 15 min. On the basis of these results, it is concluded that a 2 h preincubation is necessary to deplete the chondrocytes of the endogenous supply of UDP-N-acetyl-D-glucosamine required for optimal glycoconjugate synthesis, and that this situation results in the synthesis of a chondroitin sulphate proteoglycan with significantly altered properties, owing to the paucity of glycosaminoglycan chains; however, this condition is completely reversible if the D-glucosamine pool is replenished.

INTRODUCTION

L-Glutamine is a necessary constituent for the optimal synthesis of cartilage glycosaminoglycan (Roden, 1956; Speight *et al.*, 1978) since its amido group is required for the metabolic conversion of fructose 6-phosphate into glucosamine 6-phosphate, the latter being the direct precursor of the UDP-N-acetylhexosamine required for glycosaminoglycan assembly (Leloir & Cardini, 1953; Ghosh *et al.*, 1960). The teratogen 6-diazo-5-oxo-L-norleucine (DON) is a glutamine analogue which binds in a time-dependent manner to inactivate irreversibly glucosamine-6-phosphate synthetase, the enzyme which catalyses the above reaction (Bates & Handschumacher, 1968). As a result, DON adversely affects chondrogenesis *in vitro* by inhibiting normal proteoglycan synthesis (Greene & Kochhar, 1975; Aydelotte & Kochhar, 1975; Kochhar *et al.*, 1976; Linsenmayer & Kochhar, 1979). However, these studies were not designed to determine how cartilage proteoglycan structure was affected by DON.

A previous study from our laboratory (Clark *et al.*, 1987) addresses this question. In a dose-dependent manner, incorporation of [³⁵S]sulphate by chick-embryo chondrocytes incubated in the presence of DON is inhibited by ~ 60 %. More importantly, these results show that a portion of the newly synthesized proteoglycan contains fewer and shorter chondroitin sulphate chains compared with those synthesized in the absence of DON. Under the conditions of these experiments, however, a significant fraction of the CS-PG synthesized in the presence of DON appear to be structurally normal. It is postulated that this could be due to a time-dependent effect of DON on the endogenous hexosamine pool.

The purpose of the present investigation was to determine the

time course of DON inhibition of glycosaminoglycan synthesis in chick-embryo chondrocytes, the time course of recovery from this inhibition by the subsequent addition of exogenous D-glucosamine, and the effects of these conditions on the structure of newly synthesized CS-PG.

EXPERIMENTAL

Materials

Materials were purchased from the following commercial sources: L-glutamine from GIBCO; D-glucosamine hydrochloride and shark cartilage chondroitin sulphate from Sigma Chemical Co. All other supplies used are the same as those described previously (Iozzo & Clark, 1986; Clark *et al.*, 1987).

Chondrocyte isolation and culture

Chondrocytes were isolated from 17-day chick-embryo sterna and cultured in suspension as described previously (Clark & Richards, 1985; Clark *et al.*, 1987). In most of the experiments, the culture medium, which contained ascorbate and β-aminopropionitrile (50 µg/ml each) and 300 µM-Na₂SO₄ was further supplemented with 1 mM-L-glutamine (Speight *et al.*, 1978).

To monitor the time course of inhibition of proteoglycan synthesis, cells were incubated in the absence or presence of 100 µg of DON/ml (0.58 mM) for up to 4 h; at various times throughout this incubation period, [³⁵S]sulphate (50–80 µCi/ml) was added for 10 min intervals. Incorporation was stopped by addition of Na₂SO₄ (final concn. 100 mM), and protease inhibitors were added to final concentrations of 10 mM-EDTA, 10 mM-N-ethylmaleimide and 1 mM-phenylmethanesulphonyl fluoride; the

Abbreviations used: DON, 6-diazo-5-oxo-L-norleucine; CS-PG, the major cartilage chondroitin sulphate proteoglycan.

