Collagen Synthesis and Deposition in Cartilage during Disrupted Proteoglycan Production

By PHILIP DONDI and HELEN MUIR

Biochemistry Division, Kennedy Institute of Rheumatology, Bute Gardens, Hammersmith, London W6 7DW, U.K.

(Received 20 July 1976)

A simple system was developed to investigate *in vitro* the possible relationship between collagen and proteoglycan synthesis in cartilage. When production of complete proteoglycan molecules was effectively inhibited with 4-methylumbelliferyl β -D-xyloside collagen synthesis and distribution were virtually unaffected.

Collagen and proteoglycans are the two major macromolecular components of cartilage and are synthesized by chondrocytes, the specialized cells of the cartilage matrix. Because of the association of these molecules within the matrix and their common cellular origin, the possibility that their synthesis may be co-ordinated in some way has been examined by using 4-methylumbelliferyl β -D-xyloside to interfere with the synthesis of proteoglycan.

This problem was first studied by Bhatnagar & Prockop (1966), who showed that when collagen hydroxylation in chick-embryo tibiae was prevented by 2,2'-bipyridyl there was no apparent effect upon the synthesis of sulphated glycosaminoglycan. Similarly inhibition of glycosaminoglycan production did not decrease collagen hydroxylation. It would therefore appear that the synthetic pathways of each macromolecule may not be interrelated. On the other hand work with the compound *p*-nitrophenyl- β -D-xylose to interfere with proteoglycan synthesis in chick sternal chondrocyte and human skin fibroblast cultures (Schwartz & Dorfman, 1975*b,c*) suggests that glycosaminoglycan and procollagen synthesis are interdependent.

The effect of various β -D-xylosides upon glycosaminoglycan synthesis has been studied in several systems *in vitro*, including chick-embryo limb bones (Robinson *et al.*, 1975) and cultures of several types of connective-tissue cell (Schwartz *et al.*, 1974; Schwartz & Dorfman, 1975*a,b,c*; Galligani *et al.*, 1975). In general, it has been found that β -D-xylosides initiate glycosaminoglycan-chain synthesis and stimulate synthesis to varying degrees above normal. Chondroitin sulphate-chain synthesis (Rodén & Schwartz, 1975) can be initiated by β -D-xylosides, which act as acceptors for the galactosyltransferase that attaches the first galactose residue of the linkage region to xylose. Thus β -D-xylosides can prevent production of proteoglycan molecules by competing with the natural galactose acceptor, that is the xylosyl-

serine residue on the core protein (D. A. Lowther, personal communication).

The present paper describes the use of 4-methylumbelliferyl β -D-xyloside in a simple system *in vitro*, to determine whether disruption of proteoglycan synthesis affects the synthesis and distribution of cartilage collagen.

Materials and Methods

Incubation in vitro

Larynges from pigs (6–9 months old) were obtained fresh from the slaughterhouse. Adhering tissue and perichondrium were removed and the thick calcified regions of the thyroid plate discarded. The remaining tissue was cut into small pieces (approx. 5 mm $\times$ 1 mm $\times$ 1 mm) while kept moist with incubation medium. After removal of extraneous fluid, samples of about 8.0 g of tissue were dispensed into 50 ml conical flasks, to which was added 10 ml of Eagle's Basal Medium (Gibco Bio Cult, Paisley, Scotland, U.K.) supplemented with (final concentrations) 1.0 mM-L-glutamine, 100 μ g of ascorbic acid/ml, 10 μ g of Gentamicin/ml and 25 mM-Hepes [2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl]ethanesulphonic acid]/KOH buffer, pH 7.4. The medium was equilibrated with O₂ (Robinson *et al.*, 1975) before the tissue was added, and then each sample was preincubated at 37°C in a shaking water bath for 30 min. The medium was then quickly removed and replaced with 10 ml of fresh medium or fresh medium containing 1 mM-4-methylumbelliferyl β -D-xyloside (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) at 37°C. In both instances [6-³H]glucosamine or [³H]proline (The Radiochemical Centre, Amersham, Bucks., U.K.) was added at a concentration of 10 μ Ci/ml of medium. To determine the effect of 4-methylumbelliferyl β -D-xyloside on glycosaminoglycan synthesis incubations were continued for 6 h; effects on collagen synthesis were determined after 20 h.

