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Immunochemical analysis of cartilage proteoglycans

Cross-reactivity of molecules isolated from different species

Jörgen WIESLANDER and Dick HEINEGÅRD
Department of Physiological Chemistry 2, University of Lund, P.O. Box 750, S-220 07 Lund 7, Sweden

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Antibodies directed against whole bovine nasal-cartilage proteoglycan and against the hyaluronic acid-binding region and chondroitin sulphate peptides from the same molecule were used in immunodiffusion and immunoelectromigration experiments. Proteoglycans from bovine nasal and tracheal cartilage showed immunological identity, with all three antisera. Proteoglycans from pig hip articular cartilage, dog hip articular cartilage, human tarsal articular cartilage and rat chondrosarcoma reacted with all the antisera and showed immunological identity with the corresponding structures isolated from bovine nasal-cartilage proteoglycans. In contrast, proteoglycans from rabbit articular cartilage, rabbit nasal cartilage and cultured chick limb buds did not react with the antibodies directed against the hyaluronic acid-binding region, though reacting with antibodies raised against whole proteoglycan monomer and against chondroitin sulphate peptides. All the proteoglycans gave two precipitation lines with the anti-(chondroitin sulphate peptide) antibodies. Similarly, the proteoglycans reacting with the anti-(hyaluronic acid-binding region) antibodies gave two precipitation lines. The results indicate the presence of at least two populations of aggregating proteoglycan monomers in cartilage. The relative affinity of the antibodies for cartilage proteoglycans and proteoglycan substructures from various species was determined by radioimmunoassay. The affinity of the anti-(hyaluronic acid-binding region) antibodies for the proteoglycans decreased in the order bovine, dog, human and pig cartilage. Rat sternal-cartilage and rabbit articular-cartilage proteoglycans reacted weakly, whereas chick limb-bud and chick sternal-cartilage proteoglycans did not react. In contrast, the affinity of antibodies to chondroitin sulphate peptides for proteoglycans increased in the order bovine cartilage, chick limb bud and chick sternal cartilage, dog cartilage, rat chondrosarcoma, human cartilage, pig cartilage, rat sternal cartilage and rabbit cartilage.

The cartilage proteoglycan monomers contain a central protein core with a large number of covalently attached polysaccharide chains. In the tissue several proteoglycan monomers are bound to hyaluronic acid by a non-covalent linkage, which is stabilized by a third component, the link proteins. The interaction with hyaluronic acid is specific for one portion of the protein core, the hyaluronic acid-binding region, which contains few or no polysaccharide chains (Heinegård & Hascall, 1974). The protein core in addition contains two other regions or substructures, which contain most of the keratan sulphate chains and most of the chondroitin sulphate chains respectively (Heinegård & Axelsson, 1977; Hascall & Heinegård, 1979. The isolation of these regions or substructures (Heinegård & Axelsson, 1977; Hascall & Heinegård, 1979) made it possible to raise antibodies with specificity for each region (Wieslander & Heinegård, 1979). By immunodiffusion it was shown that the keratan sulphate region, the chondroitin sulphate peptides isolated after trypsin digestion, the hyaluronic acid-binding region and the link proteins were immunologically non-identical (Wieslander & Heinegård, 1979). With the use of the same antibodies radioimmunoassay procedures were developed for the determination of the link proteins, hyaluronic acid-binding region and chondroitin

Abbreviation used: IgG, immunoglobulin G.

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sulphate peptides in nanogram quantities (Wieslander & Heinegård, 1980).

The aim of the present study was to demonstrate whether cartilage proteoglycans from different species and locations cross-react immunologically, in analogy with many other proteins (Arons & Neurath, 1969; Pricels *et al.*, 1975).

Materials and methods

Preparation of proteoglycans

Cartilage proteoglycans were prepared essentially as described elsewhere (Wieslander & Heinegård, 1979; Heinegård & Hascall, 1979). The following tissues were collected: human tarsal articular cartilage, dog hip articular cartilage, pig hip articular cartilage, bovine nasal cartilage, bovine tracheal cartilage, rabbit hip articular cartilage, rabbit nasal cartilage, rat sternal cartilage and Swarm rat chondrosarcoma. Chick limb-bud proteoglycan monomer from day-8 cultures was a gift from Dr. V. C. Hascall (National Institutes of Health, Bethesda, MD, U.S.A.) and chick sternal-cartilage proteoglycan monomer was a gift from Dr. B. Caterson (University of Alabama, Birmingham, AL, U.S.A.).