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cells and medium were separately processed as described by Byers *et al.* (1987). Briefly, after centrifugation at 900 *g* for 10 min, the cells were extracted for 30 min at 4 °C with 2% CHAPS/50 mM-sodium acetate, pH 5.8, containing protease inhibitors. Extraction was continued for another 30 min after the addition of an equal volume of 8 M-guanidine hydrochloride in acetate buffer, and then the sample was stored at -20 °C; solid guanidine hydrochloride was added to the medium to a final concentration of 4 M, and it was stored as above. For analysis, the cell extract was thawed, centrifuged at 10000 *g* for 30 min, and the supernatant combined with the medium. Unincorporated radioactivity was recovered from combined samples either by gel filtration on Sephadex G-50 or by dialysis against 100 mM- Na_2SO_4 containing 0.01% NaN_3 .

To determine the effects of DON inhibition on proteoglycan structure, cells [$(1-2) \times 10^7$ cells/ml] were incubated in the absence or presence of DON (100 $\mu\text{g}/\text{ml}$) for 2 h before addition of [^{35}S]sulphate. In some experiments the cells were double-labelled with [^{35}S]sulphate and [^3H]serine. Incubations were continued for another 2 h in the absence or presence of DON, and the cells and medium were treated as described above.

To monitor the time course of recovery from DON inhibition, cells were incubated in the absence or presence of 100 μg of DON/ml for 2 h. At this time (0 h), D-glucosamine hydrochloride was added (final concn. 2 mM); and at various times up to 2 h after this addition, [^{35}S]sulphate (50 $\mu\text{Ci}/\text{ml}$) was added for 10 min intervals to assess total proteoglycan synthesis. Cells and medium were treated exactly as described above for the time course of inhibition experiments. It has been shown that 2 mM-D-glucosamine optimizes steady-state levels of glycosaminoglycan synthesis in chick-embryo cartilage (Kim & Conrad, 1974).

Analytical procedures and enzyme digestions

Newly synthesized macromolecules were characterized by DEAE-Sephacel chromatography, SDS/PAGE and/or Sepharose CL-4B chromatography as described previously (Clark & Richards, 1985; Clark *et al.*, 1987); the size of constituent glycosaminoglycan chains was assessed by gel filtration on CL-6B after hydrolysis in either 0.5 M-NaOH for 16 h at 4 °C (Anderson *et al.*, 1965) or 0.05 M-NaOH/1 M- NaBH_4 for 48 h at 45 °C (Carlson, 1968). Chondroitinase ABC (Saito *et al.*, 1968) and Pronase (Clark, 1979) digestions were performed as described previously.

RESULTS

Time course of inhibition of [^{35}S]sulphate incorporation by DON

Fig. 1 shows the results of a typical experiment. Incorporation of [^{35}S]sulphate into macromolecules in the presence of DON plus L-glutamine (● symbol) was inhibited in a time-dependent manner ($\text{ED}_{50} \sim 45$ min) to <15% of the control value (○ symbol at 0 h) over the course of 2 h; a further decrease to ~9% of control was observed between 2 and 4 h. The presence of L-glutamine has been shown to be necessary to maintain the rate of synthesis of glycosaminoglycans in chick cartilage *in vitro* (Speight *et al.*, 1978). Cells cultured in the absence of L-glutamine for 2 h and then labelled for 10 min (□ symbol at 0 h) incorporated significantly less [^{35}S]sulphate than did those that were cultured in its presence (○ symbol); however, after incubation for 4 h in the presence of DON, incorporation of [^{35}S]sulphate was identical in both the absence and the presence of L-glutamine (●, ■ symbols at 4 h). Therefore 1 mM-glutamine was included in the incubation medium in all of the subsequent experiments.

