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Factors Influencing Proteoglycan Size in Rachitic-Chick Growth Cartilage

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1. Proteoglycan isolated from rachitic-chick growth cartilage was of smaller size than that isolated from tissue of normal chicks. 2. The two proteoglycan populations were of similar average chemical composition and similar in the size of their chondroitin sulphate chains. 3. The size of the proteoglycans was not affected by reduction and alkylation. 4. Labelling studies *in vivo* with $Na_2{}^{35}SO_4$ and [^{3}H]leucine suggest that the difference in size in the rachitic state results from an alteration in synthesis rather than extracellular proteolytic degradation of the normal proteoglycan, but the direct cause of this alteration remains unestablished.

Vitamin D deficiency leads to a disorder of bone formation, and in the growing animal the cartilage growth plate becomes markedly enlarged. The major structural constituents of growth cartilage are collagen and proteoglycan. Studies of the former show no evidence for abnormality in the rachitic chick (Toole *et al.*, 1972). There is, however, evidence (Dickson & Roughley, 1978) that growth-cartilage proteoglycan monomer from the rachitic chick is of much smaller average hydrodynamic size than normal, as shown by Sepharose 2B column chromatography, wide-pore-gel electrophoresis and viscosity measurements.

The finding of decreased proteoglycan size in the rachitic state appeared consistent with either an altered pattern of synthesis by the chondrocytes or limited proteolytic degradation of those molecules occurring in the normal state. In order to obtain further information about the cause of these differences we have carried out a comparative investigation of the structure and composition of the proteoglycans in the rachitic and normal states, to look for differences that might suggest an altered pattern of synthesis. We have also performed labelling *in vivo* of the core protein and glycosaminoglycan side chains with radioactive isotopes to look at the size of newly synthesized proteoglycan molecules in rachitic- and normal- chick growth cartilage, to determine whether there is any evidence for initial synthesis of a large proteoglycan molecule, which is then degraded to smaller pieces by extracellular proteinases in the rachitic state.

Methods

Full details of the method for isolating the proteoglycan, agarose/polyacrylamide-gel electrophoresis, viscosity measurements and analytical methods have been given previously (Dickson & Roughley, 1978). Nomenclature for density-gradient fractions is as described by Heinegård (1972).

Radioactive labelling of newly synthesized proteoglycan

Vitamin D-deficient and vitamin D-treated chicks aged 4–5 weeks were given a single intracardial injection of 0.2ml of a sterile 0.9% NaCl solution containing 100μCi of either $Na_2{}^{35}SO_4$ or [4,5-^{3}H]-leucine (The Radiochemical Centre, Amersham, Bucks., U.K.). After 24h the chicks were bled, killed with chloroform, and the proximal tibial growth cartilage was dissected out. The cartilage from both tibiae was pooled and pulverized for 2min in a freezer mill (Spex Industries, Metuchen, NJ, U.S.A.) cooled with liquid N_2. The pulverized cartilage was extracted (48h, 5°C) with 4M-guanidinium chloride (approx. 30ml/g wet wt. of tissue) containing 0.05M-sodium acetate, pH5.0, and proteinase inhibitors [1mM-phenylmethanesulphonyl fluoride, 1mM-iodoacetic acid and 2mM-EDTA (sodium salt) as general inhibitors of serine proteinases, thiol proteinases and metalloproteinases respectively]. After centrifugation (M.S.E. 65, 10×10 rotor, for 1h at 5°C and 20000rev./min) the supernatant solution was adjusted to a density of 1.70g/ml with CsCl, and then centrifuged in the same rotor at 40000rev./min (100000$g_{av.}$) and 10°C for 48h. The gel of protein at the top of the tube was removed and the remaining solution fractionated. The fractions (0.6ml) were assayed for hexuronic acid, and those containing most hexuronic acid (approximately the bottom quarter of the contents of the tube) were pooled, dialysed against water, then against saturated NaCl

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and finally exhaustively against water before being freeze-dried.

Freeze-dried proteoglycan (1–2mg) was dissolved in buffer (0.2M-sodium acetate, pH5.5) and applied to a column (1.6cm×27cm) of Sepharose C12B (Pharmacia Products, London W5 5SS, U.K.), and eluted with the same buffer at a flow rate of 9.8ml/h. Fractions (1.8ml) were collected and portions taken for counting of radioactivity and assay of hexuronic acid.

