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Immunochemistry of Cartilage Proteoglycan

IMMUNODIFFUSION AND GEL-ELECTROPHORETIC STUDIES

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Cartilage proteoglycan is thought to be composed of subunits, core proteins with covalently attached sulphated polysaccharide side chains, which form aggregates by non-covalent association with a link protein. The new technique of non-disruptive extraction followed by fractionation in caesium chloride gradients provides a useful means of preparing relatively pure proteoglycan aggregate, subunit and link fractions. Immunological studies of these fractions led to the identification of an antigen associated with the proteoglycan subunit which was common to several species and to the demonstration of additional species-specific antigens in aggregate and link fractions derived from bovine nasal cartilage. Polyacrylamide-gel electrophoresis with sodium dodecyl sulphate of bovine proteoglycan aggregate and link fractions gave two protein bands in the gels and a protein–polysaccharide band at the origin; subunit fractions gave only the band at the origin. These results are consistent with the current concept of cartilage proteoglycan structure.

Most studies of the immunological properties of cartilage proteoglycan have utilized relatively crude material extracted by high-speed homogenization (Malawista & Schubert, 1958; Gerber *et al.*, 1960). The introduction of a non-disruptive extraction procedure (Sajdera & Hascall, 1969), coupled with fractionation by CsCl density-gradient centrifugation (Franek & Dunstone, 1967; Hascall & Sajdera, 1969), has led to the preparation of new and more clearly defined proteoglycan fractions. On the basis of physicochemical data and electron micrographs obtained by using these more highly purified proteoglycan fractions, Rosenberg *et al.* (1970*b*) have modified earlier proposed structural models for bovine nasal cartilage proteoglycan. According to their model, the proteoglycan subunit consists of a linear core protein to which sulphated polysaccharide side chains are covalently attached. In the presence of a link protein, non-covalent association of these subunits occurs, forming star-like arrays of proteoglycan aggregates.

Relatively pure preparations of aggregate, subunit and link protein fractions (Table 1) can be prepared by the method described by Hascall & Sajdera (1969). Proteoglycan components are non-disruptively extracted from cartilage by 4M-guanidine or 3M-$MgCl_2$. Lowering the molarity by dialysis results in the formation of crude proteoglycan complex. On ultracentrifugation of this material in a CsCl density gradient, purified proteoglycan complex sediments to the bottom while contaminating protein (glycoprotein-I) rises to the top. When purified proteoglycan complex is exposed to 4M-guanidine and subjected to repeat CsCl density-gradient ultracentrifugation, the complex separates into proteoglycan subunit, which sediments to the bottom of the gradient, and glycoprotein link fraction, which floats to the top. The addition of small amounts of glycoprotein link fraction to a solution of proteoglycan subunit in 0.5M-guanidine results in reaggregation as shown by an increase in viscosity and characteristic changes on analytical ultracentrifugation.

An alternative method of fractionating cartilage extracts developed by Pal *et al.* (1966) utilizes differential precipitability in various neutral salt solutions. Non-disruptively extracted proteoglycan can be separated into several subfractions (Table 1), principally a PPL3 subunit subfraction and a PPL5 aggregate subfraction, equivalent to though not identical with proteoglycan subunit and proteoglycan complex respectively (Rosenberg *et al.*, 1970*b*).

In the studies reported here, the immunological properties of bovine nasal cartilage proteoglycan components isolated by these new methods were investigated. These studies demonstrated that the proteoglycan subunit contains an antigen which is common to several species, whereas additional antigens, probably species-specific, are associated with link fraction. Polyacrylamide-gel electrophoresis of these proteoglycan components gave further evidence for the presence of proteins in link fraction and aggregates not present in the proteoglycan subunits.

Table 1. *Comparison of proteoglycan fractions isolated by different methods*

Subunits	Link	Aggregates (subunits+link)	Contaminating protein
Proteoglycan subunit*	Glycoprotein link fraction*	Crude proteoglycan complex*	Glycoprotein-I*
Subfraction PPL3†		Proteoglycan complex*	Subfraction PPL6†
		Subfraction PPL5†	
		PP-L fraction‡	

* Prepared by extraction of bovine nasal cartilage with 4M-guanidine or 3M-$MgCl_2$ followed by CsCl density-gradient centrifugation (Hascall & Sajdera, 1969).

† Prepared by extraction of bovine nasal cartilage with 3M-$MgCl_2$ followed by precipitation in neutral salt solutions (Rosenberg *et al.*, 1970*a*).

‡ Prepared by high-speed homogenization of bovine nasal cartilage followed by ultracentrifugation (Gerber *et al.*, 1960).

Materials and Methods

Antigens

PP-L fraction* was derived from bovine nasal cartilage by rapid homogenization in water followed by ultracentrifugation (Malawista & Schubert, 1958; Gerber *et al.*, 1960), and was the same preparation as that used previously in this laboratory (Sandson *et al.*, 1966).