Fig. 1. Time course of inhibition of [^{35}S]sulphate incorporation by DON and the effect of L-glutamine

Duplicate samples containing $\sim 25 \times 10^6$ cells for each time point were incubated for 20 min in 2.5 ml of medium under the conditions described in the Experimental section. To obtain the control value, [^{35}S]sulphate (50 $\mu\text{Ci}/\text{ml}$) was added to two tubes, and after a 10 min incubation, the cells and medium were processed. Total ^{35}S incorporation (cells plus medium) into macromolecules was determined by gel filtration on Sephadex G-50. At zero time, 100 μg of DON/ml was added to the remaining tubes and incubation was continued. At the time intervals shown, radioisotope was added to duplicate tubes for 10 min intervals (horizontal bars) and the samples were treated as above. The white and black symbols represent samples incubated in the absence or presence of DON respectively; the squares and circles represent samples incubated in the absence or presence of 1 mM-L-glutamine respectively. The values represent averages of two to four separate samples and are expressed as percentages of the control value (○).

Measurement of macromolecule size as a function of incubation time in the presence of DON

Sepharose CL-4B chromatography showed that the presence of DON in cartilage cell cultures resulted in the time-dependent disappearance of the normal-sized CS-PG (Fig. 2, hatched area), with the concomitant appearance of two included peaks (Fig. 2, non-hatched area). These peaks were labelled A (V_0), B ($K_{av} \sim 0.25$) and C ($K_{av} \sim 0.60$) respectively (cf. Fig. 2d).

Electrophoretic analysis of these samples (Fig. 3) showed that incubation in the presence of DON resulted in the time-dependent disappearance of material migrating at the top of the gel and the concomitant appearance of a band migrating near the 400 kDa marker. The former component represents the normal-sized CS-PG (peak A in Fig. 2), whereas the latter component represents an altered form of this macromolecule (peak B in Fig. 2). It is important to note that samples incubated for up to 4 h in the absence of DON and either the presence (Fig. 3, lane 8) or absence (Clark *et al.*, 1987) of L-glutamine did not show this component, indicating that its formation is due solely to the presence of DON. Moreover, no intermediate species migrating between the material at the top of the gel and that migrating near 400 kDa was observed.

By both techniques, therefore, it was observed that the size of the [^{35}S]sulphate-labelled components was markedly diminished with increasing incubation time in the presence of DON. This alteration, which became evident between 0.5 and 1 h and began to reach a plateau after 2 h, was in accordance with the data shown in Fig. 1. Because of the short pulse period, it is unlikely that these alterations are due to proteoglycan degradation. The results thus confirm that, under these incubation conditions *in vitro*, a significant effect of DON on the synthesis and structure of the CS-PG can be observed by 2 h.

Fig. 2. Gel-filtration analysis of newly synthesized cartilage macromolecules as a function of incubation time in the presence of DON

Portions (20000 c.p.m.) of samples isolated after incubation for various times in the presence of DON (cf. Fig. 1) were dialysed against deionized water, freeze-dried, redissolved in 500 μl of 4 M-guanidine HCl/0.1 M-Tris/0.1 M- Na_2SO_4 /20 mM-EDTA/0.2% Triton X-100, pH 7.0, and applied to a 1 cm $\times$ 110 cm column of Sepharose CL-4B equilibrated with the same buffer. Fractions (1 ml) were collected and quantified in a scintillation counter. The total column volume was measured by using $^3\text{H}_2\text{O}$, and the void volume by cartilage proteoglycan. The times of incubation with DON and the percentage of the total radioactivity eluted in the void volume (hatched area) are shown in each panel. The peaks labelled A, B and C (panel d) are discussed in the text.