After each incubation, medium and tissue were separated by filtration through a sintered-glass funnel. The tissue was washed and then rapidly frozen. The medium and washings were combined and exhaustively dialysed against water at 4°C, after which a sample was removed and stored at -20°C and the remainder freeze-dried and weighed. The cartilage pieces were extracted for 24h with 4M-guanidine hydrochloride in sodium citrate/sodium phosphate buffer (McIlvaine, 1921), pH4.5, by using 10ml of solution/g of wet tissue. The extract was dialysed exhaustively against water at 4°C and a sample stored at -20°C. The remainder was freeze-dried and weighed. The residue from the extractions was thoroughly washed with water at 4°C, freeze-dried, weighed and stored at -20°C. Thus each incubation consisted of three fractions: medium, extract and residue.

Measurement of glycosaminoglycan and collagen synthesis

To determine the distribution of [^3H]glucosamine-labelled material, samples of residue were digested overnight with activated crystalline papain (Hardingham & Muir, 1974) to release glycosaminoglycans from proteoglycan remaining in the residue. The uronic acid content of the three fractions from each incubation was then determined in duplicate samples by an automated modification (Heinegård, 1973) of the method of Bitter & Muir (1962). The proteoglycans and glycosaminoglycans in each fraction were purified by precipitation with cetylpyridinium chloride, converted into the sodium salt, washed and dried *in vacuo* (Hardingham & Muir, 1974). Duplicate samples were then dissolved in water or 0.2M-sodium acetate, pH6.8, and samples of 50–100 μl placed in 10ml of toluene/2-methoxyethanol (3:2, v/v) scintillation fluid containing 80g of naphthalene and 4g of BBOT [2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen]/litre. Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer.

To determine the distribution of newly synthesized collagen in the medium, extract and residue, duplicate samples of each were hydrolysed in 6M-HCl at 105°C for 24h. Hydroxyproline was separated from proline by chromatography on a column (12.0cm $\times$ 1.0cm) of Dowex-50 X8 resin eluted with 0.2M-sodium citrate buffer, pH3.2. Fractions of 2.2–2.6ml were collected and samples of 1.5 or 2.0ml were mixed with 10ml of xylene/Triton 114 (3:1, v/v) scintillation fluid containing 6g of PPO (2,5-diphenyloxazole) and 0.2g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]/litre. Hydroxyproline was eluted between 19.0 and 29.0ml, and proline between 33.0 and 53.0ml. The total radioactivity of hydroxyproline in each incubation fraction was thus determined relative to the dry weight of tissue.

Sephadex G-200 chromatography

Material purified by cetylpyridinium chloride precipitation was examined on a column (91.0cm $\times$ 1.5cm) of Sephadex G-200, eluted with 0.2M-sodium acetate, pH6.8, at the rate of 7.0ml/h by using a Gilson Minipuls II peristaltic pump. Fractions of 3.2ml were collected and 400 μl was removed from each for the determination of radioactivity with toluene/2-methoxyethanol scintillation fluid. Uronic acid contents were determined as described above.

The fluorescence of each fraction due to the presence of 4-methylumbelliferyl groups was determined by using an Aminco-Bowman spectrofluorimeter with excitation and emission wavelengths of 318 and 367nm respectively.

Results and Discussion

Effect of 4-methylumbelliferyl β -D-xyloside on the synthesis and distribution of glycosaminoglycans

The synthesis of glycosaminoglycans in this system *in vitro* was followed for various times up to 6h in a series of incubations and the incorporation of [^3H]glucosamine into glycosaminoglycans was linear, as has previously been demonstrated with the same tissue by using $^{35}\text{SO}_4^{2-}$ (Hardingham & Muir, 1972). In the presence of the 4-methylumbelliferyl β -D-xyloside the results were similar to those reported by Galligani *et al.* (1975) using cell cultures. The results reported in the present paper were obtained from one 6h incubation period and the effects observed are representative of previous experiments.