The cartilage pieces were either ground in liquid N_2 or sliced with a Surform blade. Tissues were extracted under dissociative conditions at 4°C for 20h with 4M-guanidinium chloride containing proteinase inhibitors (Oegema *et al.*, 1975), reassociated by dialysis and centrifuged in CsCl density gradients to prepare the aggregate (A1) fraction as described elsewhere (Heinegård & Hascall, 1979). The proteoglycan monomers (A1-D1 fraction) were prepared from the A1 fraction by CsCl-density-gradient centrifugation under dissociative conditions (Heinegård, 1972). Alternatively the monomers were isolated by chromatography of the A1 fraction on Sephadex G-200 eluted with 4M-guanidinium chloride. The proteoglycan monomers were recovered in the void volume (A1-G200-1 fraction), whereas the link proteins were retained on the column (Heinegård & Hascall, 1974). The proteoglycans from human articular cartilage, rabbit nasal cartilage and rabbit articular cartilage were prepared by the chromatographic procedure, and all other proteoglycans were A1-D1 fractions.

All proteoglycan samples were digested with chondroitinase ABC before immunochemical analysis. The digestion was done in 0.1M-sodium acetate/0.1M-Tris/HCl buffer, pH 7.3, at a concentration of 1mg/ml with 0.01 unit (nominal) of the enzyme/ml for 4h at 37°C. In some cases a sample of the antigen was further digested with trypsin (diphenylcarbamoyl chloride - treated; Sigma Chemical Co., St. Louis, MO, U.S.A.), 5μg of enzyme/mg of sample being used at 37°C for 3h.

Preparation of antisera

All antisera were directed against preparations isolated from bovine nasal-cartilage proteoglycans and were raised in rabbits as described elsewhere (Wieslander & Heinegård, 1979). The rabbits were immunized with proteoglycan monomer (A1-D1 fraction), the fraction A1-T-A1 (mainly chondroitin sulphate peptides) and the hyaluronic acid-binding region (A1-T-A3-2B1-G-200-2 fraction). All antigens had been rechromatographed on Sephadex G-200 in 4M-guanidinium chloride before immunization. The rabbits were bled every second week, and the IgG was purified by the procedure of Steinbuch & Audran (1969). The anti-(A1-T-A1 fraction) serum was further purified by passage through a column of hyaluronic acid-binding region covalently attached to Sepharose Cl-4B (Wieslander & Heinegård, 1980). Previous findings indicate that the purified antibodies are directed against chondroitin sulphate peptides and do not react with keratan sulphate peptides (Wieslander & Heinegård, 1979).

Immunological methods

Antigen identities were demonstrated with the immunoelectromigration procedure of Grubb (1972), a modification of the electroimmunoassay 'rocket' procedure of Laurell (1966). Double immunodiffusion was done as described by Ouchterlony (1962). Immunoelectrophoresis was done in 1% agarose gels containing 24mM-barbital buffer, pH 8.6, as described elsewhere (Wieslander & Heinegård. 1979).

Radioimmunoassay procedures for quantification of hyaluronic acid-binding region and chondroitin sulphate peptides (Wieslander & Heinegård, 1980) were used to measure affinity of the anti-(bovine nasal-cartilage proteoglycan) antibodies for the various proteoglycan preparations, or trypsin digests thereof. Usually 50μl of antigen was incubated with 50μl of diluted rabbit antibody and 50μl of ^{125}I-labelled antigen for 24h at 4°C. Pig anti-(rabbit IgG) antibody was added, and after incubation at 4°C for 16h the precipitate was collected by centrifugation and its radioactivity subsequently counted in a γ-radiation counter.