Structural analysis of newly synthesized CS-PG

On the basis of the above observations, the remaining experiments were performed by using a 2 h preincubation period in the absence or presence of DON followed by a 2 h pulse label with ^{35}S -sulphate. The ^{35}S -labelled macromolecules were characterized by ion-exchange and size-exclusion chromatography. The Sepharose CL-4B profiles were similar to those shown previously for the control and DON-treated samples (see

Fig. 3. Fluorescence autoradiographic analysis of newly synthesized cartilage macromolecules as a function of incubation time in the presence of DON

Portions (~ 3000 c.p.m.) of samples isolated after incubation for various times in the presence of DON (cf. Fig. 1) were dialysed against 0.4 M-NaCl/0.1 M-Tris, pH 7.4, containing protease inhibitors, precipitated on ice by addition of 0.1 vol. of 100% trichloroacetic acid, and the precipitate was analysed on an SDS/5%-polyacrylamide gel under reducing conditions. The time of incubation before the addition of radioisotope, and the absence (-) or presence (+) of 1 mM-L-glutamine (Gln) and/or 0.58 mM-DON are shown at the bottom; the migration position of the large subunit of laminin (400 kDa) is indicated on the left.

Figs. 2a and 2f respectively; results not shown). The DEAE-Sephacel profiles (Fig. 4) showed that, compared with control ($\circ$), all of the proteoglycans synthesized in the presence of DON ($\bullet$) was now eluted at a lower ionic strength. The radioactivity which bound to the column ($> 98\%$ of total) was divided into two pools: pool II contained components which were eluted early in the gradient and represented 3–5% of the radioactivity recovered from control samples, compared with 6–10% from the DON sample; pool III contained components which were eluted in the region usually occupied by normal or structurally altered CS-PG and always represented the majority of the ^{35}S -sulphate. Interestingly, pool III was usually eluted in a biphasic manner in the DON-treated sample; and in some cases, pool III-A and pool III-B (Fig. 4) were analysed separately (see below). The nature of the sulphated material eluted from DEAE-Sephacel in Pool II requires investigation.

Initial experiments on the nature of the material eluted in pool III showed that the inhibition of glycosaminoglycan chain substitution by DON (55% of control) was of the same magnitude as that determined previously (Clark *et al.*, 1987), suggesting that there was no further inhibition of core-protein substitution under the present incubation conditions. In addition, there was virtually no difference in the amount of ^{35}S -serine incorporated into the CS-PG, consistent with the report that DON does not affect cartilage core-protein synthesis (Schwartz, 1981) or heparan sulphate core-protein synthesis (Iozzo & Clark, 1987). Thus the observed alterations in CS-PG structure were not due to a non-specific cellular toxicity (results not shown).

Fig. 4. DEAE-Sephacel chromatogram of [^{35}S]sulphate-labelled macromolecules synthesized under conditions of maximum inhibition by DON

Suspension cultures were incubated for 2 h in the absence ($\circ$) or presence ($\bullet$) of DON (0.58 mM). After labelling with [^{35}S]sulphate (80 $\mu\text{Ci}/\text{ml}$) for a further 2 h, the cells and medium were processed as described in the Experimental section. Samples (1.3×10^6 c.p.m.) of medium plus cell extract were applied to a $1 \text{ cm} \times 10 \text{ cm}$ column of DEAE-Sephacel equilibrated in 50 mM-Tris/0.1 M-NaCl/8 M-urea/0.2% CHAPS, pH 8.0, followed by washing with 2 column volumes of this buffer. Pool I (not shown) represented 1% and 2%, respectively, of the recovered radioactivity in control and DON-treated samples. Elution was then continued by the initiation of a linear gradient (G) of NaCl from 0.1 to 0.7 M in a total volume of 100 ml. Fractions (1.1 ml) were collected at a flow rate of 3 ml/h, and were analysed for total radioactivity and for conductivity ($\star$). The gradient fractions were pooled as indicated by the horizontal bars: pool II, minor peak eluted between 0.15 M- and 0.3 M-NaCl; pool III (pool III-A and III-B), major peak(s) eluted between 0.3 M- and 0.6 M-NaCl.