In the control incubation the majority of newly synthesized glycosaminoglycans remained in the tissue with only 1% of the total radioactivity in the medium, whereas in the presence of 1mM-4-methylumbelliferyl β -D-xyloside as much as 51% of the newly synthesized material was present in the medium. However, the proportion of total radioactivity extracted from the tissue with 4M-guanidine hydrochloride was very similar (Table 1).

Table 1. *Distribution of radioactivity between glycosaminoglycan fractions*

Fractions were isolated from medium, cartilage extract and residue after 6h incubation of diced cartilage with [^3H]glucosamine in the absence and presence of 1mM-4-methylumbelliferyl β -D-xyloside. See the text for details.

Fraction	Distribution of total radioactivity (%)	
	Control	4-Methylumbelliferyl β -D-xyloside-treated
Medium	1	51
4M-Guanidine hydrochloride extract	47	46
Residue	52	3

Fig. 1. Gel chromatography on Sephadex G-200 of material isolated after a 6 h incubation with [^3H]glucosamine

Material was isolated by cetylpyridinium chloride precipitation from the control experiment [(a) medium; (c) extract; (e) digested residue] and from the experiment in the presence of 4-methylumbelliferyl β -D-xyloside [(b) medium; (d) extract; (f) digested residue]. Samples of 0.5 mg of uronic acid (a-d) or 1.0 mg of uronic acid (e-f) were applied to a column (91 cm $\times$ 1.5 cm) of Sephadex G-200 and eluted with 0.2M-sodium acetate, pH 6.8, as described in the text. —, Uronic acid; ----, radioactivity; ····, fluorescence (note that the scale of radioactivity is different for each pair of chromatograms). V_0 , void volume; V_t , total volume.

The molecular size of newly synthesized material was examined by gel chromatography on Sephadex G-200. In the control medium the uronic acid and radioactivity were eluted together in the void volume (Fig. 1a). However, when 4-methylumbelliferyl β -D-xyloside was present during the incubation, the majority of the radioactivity was eluted much later than uronic acid, which as before was eluted in the void volume (Fig. 1b).

A similar result was obtained when 4M-guanidine hydrochloride extracts of cartilage were examined in the same way. In the control experiment, radioactivity and uronic acid were eluted together in the void volume (Fig. 1c), whereas in the presence of the xyloside the majority of the radioactivity was retarded by the gel, whereas the uronic acid, representing pre-existing proteoglycan, was eluted in the void volume. From these chromatograms it could be calculated that in the presence of 4-methylumbelliferyl β -D-xyloside at least 80% of the radio-

activity was incorporated into glycosaminoglycans of low molecular weight.

Approximate molecular weights were calculated from elution volumes on columns of Sephadex G-200 (Hopwood & Robinson, 1973). The non-radioactive glycosaminoglycans obtained after proteolysis of cartilage residues had mol.wts. of about 20000 in both control and treated tissue. However, elution of radioactivity showed that glycosaminoglycans of somewhat greater chain length than those pre-existing had been synthesized in control tissue (Fig. 1e), whereas in the presence of the β -xyloside the newly synthesized chains had mol.wts. of only 8000–10000 (Figs. 1b, 1d and 1f).

Fluorescence was detected only in column effluents of material from the medium of the xyloside-treated tissue, although the peak of fluorescence (Fig. 1b) was eluted some three fractions later than the peak of radioactivity. The fluorescent aglycone group of 4-methylumbelliferyl β -D-xyloside was thus closely

Table 2. *Distribution of [^3H]hydroxyproline between medium, cartilage extract and residue*

Diced cartilage was incubated for 20h with [^3H]proline in the absence and presence of 1 mM-4-methylumbelliferyl β -D-xyloside. See the text for details.