Gel chromatography

Samples of rabbit articular-cartilage proteoglycan monomers were chromatographed on a Sepharose 2B column (140cm × 0.8cm) eluted with 0.5M-sodium acetate buffer, pH 7.0. One sample (0.5mg) was reduced and alkylated as described elsewhere (Heinegård, 1977) to abolish interaction with hyaluronic acid. Another sample was incubated with 1% (w/w) hyaluronic acid to allow formation of aggregates (Hardingham & Muir, 1972).

Results and discussion

Assay for precipitating antigen–antibody complexes

Antigens were characterized by double immunodiffusion and immunoelectromigration (Figs. 1, 2 and 3). Bovine nasal-cartilage and tracheal-cartilage proteoglycan monomers reacted with all antisera and gave lines of identity.

All cartilage proteoglycan samples reacted with purified anti-(A1-D1 fraction) IgG and gave one precipitation line (Fig. 1). They all contained immunologically identical antigenic sites, as was demonstrated by the fused precipitation lines obtained on immunoelectromigration (Fig. 1). The bovine cartilage proteoglycans in addition contained antigenic sites that were not shared by the other proteoglycans, as indicated by the spur on double diffusion against anti-(A1-D1 fraction) IgG (results not shown). Neither the chick limb-bud nor the rabbit cartilage proteoglycans reacted in double diffusion, whereas immunoelectromigration (Fig. 1) indicated a reaction of identity with bovine proteoglycans. The absence of reactivity in the double diffusion may result from a suboptimal antigen/antibody ratio. The number of lines obtained on electroimmunomigration with the various antisera are outlined in Table 1.

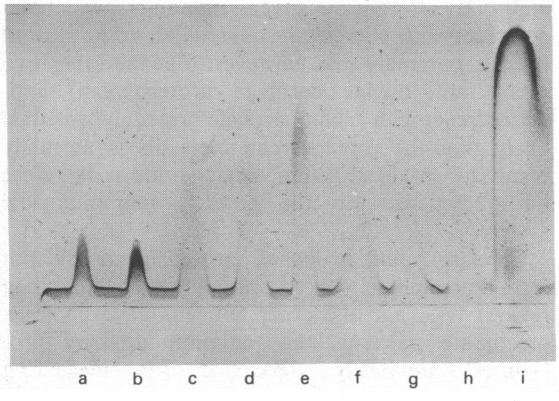

Fig. 1. *Immunoelectromigration of proteoglycans from different species against anti-(A1-D1 fraction) IgG*
The well contained bovine nasal-cartilage proteoglycan A1-D1 fraction and the slits the following antigens: a, bovine tracheal cartilage; b, bovine nasal cartilage; c, pig articular hip cartilage; d, dog articular hip cartilage; e, human articular cartilage, f, rabbit articular cartilage; g, rabbit nasal cartilage; h, chick limb bud; i, rat chondrosarcoma. All antigens were digested with chondroitinase ABC before electrophoresis. For full experimental details see the text.

All the proteoglycan samples also reacted with anti-(A1-T-A1 fraction) IgG. With this antiserum two precipitation lines were observed with all the proteoglycans except for those prepared from rabbit cartilage, which gave only one distinct line. A second very faint line was possibly present. All the proteoglycans contain immunologically identical antigenic sites reacting with the anti-(A1-T-A1 fraction) IgG, as was demonstrated in double diffusion (results not shown) and by the fused 'rockets' on immunoelectromigration (Fig. 2 and Table 1).

The only antiserum that did not react with all the proteoglycan samples was that directed against the hyaluronic acid-binding region. The proteoglycans prepared from bovine, pig, dog and human cartilages and from rat chondrosarcoma reacted and gave two precipitation lines (Fig. 3). The antigenic sites were shown to be immunologically identical by double diffusion (results not shown) and by immunoelectromigration (Fig. 3). The proteoglycans from the rabbit cartilage and the chick limb-bud cultures, however, did not form immunoprecipitates with the anti-(hyaluronic acid-binding region) antibody in either procedure.

The two precipitation lines observed when the proteoglycans were allowed to react with anti-(A1-T-A1 fraction) IgG and anti-(hyaluronic acid-binding region) IgG respectively may represent two

Table 1. *Cross-reaction and number of precipitation lines in immunoelectromigration of proteoglycans from different species against the different antisera*
Experimental details are given in the text. Each line is represented by a + sign.