The pool III material was further characterized with respect to chain size by Sepharose CL-6B chromatography after alkaline hydrolysis (Fig. 5). In the control sample, the chains were eluted in a polydisperse peak with $K_{av} \sim 0.6$ (Fig. 5a), whereas in the DON-treated sample, pool III-A was eluted with $K_{av} \sim 0.85$ (Fig. 5b) and pool III-B was eluted with $K_{av} \sim 0.80$ (Fig. 5c). Based on the calibration described by Wasteson (1971), the estimated molecular mass of the peak fraction of the control chains was ~ 12 kDa, whereas those of the DON-treated samples ranged from 4 (pool III-B) to 2.4 (pool III-A) kDa. These results showed that all of the chains synthesized in the presence of DON were significantly shorter than those in the control sample, and that the difference in anionic properties between the latter pools (cf. Fig. 4, $\bullet$) was likely to be due to the presence of shorter chondroitin sulphate chains in pool III-A as compared with pool III-B. Subsequent experiments showed that all chains were completely digested by either chondroitinase ABC or AC II (results not shown). It should be reiterated that the difference in chain size between pools III-A and III-B could not be discerned by electrophoretic analysis of the intact proteoglycan (results not shown). Thus, even though these two altered proteoglycan populations contained a different average chain length which could be easily detected by ion-exchange chromatography, they could not be distinguished by size-exclusion methods.

Consistent with our previous findings, most ($\sim 84\%$) of the

Fig. 5. Gel-filtration analysis of DEAE-Sephacel pool III components after alkaline treatment

Samples (50000 c.p.m.) of DEAE-Sephacel pool III (Fig. 4, $\circ$) (a), pool III-A and pool III-B (Fig. 4, $\bullet$) (b and c, respectively) were alcohol-precipitated overnight at -20°C with 3 vol. of ice-cold ethanol/1.3% potassium acetate. The precipitates were made 0.5 M in NaOH, incubated overnight at 4°C , neutralized with acetic acid, and applied to a $0.6 \text{ cm} \times 50 \text{ cm}$ column of Sepharose CL-6B equilibrated and eluted with 4 M-guanidine HCl/0.1 M-Tris/0.1 M- Na_2SO_4 /20 mM- Na_2EDTA /0.2% Triton X-100, pH 7.0.

alkali-releasable chains in the control sample were excluded from a Sephadex G-50 column (> 10000 Da), in contrast with the DON-treated sample, where most ($\sim 90\%$) of the chains were included in two peaks with $K_{av} = 0.55\text{--}0.60$ and $K_{av} \sim 0.75$ (results not shown). These last two peaks have been shown to contain shortened chondroitin sulphate chains and the linkage-region oligosaccharide respectively (Clark *et al.*, 1987). The smaller peak was predominant in pool III-A, and the larger was predominant in pool III-B (results not shown).

Fig. 6. Time course of recovery of $[^{35}\text{S}]$ sulphate incorporation by addition of D-glucosamine after DON inhibition

Cartilage cells were isolated and apportioned as described in the legend to Fig. 1. Duplicate samples were incubated for 2 h in either the absence or the presence of 0.58 mM-DON. At this time (0 h), one set (e.g. $\pm$ DON) of duplicates was incubated with $[^{35}\text{S}]$ sulphate (50 $\mu\text{Ci}/\text{ml}$) for 10 min and processed as described in the legend to Fig. 1. D-Glucosamine (2 mM final concn.) was added to the remaining duplicate sets and incubation was continued for another 2 h. At the times shown, radioisotope was added to a set of duplicates for a 10 min interval (short horizontal lines), and the samples were treated as above. The open and closed symbols represent samples incubated in the absence or presence of DON, respectively; the squares and circles represent samples incubated in the absence or presence of D-glucosamine hydrochloride (GlcN HCl), respectively. For each time point during recovery, the plotted value represents the percentage of the corresponding control (minus DON) value.