Incubation no.	Fraction	Distribution of hydroxyproline radioactivity (%)	
		Control	4-Methylumbelliferyl β -D-xyloside-treated
1	Medium	8.0	10.0
	Extract	15.0	16.0
	Residue	77.0	74.0
2	Medium	4.5	7.0
	Extract	7.5	9.5
	Residue	88.0	83.5
3	Medium	5.0	6.5
	Extract	7.5	8.5
	Residue	87.5	85.0
4	Medium	5.0	6.0
	Extract	6.0	9.0
	Residue	89.0	85.0

associated with the newly synthesized glycosaminoglycan chains, indicating involvement with chain initiation.

Effect of 4-methylumbelliferyl β -D-xyloside on collagen synthesis and distribution

The production and distribution of non-diffusible [^3H]hydroxyproline was used as a measure of collagen synthesis, and the results from four experiments are shown in Table 2. Prolonged incubations were adopted to obtain a high incorporation of label into hydroxyproline.

The majority of newly synthesized collagen remained in the cartilage residue in both control and β -xyloside-treated tissue (Table 2).

In both the medium and extract fractions from the xyloside incubations there was an increase in the amount of non-diffusible [^3H]hydroxyproline above control values. However, the concentration of non-diffusible [^3H]proline also increased by a similar amount, indicating a general increase in the amount of total soluble protein, of which collagen was only a small proportion.

In the residue fractions of control and xyloside incubations the concentrations of radioactivity in hydroxyproline and proline were, however, very similar, and, as the residue fractions represented the majority of newly synthesized collagen (Table 1b), 4-methylumbelliferyl β -D-xyloside did not specifically

affect collagen synthesis and distribution, even when the formation of completed proteoglycans was largely prevented.

The results are in general agreement with those of Bhatnagar & Prockop (1966), but differ from the observations of Schwartz & Dorfman (1975b,c), who used cultured cells (chick-embryo sternal chondrocytes and human skin fibroblasts) and a different β -xyloside and who found that collagen synthesis was decreased. In the present experiments whole cartilage pieces were used in which the cells were surrounded by a normal matrix and thus might be expected to be less affected by disruption of proteoglycan synthesis over the short periods of time used here. Embryonic chondrocytes, particularly those in culture, are more active in synthesizing collagen than are chondrocytes in adult tissue and hence any effects on collagen synthesis might be more apparent. Prolonged disruption of proteoglycan synthesis might eventually affect collagen synthesis indirectly even by cells within cartilage matrix. The present results show, however, that collagen and proteoglycan synthesis are not closely integrated processes in adult cartilage.

We are grateful to T. Wall and Son, London N.W.10, U.K., for the supply of tissue for this study. We thank the Medical Research Council for the support of P. G. D. and the Arthritis and Rheumatism Council for general support.

References

- Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330-334
- Bhatnagar, R. S. & Prockop, D. J. (1966) *Biochim. Biophys. Acta* **130**, 383-392
- Galligani, L., Hopwood, J., Schwartz, N. B. & Dorfman, A. (1975) *J. Biol. Chem.* **250**, 5400-5406
- Hardingham, T. E. & Muir, H. (1972) *Biochem. J.* **126**, 791-803
- Hardingham, T. E. & Muir, H. (1974) *Biochem. J.* **139**, 565-581
- Heinegård, D. (1973) *Chim. Scripta* **4**, 199-201
- Hopwood, J. J. & Robinson, H. C. (1973) *Biochem. J.* **135**, 631-637
- McIlvaine, T. C. (1921) *J. Biol. Chem.* **49**, 183-186
- Robinson, H. C., Brett, M. J., Tralaggaan, P. J., Lowther, D. A. & Okayama, M. (1975) *Biochem. J.* **148**, 25-34
- Rodén, L. & Schwartz, N. B. (1975) *Biochem. Ser. One* **5**, 95-152
- Schwartz, N. B. & Dorfman, A. (1975a) *Conn. Tiss. Res.* **3**, 115-122
- Schwartz, N. B. & Dorfman, A. (1975b) *J. Cell Biol.* **67**, 390a
- Schwartz, N. B. & Dorfman, A. (1975c) *Biochem. Biophys. Res. Commun.* **67**, 1108-1113
- Schwartz, N. B., Galligani, L., Ho, P.-L., & Dorfman, A. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4047-4051