Preparation	Anti-(A1-D1 fraction) IgG	Anti-(A1-T-A1 fraction) IgG	Anti-(hyaluronic acid-binding region) IgG
Bovine nasal cartilage	+	++	++
Bovine tracheal cartilage	+	++	++
Pig articular cartilage	+	++	++
Dog articular cartilage	+	++	++
Human articular cartilage	+	++	++
Rat chondrosarcoma	+	++	++
Rabbit articular cartilage	+	+(+)	−
Rabbit nasal cartilage	+	+(+)	−
Chick limb bud	+	++	−

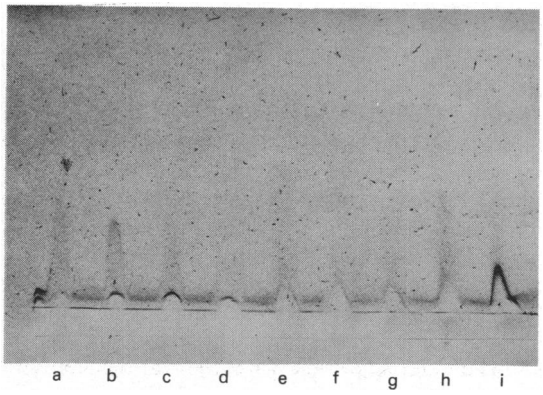

Fig. 2. *Immunoelectromigration of proteoglycans from different species against anti-(A1-T-A1 fraction) IgG*
The well contained bovine nasal-cartilage proteoglycan A1-D1 fraction and the slits antigens a–i as in Fig. 1. All antigens were digested with chondroitinase ABC before electrophoresis. For full experimental details see the text.

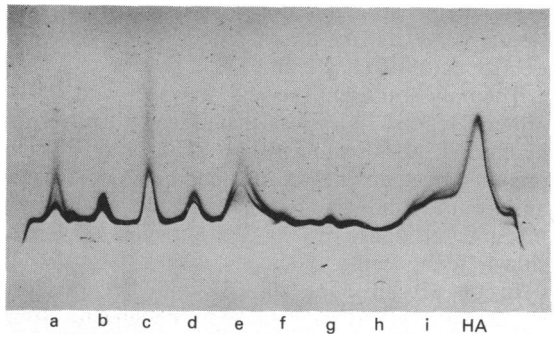

Fig. 3. *Immunoelectromigration of proteoglycans from different species against anti-(hyaluronic acid-binding region) IgG*
The well contained hyaluronic acid-binding region prepared from bovine nasal cartilage A1 fraction and in the slits antigens a–i as in Fig. 1. HA represents isolated hyaluronic acid-binding region. For full experimental details see the text.

populations of the proteoglycan monomer (Figs. 2 and 3). It is known that there are at least two distinct populations of high-molecular-weight proteoglycans in cartilage extracts, the aggregating and the non-aggregating proteoglycans (Heinegård & Hascall, 1979). Electrophoresis of cartilage proteoglycan monomers on polyacrylamide/agarose gels demonstrate two metachromatic components with somewhat different mobilities (Stanescu *et al.*, 1977; Pearson & Mason, 1977). The two precipitation lines discussed above probably do not represent aggregating and non-aggregating proteoglycans, since the non-aggregating proteoglycans do not react with the anti-(hyaluronic acid-binding region) serum (J. Wieslander & D. Heinegård, unpublished work). The results therefore indicate the presence of two antigenically different subpopulations among the aggregating proteoglycans.

When the same amounts of the antigens were used for immunoelectromigration (Figs. 2 and 3), the relative heights of the two precipitation lines varied for the different proteoglycan preparations, perhaps indicating different proportions or reactivities of the two antigenic components. It is possible that the rabbit cartilage proteoglycans actually contained both components, although they gave the same height of precipitation line. It appears that all the proteoglycan samples were immunologically identical, with the exception of rabbit cartilage and chick limb-bud culture proteoglycan monomers, which did not react with anti-(hyaluronic acid-binding region) IgG. It is remarkable, however, that the other two rabbit anti-(bovine cartilage proteoglycan) antibodies react with rabbit cartilage proteoglycans. It is likely that the proteoglycan molecule is normally compartmentalized in the cartilage and is therefore not recognized by the animals immunological system.