Fig. 7. Gel-filtration analysis of newly synthesized glycosaminoglycans as a function of recovery time in the presence of D-glucosamine

Portions (~ 10000 c.p.m.) of samples isolated after incubation in the absence or presence of 2 mM-D-glucosamine hydrochloride (cf. Fig. 6) were dialysed against water, freeze-dried, treated with alkaline borohydride, neutralized with 10 M-acetic acid, and chromatographed on Sepharose CL-6B as described in the legend to Fig. 5. (a) Incubation for 2 h in the absence ($\circ$) or presence ($\bullet$) of DON (0 h of recovery); (b) incubation as above followed by 15 min in the presence of D-glucosamine hydrochloride (GlcN).

Time course of recovery after inhibition of $[^{35}\text{S}]$ sulphate incorporation

Cells were incubated in the presence of 100 μg of DON/ml for 2 h, and the effect of the subsequent addition of a 3.4-fold molar excess of D-glucosamine hydrochloride on proteoglycan synthesis was monitored by measuring total macromolecular $[^{35}\text{S}]$ sulphate in the cells plus medium (Fig. 6). As expected, incorporation after 2 h in the presence of DON ($\blacksquare$ at 0 h) was $< 10\%$ of the control value ($\square$ at 0 h). However, subsequent addition of the D-glucosamine hydrochloride resulted in a time-dependent restoration of $[^{35}\text{S}]$ sulphate incorporation over the course of 2 h ($\bullet$).

Measurement of proteoglycan and glycosaminoglycan size as a function of recovery time after the addition of D-glucosamine

Analysis of macromolecular size by either SDS/PAGE or Sepharose CL-4B chromatography showed that, as early as 15 min after the addition of D-glucosamine, virtually all molecules behaved identically with those synthesized in the absence of DON. Similar to the observations made during inhibition by DON (Clark *et al.*, 1987), electrophoretic analysis of the proteoglycans synthesized during recovery from inhibition did not show the presence of intermediates with mobilities between ~ 400000 kDa and the top of the gel (results not shown).

Since it is possible that only a few normal-sized glycosaminoglycan chains could confer on the newly synthesized proteoglycans a sufficiently large hydrodynamic size to be excluded from either polyacrylamide gels or Sepharose CL-4B, the constituent side chains were released by alkaline-borohydride treatment and analysed by Sepharose CL-6B chromatography (Fig. 7). The results confirmed that a normal chondroitin sulphate size distribution was attained within 15 min after addition of D-glucosamine hydrochloride to by-pass the DON inhibition.

DISCUSSION

The results of the present study showed that the synthesis and structure of the CS-PG glycosaminoglycan (as measured by $[^{35}\text{S}]$ sulphate incorporation) could be relatively rapidly and efficiently perturbed in a time-dependent manner in the presence of DON, an L-glutamine analogue. Specifically, in the presence of 0.58 mM-DON (Clark *et al.*, 1987), a significant decrease in $[^{35}\text{S}]$ sulphate incorporation was detected at the earliest time measured (15 min) and appeared to reach a plateau by 2 h of incubation ($< 15\%$ of control). It should be noted that the same level of inhibition was attained whether incubations were performed in the absence or presence of exogenous 1 mM-L-glutamine, a concentration found to promote optimal glycosaminoglycan synthesis in chick epiphyseal chondrocytes (Speight *et al.*, 1978). These results demonstrated the efficacy of DON in blocking L-glutamine utilization and thereby progressively decreasing the intracellular pool of UDP-N-acetylhexosamine (Bates *et al.*, 1966; Clark *et al.*, 1987; Iozzo & Clark, 1987). Thus, under the incubation conditions described here, it can be concluded that at least 2 h is required to deplete chick-embryo chondrocytes of an endogenous hexosamine pool sufficient to maintain a normal level of glycosaminoglycan synthesis. A similar time-dependent inhibition of $[^{35}\text{S}]$ sulphate incorporation by DON has been reported for cultures of chick heart fibroblasts (Spooner & Conrad, 1975).