Crude proteoglycan complex, extracted from bovine nasal cartilage with 3M-$MgCl_2$ (Sajdera & Hascall, 1969), and subfractions PPL3 and PPL5, derived from crude proteoglycan complex by the method of Pal *et al.* (1966), were generously provided by Dr. Lawrence Rosenberg.

Proteoglycan complex, glycoprotein-I, proteoglycan subunit and glycoprotein link fraction were derived from crude proteoglycan complex by the method of Hascall & Sajdera (1969). In brief, crude proteoglycan complex was dissolved in 0.5M-guanidine (ultra-pure grade; Mann Research Laboratories, Orangeberg, N.Y., U.S.A.), 0.05M-sodium acetate. CsCl (99% grade; Gallard–Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y., U.S.A.) was added to give a density of 1.69g/ml. The solution was centrifuged at 34000rev./min in a Spinco type 40 angle rotor for 42h at 4°C. The density gradient was fractionated from below with a timed peristaltic pump into five fractions of equal volume, the two bottom fractions containing proteoglycan complex and the top one glycoprotein-I. Additional guanidine was then added to the purified proteoglycan complex to bring the solution to 4M-guanidine. The CsCl concentration was adjusted to give a density of 1.50g/ml and the solution was again centrifuged at 34000rev./min for 42h. Five fractions of equal volume were separated by suction from below with the top fraction containing glycoprotein link fraction and the bottom two fractions containing proteoglycan subunit. The fractions were dialysed against water and freeze-dried before use as antigens.

Human proteoglycan was extracted from human cartilage obtained at autopsy with 4M-guanidine by the method of Sajdera & Hascall (1969).

Chicken proteoglycan was extracted with EDTA from chicken xyphoid cartilage as described by Shulman & Meyer (1970).

Porcine cartilage proteoglycan and rat chrondrosarcoma proteoglycan (Choi *et al.*, 1971) were gifts from Dr. Karl Meyer.

* Abbreviation: PP-L fraction, protein–polysaccharide light fraction.

Immunization

Rabbits were immunized with 5mg (2mg/ml) of proteoglycan components mixed with an equal volume of Freund's complete adjuvant. Booster injections of 2.5mg were given 4 weeks after the start of immunization and approximately every 4 weeks thereafter. Blood was drawn 10–14 days after booster injections, the serum separated, preserved with merthiolate and stored at 4°C. Recently antisera have been obtained with the use of as little as 1mg of proteoglycan antigen by simultaneously injecting 0.25ml of pertussus vaccine (Eli Lilly and Co., Indianapolis, Ind., U.S.A.) at the time of initial immunization (Dresser *et al.*, 1970). Before use antisera were concentrated three times by vacuum ultrafiltration and absorbed with freeze-dried bovine serum until no precipitin line was noted on immunodiffusion against bovine serum. These antisera did not form lines of precipitation on immunodiffusion against hyaluronidase.

Immunoelectrophoresis and immunodiffusion

Immunoelectrophoresis and immunodiffusion were performed in 0.6% agarose at pH8.6 according to standard methods (Grabar & Williams, 1955; Ouchterlony, 1961). Antigen concentration was

10mg/ml except for glycoprotein link fraction, which was used at 5mg/ml. All antigens were digested with testicular hyaluronidase before use in these systems.

Enzymic digestion

A 10mg portion of proteoglycan component was dissolved in 0.9ml of water. To this was added 0.1ml of 0.1M-acetate buffer, pH5, containing 750 U.S.P. units of bovine testicular hyaluronidase (Worthington HSEA; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) and the solution incubated at 37°C for 18h.

Polyacrylamide-gel electrophoresis

Electrophoresis in basic 5% (w/v) polyacrylamide gels in 0.035M-tris–glycine buffer, pH8.8, and in neutral 5% (w/v) polyacrylamide gels with 0.1% sodium dodecyl sulphate in 0.01M-phosphate buffer, pH6.8, was performed by using 10cm analytical gels according to standard procedures (Maizel, 1969). Samples of proteoglycan fractions (1–2mg) or of glycoprotein link fraction (0.1–0.2mg) in buffer and 1% sodium dodecyl sulphate were heated in a boiling-water bath for 1min before application to the sodium dodecyl sulphate gels in 10% sucrose solutions.

Results

Immunization of rabbits with crude proteoglycan complex, subfraction PPL3 and glycoprotein link fraction resulted in the production of antibodies demonstrable by double immunodiffusion in agarose against hyaluronidase-digested proteoglycan components.

Antiserum to subfraction PPL3 gave a single line of complete fusion on diffusion against subfraction PPL3, subfraction PPL5, proteoglycan subunit, crude proteoglycan complex, proteoglycan complex and glycoprotein link fraction (Fig. 1*a*). Proteoglycan preparations from human, chicken and porcine cartilage and from a rat chondrosarcoma were found to react with the anti-(subfraction PPL3) serum forming single lines that fused with the single lines formed against bovine proteoglycan components (Fig. 1*b*).