Chondroitinase degradation of proteoglycans

Proteoglycan was dissolved in 0.1M-Tris/HCl/0.1M-sodium acetate, pH7.30, at a concentration of 8mg/ml. Chondroitinase ABC (10μl; 5 units/ml; Miles Laboratories, Elkhart, IN ,U.S.A.) was added per mg of proteoglycan (Hascall & Heinegård, 1974*a*). The mixture was incubated at 40°C for 2h, and then heated at 100°C for 10min. The unsaturated disaccharides produced by this procedure were separated by t.l.c. as described by Wasserman *et al.* (1977): 25μl of the degradation mixture was spotted on to a 250μm cellulose plate containing Avicel F fluorescent indicator (Mandel Scientific Co., Montreal, Que., Canada). This plate was eluted, firstly with butan-1-ol/ethanol/water (13:8:4, by vol.), and then with butan-1-ol/acetic acid/1M-NH_3 (2:3:1, by vol.). The separated disaccharides were detected by u.v. irradiation, and the degradation products eluted from the cellulose by incubation with 0.5ml of water at 40°C for 16h. A portion (0.2ml) of this solution was assayed by the procedure of Koseki *et al.* (1978), with reference disaccharides (Miles Laboratories) as standards.

Reduction and alkylation of proteoglycans

Reduction and alkylation of the proteoglycan was carried out under conditions similar to those described by Heinegård (1977). Proteoglycan was dissolved in 0.05M-Tris/HCl, pH7.25, containing 4M-guanidine hydrochloride and 5mM-dithiothreitol, at a concentration of 2mg/ml. The mixture was incubated at 40°C for 4h. Iodoacetic acid was then added to give a final concentration of 15mM, and the mixture incubated at 25°C for a further 16h. The proteoglycan was dialysed against 1000vol. of distilled water, converted to its potassium salt by dialysis against 1000vol. of 0.1M-potassium acetate, and then exhaustively dialysed against distilled water, and the resulting solution freeze-dried. The reduced and alkylated proteoglycan was dissolved in 0.2M-sodium acetate, pH5.50, at 2mg/ml for viscometric analysis and Sepharose 2B chromatography. A 2mg/ml solution in water was used for agarose/polyacrylamide-gel electrophoresis.

Proteolytic degradation of proteoglycan

Proteoglycan was dissolved in 0.2M-sodium acetate, pH5.00, at a concentration of 2mg/ml. This solution was subjected to proteolytic degradation by either pepsin (Worthington, Freehold, NJ, U.S.A.) or papain (Sigma Chemical Co., St.Louis, MO, U.S.A.) at 40°C. For degradation by papain, the incubation buffer was supplemented with EDTA and cysteine (each at 5mM). The proteinase was added at a concentration of 10μg/mg of proteoglycan, and two equal additions were made, one at the commencement of incubation and the second after 4h. Incubation was carried out for 24h, by which time proteolytic degradation was complete. Remaining pepsin activity was inhibited by the addition of pepstatin (0.5μg/μg of pepsin) and papain activity by the addition of iodoacetic acid (final concentration 10mM).

The degradation mixture was investigated directly by Sepharose 4B chromatography and was also subjected to electrophoresis in agarose/polyacrylamide gels after dialysis against 100vol. of distilled water. Hexosamine analysis or amino acid analysis was performed on the chondroitin sulphate-rich fragments present in the degradation mixture after their precipitation by cetylpyridinium chloride (Roughley & Barrett, 1977; Heinegård & Hascall, 1974) and conversion to their potassium salts. Electrophoresis was also performed on this product, and the staining patterns obtained were always identical with those from unpurified degradation mixtures.

Sepharose 2B and 4B chromatography

The intact proteoglycans were subjected to chromatography on Sepharose 2B, and the proteolytic degradation products to chromatography on Sepharose 4B. In either case the column (1.0cm×53cm) was equilibrated and eluted with 0.2M-sodium acetate, pH5.50. The sample (1ml), containing about 2mg of the proteoglycan, was chromatographed by downward elution at 4°C with a flow rate of 8ml/h, and fractions of volume 1ml were collected. The hexuronic acid content of the eluate was determined by the method of Bitter & Muir (1962).