The observed inhibition of $[^{35}\text{S}]$ sulphate incorporation in the presence of DON was due to the synthesis of a CS-PG containing fewer and smaller glycosaminoglycan chains. Similar conclusions have been recently reported for another glutamine analogue, azaserine, which is used to block L-glutamine utilization in

day-4 chick embryos *in ovo*; chondrogenesis was subsequently inhibited and micromelia induced owing to the interference with normal cartilage proteoglycan synthesis (Honda *et al.*, 1989). Thus a major effect of glutamine analogues both *in vitro* and *in vivo* is an inhibition of glycosaminoglycan synthesis, resulting in a characteristic alteration in the CS-PG structure.

The advantage gained by increasing the preincubation period from 30 min (Clark *et al.*, 1987) to 2 h in the present experiments was that virtually all of the newly synthesized major cartilage proteoglycan was affected; little normal-sized monomer could be detected. However, two populations of proteoglycan with shortened chondroitin sulphate chains were easily distinguishable by ion-exchange chromatography; subsequent analyses of the constituent glycosaminoglycan chains suggested that this is due to differences in average chain size as opposed to chain number. The principal effect of the new incubation conditions, therefore, was to decrease further the average size of the newly synthesized glycosaminoglycan chains; the average number of chains per protein core was not decreased below that found previously (Clark *et al.*, 1987). The significance of the latter observation is unknown. It is important to emphasize, however, that the decrease in [³⁵S]sulphate incorporation owing to the absence of exogenous L-glutamine in the culture medium did not result in the synthesis of shortened chondroitin sulphate chains, even though this condition also is reported to lower intracellular concentrations of UDP-N-acetylhexosamine (Speight *et al.*, 1978); altered chains were found only after incubation in the presence of DON. This suggests that, during the synthesis of glycosaminoglycan chains, the chondrocyte responds differently to the lack of exogenous L-glutamine than to the lack of the ability to utilize it. This may be due to the fact that chick-embryo cells have been reported to contain low levels of glutamine synthetase, and thus may be able to synthesize small amounts of L-glutamine (Rudnick *et al.*, 1954).

In a series of experiments *in vitro* using chick-embryo limb buds, Aydelotte & Kochhar (1975) show that the simultaneous presence of a > 200-fold molar excess of L-glutamine over DON is required to minimize the morphological changes seen in these chondrogenic tissues. Our previous results show that incubation of chick cartilage cells in the simultaneous presence of DON and a > 100-fold molar excess of L-glutamine only restores [³⁵S]sulphate incorporation to 67% of control, but the presence of a 77-fold molar excess of D-glucosamine maintains incorporation at 93% of control (Clark *et al.*, 1987). In the present experiments, addition of only a 3.4-fold molar excess of exogenous D-glucosamine after inhibition had occurred in the presence of DON resulted in a rapid restoration of the synthesis of normal-sized glycosaminoglycan chains and proteoglycans. These results showed that, in chick chondrocyte cultures, D-glucosamine, but not L-glutamine, is capable of completely releasing the metabolic block by DON. This is consistent with the postulated mechanism for DON-induced inhibition by irreversibly binding to glucosamine-6-phosphate synthetase

(Bates & Handschumacher, 1968). It has also been reported that D-glucosamine can overcome the azaserine-induced inhibition of chick CS-PG synthesis *in ovo* (Honda *et al.*, 1989).

In summary, the L-glutamine analogue DON has been shown to inhibit the synthesis of chondroitin sulphate in a time-dependent manner, resulting in the elaboration of a CS-PG in which virtually all of the glycosaminoglycan chains are significantly shorter than normal. Moreover, this inhibition and its structural consequences could be immediately reversed by the addition of exogenous D-glucosamine, but not L-glutamine. Thus glutamine analogues would be useful tools in probing the mechanism of cartilage proteoglycan synthesis and the role of these macromolecules in chondrogenesis *in vitro* and *in vivo*.

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