Because rabbit and chick proteoglycans did not form a precipitate with anti-(hyaluronic acid-binding region) IgG, and the other antigens gave complex precipitate patterns, the radioimmunoassay procedures previously developed for measuring hyaluronic acid-binding region and chondroitin sulphate peptides were used as a complement to double diffusion and immunoelectromigration. In radioimmunoassay all antibodies, precipitating and non-precipitating, participate in the reaction.

The radioimmunoassay of the hyaluronic acid-binding region indicated that the antibodies had the same affinity for bovine nasal-cartilage and tracheal-cartilage proteoglycans, when measured as capability of the proteoglycan to inhibit the reaction with bovine nasal-cartilage proteoglycans (results not shown). The affinity to cartilage proteoglycans from the other species was lower, when determined as inhibition by a constant amount of proteoglycan (15.6μg/ml) compared with the same amount of bovine cartilage proteoglycan (Fig. 4). The lower affinity to these proteoglycans indicates structural differences. The inhibition curves for increasing amounts of proteoglycans (Fig. 5) also indicate a lower degree of reactivity. All the mammalian proteoglycans showed some cross-reactivity, although it was minimal for rabbit cartilage (Fig. 5). The chick cartilages showed no inhibitory capacity, corroborating the results obtained with the precipitation techniques discussed above.

The radioimmunoassay for the chondroitin sulphate peptides with anti-(bovine A1-T-A1 fraction) IgG showed the same degree of inhibition for the

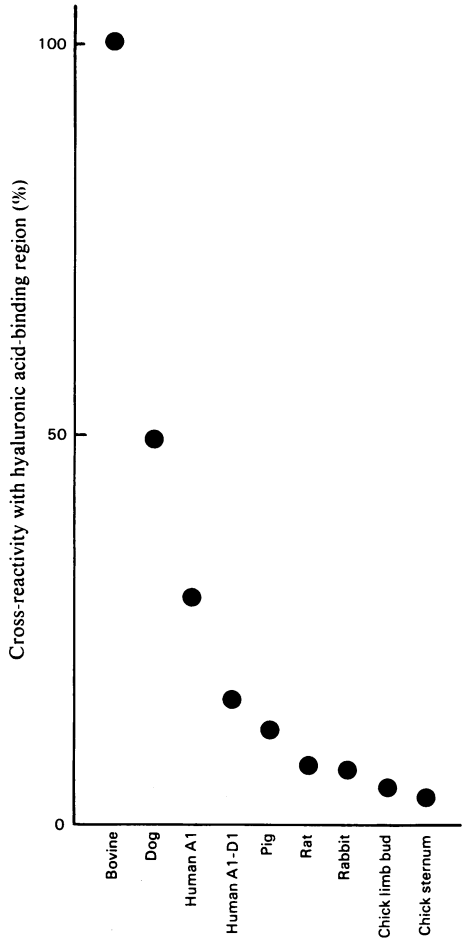

Fig. 4. *Cross-reactivity of hyaluronic acid-binding region isolated from different species*

The inhibition obtained with 15.6 μg of each cartilage proteoglycan preparation/ml relative to that of 15.6 μg of bovine nasal-cartilage proteoglycans/ml is indicated. For full experimental details see the text.