Viscometry

The proteoglycan was dissolved in 0.2M-sodium acetate, pH5.50, at 2mg/ml. The specific viscosity of the sample (1ml) relative to buffer was determined at 25°C in a Cannon–Manning semi-micro viscometer (flow time 34s with buffer; Cannon Instrument Co., State College, PA, U.S.A.). Interaction with hyaluronic acid was studied by the addition of 10μl portions of a 2mg/ml solution of hyaluronic acid (Sigma) in the same buffer. The hyaluronic acid was purified before use by the method of Cleland & Sherblom (1977).

Results

Radioactive labelling of newly synthesized proteoglycan

When growth-cartilage proteoglycan, from rachitic and normal chicks given a dose of $Na_2{}^{35}SO_4$ 24h before being killed, was applied to a Sepharose 2B column, the elution profiles of ^{35}S-bound proteoglycan, representing newly synthesized molecules, were very similar to the corresponding profiles for hexuronic acid, representing new as well as older proteoglycan molecules (Figs. 1 and 2). These results imply that the size of the most recently synthesized proteoglycan molecules was identical with that of all the proteoglycan molecules in the cartilage. The ^{35}S profile for proteoglycan from chicks given the radioactive isotope 6h before being killed was also identical with the hexuronic acid profile (results not shown). There was no evidence for the existence of large newly synthesized proteoglycan molecules in the rachitic chick at 24 or 6h.

When [^{3}H]leucine was given to chicks 24h before they were killed and the growth-cartilage proteoglycan chromatographed on Sepharose 2B, the ^3H elution profile was similar to that for hexuronic acid (Fig. 3).

Chemical composition of the glycosaminoglycan chains

The proteoglycans were investigated by hexosamine analysis and chondroitinase ABC digestion. The galactosamine/glucosamine molar ratios indicated a predominance of chondroitin sulphate over keratan sulphate in both the normal and rachitic preparations, and little difference in the absolute amounts of either glycosaminoglycan (Table 1). The unsaturated disaccharides obtained after chondroitinase digestion

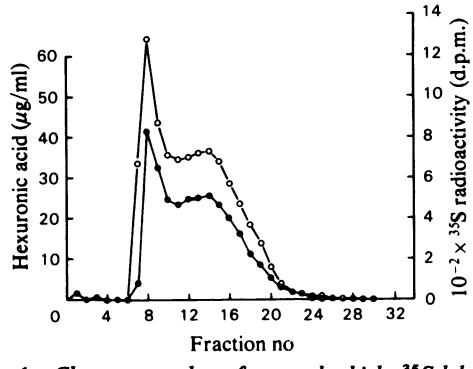

Fig. 1. *Chromatography of normal chick ^{35}S-labelled growth-cartilage proteoglycan on a Sepharose 2B column*
$Na_2{}^{35}SO_4$ was injected intracardially 24h before the chick was killed. For details of procedure and isolation and chromatography of proteoglycan see the Methods section. ●, Hexuronic acid elution; ○, ^{35}S radioactivity elution.

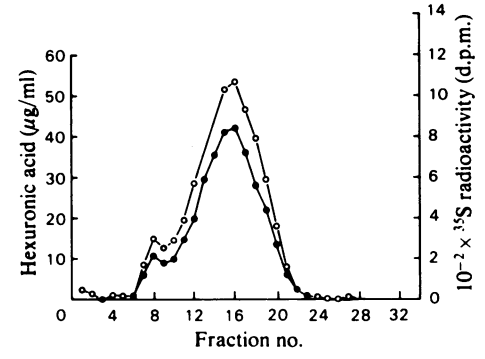

Fig. 2. *Chromatography of rachitic-chick ^{35}S-labelled growth-cartilage proteoglycan on a Sepharose 2B column*
Details are as for Fig. 1.

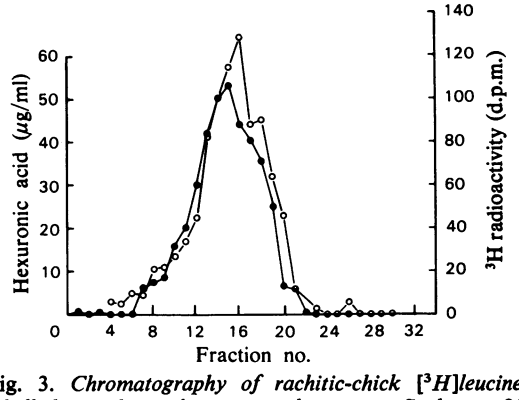

Fig. 3. *Chromatography of rachitic-chick [^3H]leucine-labelled growth-cartilage proteoglycan on a Sepharose 2B column*
Details are as for Fig. 1.

Table 1. *Hexosamine composition and degree of sulphation of proteoglycan from normal (A1D1-N) and rachitic (A1D1-R) chicks*
See the Methods section for details of the experimental procedure.

	Composition and sulphation (residues/100 residues)	
	A1D1-N	A1D1-R
Hexosamine composition		
Galactosamine	94	93
Glucosamine	6	7
Galactosamine sulphation		
0-Sulphate	12	12
4-Sulphate	66	64
6-Sulphate	22	24

of the proteoglycan indicated that both preparations possessed similar distributions with respect to the position of sulphation along chondroitin sulphate chains (Table 1). In both cases 4-sulphation was most prevalent with smaller quantities of 6-sulphation (4S/6S about 3:1) and non-sulphation (4S/0S about 5:1). Disulphated disaccharides were not detected in either preparation. Thus in both the normal and rachitic animals chondroitin sulphate and keratan sulphate were present to a similar degree and the former was sulphated in the same manner.

Ability of proteoglycan to interact with hyaluronic acid

The interaction between proteoglycan and hyaluronic acid was measured by viscometry. Hyaluronic acid was added to a solution of the proteoglycan subunits in successive portions to give proteoglycan/hyaluronic acid weight ratios of 100:1 and 50:1. Interaction between the two species was indicated by an increase in viscosity above that obtained by the addition of hyaluronic acid to the buffer alone. The results indicate that both the normal and rachitic subunit preparations interacted with hyaluronic acid (Table 2), though the extent of interaction was not as great as one would have expected if all the proteoglycan subunits were capable of the specific aggrega-

Table 2. *Specific viscosity of proteoglycan subunit preparations in the absence and presence of hyaluronic acid*

The proteoglycan solutions were 2mg/ml in 0.2M-sodium acetate, pH5.5. Hyaluronic acid (10 or 20μl; 2mg/ml in the same buffer) was added to 1ml of proteoglycan solution. Measurements of specific viscosity were made at 25°C. A1D1-N and A1D1-R are proteoglycan subunits from normal and rachitic chicks respectively.

	Specific viscosity		
Hyaluronic acid added (μg) ...	0	20	40
Sample			
A1D1-N	0.40	0.60	0.66
A1D1-R	0.31	0.44	0.50
Buffer	0	0.04	0.07

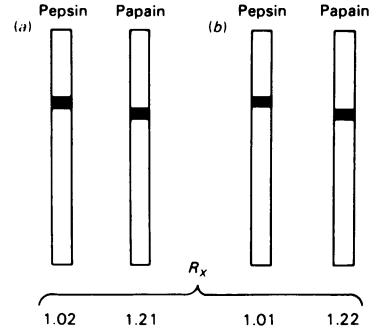

Fig. 5. *Agarose/polyacrylamide-gel electrophoresis of normal (a) and rachitic (b), chick growth-cartilage proteoglycan preparations after pepsin or papain digestion*

The bands represent material that stains with Tolidine Blue, and R_x values were calculated relative to the mobility of Bromophenol Blue. See the Methods section for details of the experimental procedure.