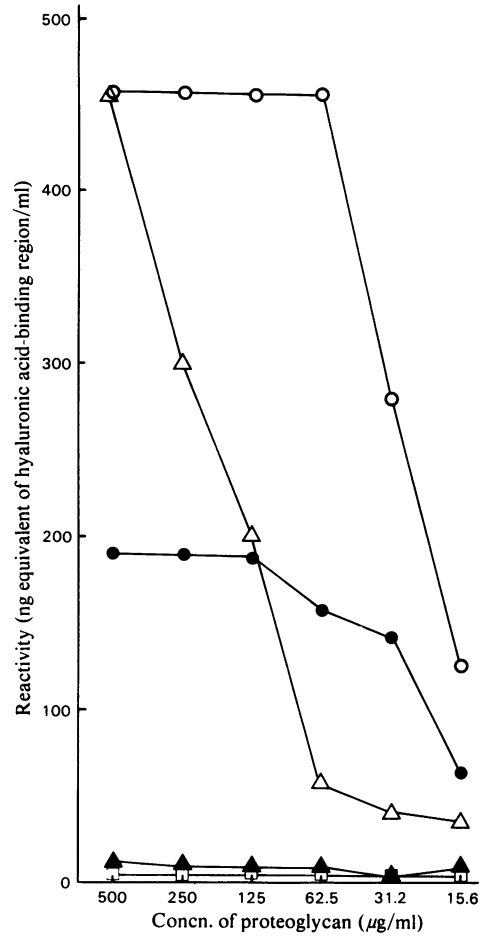

Fig. 5. *Radioimmunoassay of hyaluronic acid-binding region*

The inhibition obtained with increasing concentrations of proteoglycans from bovine (○), human (△), rabbit (▲), chick (□) and dog (●) cartilage is given as the equivalent amount of isolated hyaluronic acid-binding region from bovine proteoglycans. For full experimental details see the text.

two bovine preparations (results not shown). Cartilage proteoglycans from the other species tested gave a higher degree of inhibition than did the bovine proteoglycans, when a constant amount of proteoglycan (1.25 μg/ml) (Fig. 6) was tested. Corroborating results were obtained by inhibition with increasing amounts of proteoglycans (Fig. 7). The antibodies had stronger affinity for proteoglycans from species other than that used for immunization. This could be due to a larger number of available antigenic sites, as a variable number of antigenic sites along the chondroitin sulphate-rich region of the proteoglycan may be masked by glycosaminoglycan side chains. The bovine proteoglycan, then, would have the highest substitution with side chains, whereas rabbit proteoglycans would have the lowest substitution. Alternatively, the antibodies could have a higher affinity for one of the two subpopulations, seemingly present in different proportions in all the samples. The bovine preparations, then, would contain the highest proportion of the low-affinity proteoglycans, whereas the rabbit preparation would contain the highest proportion of the high-affinity proteoglycans.

The absent or weak reaction of the rabbit cartilage proteoglycans with the anti-(hyaluronic acid-binding region) IgG is puzzling, as anti-(bovine cartilage proteoglycan) antibodies react with other substruc-

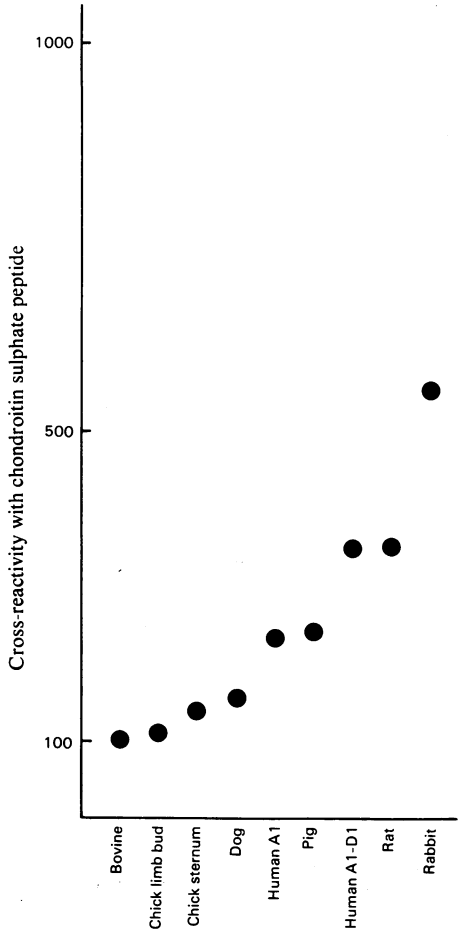

Fig. 6. *Cross-reactivity of chondroitin sulphate peptides isolated from different species*
The inhibition obtained with 1.25 μg of each cartilage proteoglycan preparation/ml relative to that of 1.25 μg of bovine nasal cartilage proteoglycans/ml is indicated. For full experimental details see the text.