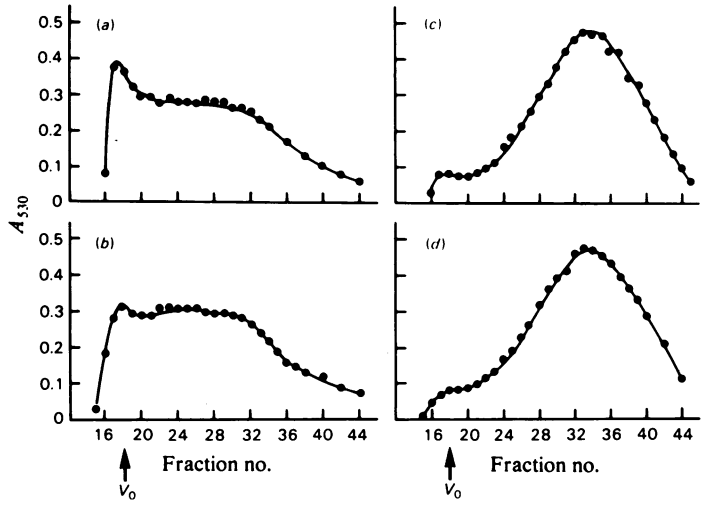

Fig. 4. *Elution profiles of hexuronic acid from a Sepharose 2B column after application of proteoglycan subunit, isolated from normal (a,c) and rachitic (b,d) chicks before (a,b) and after (c,d) reduction and alkylation*

See the Methods section for details of the experimental procedure. V_0 is the void volume of the column.

Table 3. *Amino acid and hexosamine composition of the products of papain and pepsin digestion of proteoglycan from growth cartilage from normal (A1D1-N) and rachitic (A1D1-R) chicks*

The degradation products were those containing chondroitin sulphate chains that were precipitable in the presence of cetylpyridinium chloride. See the Methods section for details of the experimental procedure.

	Composition (residues/1000 residues)			
	Papain		Pepsin	
Amino acid	A1D1-N	A1D1-R	A1D1-N	A1D1-R
Asp	40	37	41	40
Thr	67	54	95	93
Ser	246	211	166	173
Glu	120	115	156	150
Pro	65	76	83	86
Gly	160	182	138	146
Ala	55	62	74	72
Val	39	39	61	62
Met	5	5	5	4
Ile	39	33	52	50
Leu	56	50	56	58
Tyr	11	10	12	11
Phe	21	19	34	34
Lys	63	91	7	5
Arg	14	16	20	17
GalN/GlcN molar ratio	>100	>100	28.9	26.5

tion described for bovine nasal cartilage or porcine laryngeal cartilage (Hascall & Heinegård, 1974*b*; Hardingham & Muir, 1974).

Reduction and alkylation of the proteoglycans

The dissociated proteoglycan preparations were reduced in the presence of dithiothreitol and the resulting cysteine residues alkylated with iodoacetic acid. In this manner protein–protein interactions involving disulphide bonds are eliminated, as is the functional capacity of the hyaluronic acid-binding region (Hardingham *et al.*, 1976). The proteoglycans recovered after this procedure were subjected to Sepharose 2B chromatography, and the resulting hexuronic acid profiles were found to be in close agreement with those of the untreated samples (Fig. 4). Similarly, there was little change in either the viscosity or electrophoretic mobility of the reduced and alkylated samples. Thus neither the normal nor the rachitic-chick proteoglycan subunits were present in the form of disulphide-bridged aggregates or hyaluronic acid-bound aggregates.

Proteolytic degradation of the proteoglycans

The proteoglycans were subjected to proteolytic degradation by either pepsin or papain, and the degradation was allowed to proceed until no further proteolytic cleavages could occur. It has been shown that under such conditions bovine nasal cartilage

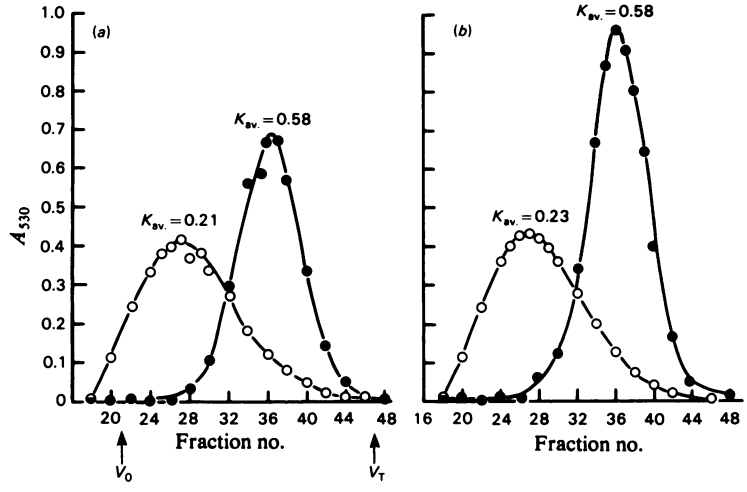

Fig. 6. *Chromatography on Sepharose 4B of normal (a) and rachitic (b) chick growth-cartilage proteoglycan preparations after pepsin (○) or papain (●) digestion*

See the Methods section for details of the experimental procedure. V_0 and V_T are the void volume and total volume of the column and $K_{av.}$ is the partition efficient between the liquid phase and the gel phase.

proteoglycan was degraded to multichain glycosaminoglycan–peptides by pepsin and to single-chain glycosaminoglycan–peptides by papain (Roughley, 1977). The normal and rachitic-chick proteoglycans appeared to behave in a similar manner towards proteolytic degradation. Papain produced a product that migrated as a single band on agarose/polyacrylamide-gel electrophoresis (Fig. 5). Its mobility was consistent with that of single chondroitin sulphate chains, and the hexosamine analysis indicated that keratan sulphate no longer remained bound to the chondroitin sulphate (Table 3). Chromatography on Sepharose 4B indicated that the chondroitin sulphate chains from both the normal and rachitic-chick proteoglycans had similar average chain lengths (Fig. 6). In contrast, pepsin degradation appeared to produce multichain glycosaminoglycan–peptides. A single band of staining was observed on agarose/polyacrylamide-gel electrophoresis at a slower mobility than that expected for single chondroitin sulphate chains (Fig. 5). Hexosamine analysis indicated that about half the keratan sulphate chains were still associated with the chondroitin sulphate (Table 3). The hexuronic acid elution profiles on Sepharose 4B indicated that the degradation products from both the normal and rachitic-chick proteoglycans were of similar average hydrodynamic size (Fig. 6). Further, the fragments containing chondroitin sulphate possessed similar amino acid compositions (Table 3). Thus it is possible that there are analogies between the normal and rachitic-chick core protein sequences.

Discussion

It has been demonstrated (Dickson & Roughley, 1978) that the proteoglycan subunits present in the growth cartilages of rachitic chicks differ in structure from those present in the growth cartilages of normal chicks. The normal proteoglycan subunits are larger in size than their rachitic-chick counterparts, and this is reflected in their lower mobility on agarose/polyacrylamide-gel electrophoresis. However, the chemical composition of the two species is very similar with respect to amino acid and glycosaminoglycan content.

In the present paper we have further investigated the composition of the two proteoglycan subunits. Chondroitinase ABC was used to determine the degree and position of sulphation of the chondroitin sulphate. Seno *et al.* (1975) have shown that a single chain may bear sulphate at both the 4 and 6 positions of their galactosamine residues. In both cases a similar pattern of sulphation was obtained with the amount of sulphation increasing in the order, non-sulphated < 6-sulphate < 4-sulphate. Thus it would appear that the enzyme systems responsible for sulphation are unaffected in the rachitic state.

The susceptibility of the proteoglycan subunits to proteolytic degradation by papain or pepsin was also studied. Papain has been shown to produce only single chondroitin sulphate chains from bovine nasal cartilage proteoglycan, probably by cleavage in the amino acid sequence surrounding the glycosaminoglycan linkage sites (Roughley, 1977). An identical result was obtained for proteoglycans from both normal and rachitic chicks, suggesting that all three preparations may have similar chondroitin sulphate-linkage sequences (Isemura & Ikenaka, 1975). Analysis of the degradation products by Sepharose 4B chromatography indicated that both the normal and rachitic subunits possessed chondroitin sulphate of similar average chain length. Thus it would appear that the enzyme systems responsible for chondroitin sulphate initiation, elongation and termination are also unaffected in the rachitic state.

Degradation of the proteoglycan subunits by pepsin produced larger degradation products than those produced by papain, with only multichain glycosaminoglycan–peptides being detected. The fragments from subunits from both normal and rachitic chicks were similar with respect to amino acid composition, hexosamine composition, electrophoretic mobility and elution from Sepharose 4B. This suggests that the two species may possess similar arrangements of glycosaminoglycan chains and similar core-protein sequences. It is noteworthy that about one-half of the keratan sulphate present in the intact proteoglycan remains bound to the fragments containing chondroitin sulphate. This is less than was obtained by similar pepsin degradation of the proteoglycan from bovine nasal cartilage (Roughley, 1978), though the two species contain similar amounts of chondroitin sulphate and keratan sulphate. This may therefore reflect differences in the arrangement of keratan sulphate or in core-protein sequences between bovine and chick, and this would be in agreement with the different amino acid compositions noted for the two species (Dickson & Roughley, 1978).