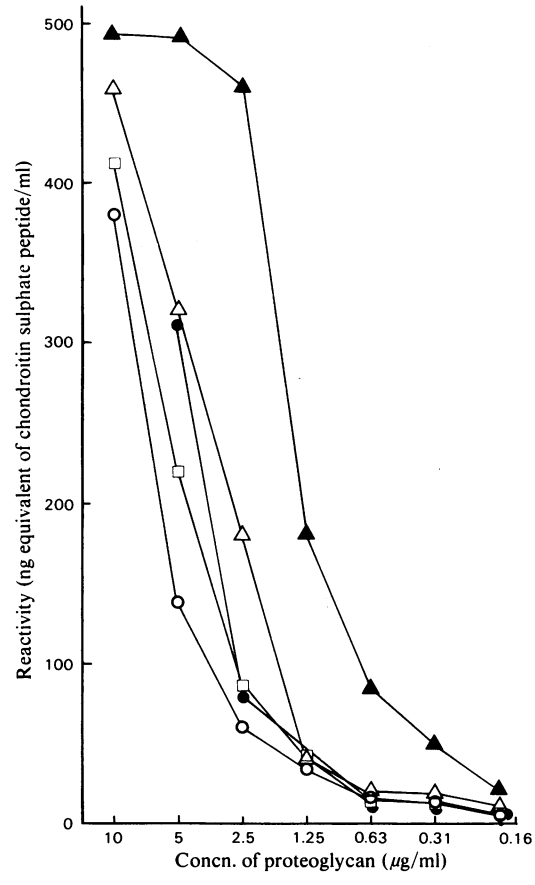

Fig. 7. *Radioimmunoassay of chondroitin sulphate peptides*
The inhibition obtained with increasing concentrations of proteoglycans from bovine (○), human (△), rabbit (▲), chick (□) and dog (●) cartilage is given as the equivalent amount of isolated chondroitin sulphate peptide (A1-T-A1 fraction) from bovine proteoglycans. For full experimental details see the text.

tures in rabbit proteoglycans. It may be that the structure of this portion of the molecule varies more markedly between the species, compared with the structure of the rest of the proteoglycan. In a separate experiment it was shown by Sepharose 2B gel chromatography that rabbit articular-cartilage proteoglycans can aggregate with hyaluronic acid and that this ability is abolished when the proteoglycans are reduced and alkylated (results not shown). The functional properties of the hyaluronic acid-binding region then, are, similar to those of bovine proteoglycans (Heinegård, 1977). Similarly, the chick limb-bud-culture proteoglycans are also able to interact with hyaluronic acid (DeLuca *et al.*, 1977) and could therefore be expected to contain the hyaluronic acid-binding region. The proteoglycans from this source did not react with the anti-(hyaluronic acid-binding region) IgG either. The chick is evolutionarily distant from the cow, and the structure of its hyaluronic acid-binding region may differ from that of the mammals. Minor structural differences between species of the mammals were indicated by different degrees of cross-reactivity.

The structure of the chondroitin sulphate-rich region of the core protein appears to be well conserved, since antibodies to this structure reacted with all species tested.

The antigenic structures reacting with anti-(A1-D1 fraction) IgG may represent antigenic sites of the

hyaluronic acid-binding region and/or the keratan sulphate region. These sites then would also be species- and class-common.

The present investigation illustrates the necessity of using antisera directed against well-defined antigenic sites when cross-reactivities are studied, to prevent cancelling of increased affinity for some sites by decreased affinity for other sites. Several attempts have been made to characterize cartilage proteoglycans immunologically (Baxter & Muir, 1972; Keiser & DeVito, 1974). Antibodies were prepared in rabbits and a number of mammalian species, and were tested for cross-reactivities. Species common antigenic determinants, resistent to trypsin digestion, as well as species-specific antigenic determinants sensitive to trypsin digestion, were demonstrated. Sugahara & Dorfman (1979), in a study of reactivity of chick epiphysial-cartilage and rat chondrosarcoma proteoglycans with antibodies raised in rabbits, demonstrated species-specific antigenic sites sensitive to reduction and species-common sites insensitive to reduction.

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