Another difference between the chick and bovine proteoglycans was illustrated by viscometry. As expected the proteoglycan subunits from rachitic chick had lower viscosity than their normal counterparts, though both preparations demonstrated some ability to interact with hyaluronic acid.

The results described so far are all consistent with the proteoglycan in rachitic chicks being derived from the normal proteoglycan by limited degradation *in vivo*. If this is true, then newly synthesized subunits in the rachitic-chick growth cartilage should be larger in average size than those that have resided in the matrix from some time. However, labelling studies *in vivo* with [^{35}S]sulphate and [^{3}H]leucine followed by chromatography on Sepharose 2B indicate that the newly synthesized molecules in both

the rachitic and normal cartilage are of the same size as the molecules already present. This implies that the difference between the two subunits arises before secretion into the matrix and is most likely synthetic in origin.

It could be postulated that the larger normal proteoglycan subunit is in fact an aggregate of smaller molecules. To investigate this possibility the subunits were reduced with dithiothreitol and then alkylated with iodoacetic acid. In this manner protein–protein interactions via disulphide bridges are eliminated, and the functional capacity of the hyaluronic acid-binding region of the subunit is destroyed. When the reduced and alkylated subunits were chromatographed on Sepharose 2B the hexuronate-elution profiles were similar to those of the untreated subunits, indicating that the subunits from neither the normal nor rachitic chicks were composed of aggregates of smaller molecules stabilized by disulphide bonds. Treatment of the subunits with 4M-guanidinium chloride, and subsequent chromatography on Sepharose 2B in the presence of 4M-guanidinium chloride, also failed to produce a decrease in size in the two species, indicating that aggregates stabilized by ionic forces are not present (results not shown).

The difference in proteoglycan size is not related directly to the vitamin D status of the chick, but is related to the concentration of plasma Ca^{2+} (I. Dickson, unpublished work). Intestinal Ca^{2+} transport is stimulated by the active form of vitamin D_3, 1,25-dihydroxycholecalciferol (Lawson, 1978), and, as a consequence, vitamin D deficiency in the chick is usually accompanied by hypocalcaemia. This hypocalcaemia can be corrected by elevating the Ca^{2+} concentration of the diet, and in these circumstances large proteoglycan is formed in the growth cartilage. If fed on a diet deplete in calcium but replete in vitamin D, the resulting hypocalcaemic chick forms small growth-cartilage proteoglycan. These findings do not necessarily implicate plasma Ca^{2+} concentrations directly in the proteoglycan-size change, since a disturbance in Ca^{2+} homoeostasis leads to an alteration of a number of factors, particularly secretion of parathyroid hormone.

The decreased proteoglycan size in the rachitic state may, in the chick, be a peculiarity of growth cartilage, since size differences have not been observed in articular cartilage or cartilage of the xiphoid process; in these tissues the size range of the proteoglycan subunits is similar to that in rachitic-chick growth cartilage (P. Roughley & I. Dickson, unpublished work). From this context the large growth-cartilage proteoglycan molecules seem abnormal and it will be of interest to establish whether such large molecules are a feature of any mammalian growth-cartilage proteoglycans.

Thus, to summarize, it would appear that the small proteoglycan subunits characteristic of rachitic-chick growth cartilage and the large subunits characteristic of the normal growth cartilage are similar not only in chemical composition but also in the structure of their chondroitin sulphate chains. Further, their behaviour towards proteinases suggests that they bear some analogy in their core-protein structure. The smaller size of the rachitic-chick subunit does not appear to arise from proteolytic degradation of the larger normal subunit after its secretion from the matrix, and the larger subunit does not appear to be an aggregate of smaller units stabilized by disulphide or ionic bonds. The size difference in the rachitic state can be related to the hypocalcaemia accompanying rickets.

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