Antiserum to crude proteoglycan complex that had reacted against crude proteoglycan complex gave at least three precipitin lines (Fig. 2). The line nearest the antibody well fused completely with the lines formed by the reaction of this antiserum with subfraction PPL3, proteoglycan subunit, subfraction PPL5, glycoprotein link fraction, proteoglycan complex and with proteoglycan preparations from other species. A second precipitin line of fusion was demonstrated on the diffusion of this antiserum against proteoglycan complex, subfraction PPL5 and glycoprotein link fraction but not against subfraction PPL3, proteoglycan subunit or non-bovine proteoglycans.

The antiserum prepared to glycoprotein link fraction resembled anti-(crude proteoglycan complex) in its reactivity to proteoglycan components. As shown in Fig. 3(*a*), a precipitin line of fusion near the antibody well formed on reaction against crude and purified proteoglycan complex, glycoprotein link

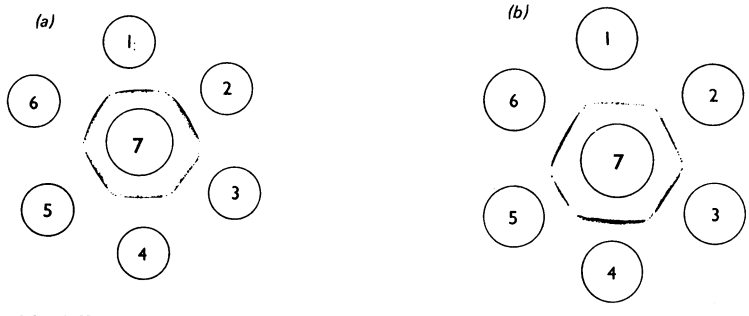

Fig. 1. *Double diffusion in agarose with an antiserum to a bovine proteoglycan subunit fraction*

Both centre wells contained antiserum to a proteoglycan subunit fraction derived from bovine nasal cartilage. (*a*) The circumferential wells contained the indicated hyaluronidase-digested proteoglycan fractions derived by various methods from bovine nasal cartilage (see Table 1). 1, Subfraction PPL3; 2, proteoglycan subunit; 3, crude proteoglycan complex; 4, proteoglycan complex; 5, glycoprotein link fraction; 6, subfraction PPL5; 7, anti-(solution PPL3) serum. (*b*) The circumferential wells contained hyaluronidase-digested proteoglycan preparations from the indicated species. 1, Human proteoglycan complex; 2, chicken proteoglycan; 3, pig proteoglycan; 4, bovine proteoglycan subunit; 5, rat chondrosarcoma proteoglycan; 6, bovine proteoglycan complex; 7, anti-(subfraction PPL3) serum.

fraction, subfraction PPL5, subfraction PPL3 and proteoglycan subunit. Additional common lines formed on reaction with crude and purified proteoglycan complex, glycoprotein link fraction and inconsistently with subfraction PPL5. When anti-(glycoprotein link fraction) serum was absorbed with proteoglycan subunit (5mg/ml), the line nearest the antibody well was abolished with two additional precipitin lines persisting (Fig. 3*b*).

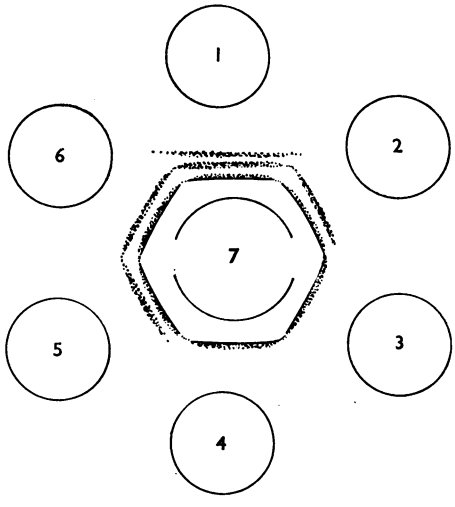

Fig. 2. *Double diffusion in agarose with antiserum to a bovine proteoglycan aggregate fraction*

The centre well (7) contained antiserum to crude proteoglycan complex extracted from bovine nasal cartilage with $MgCl_2$. The circumferential wells contained the indicated hyaluronidase-digested proteoglycan fractions (see Table 1). 1, Crude proteoglycan complex; 2, subfraction PPL5; 3, subfraction PPL3; 4, proteoglycan subunit; 5, glycoprotein link fraction; 6, proteoglycan complex; 7, anti-(crude proteoglycan complex) serum.

Anti-(glycoprotein link fraction) serum was also tested by immunodiffusion against PP-L fraction, a cartilage homogenate proteoglycan preparation used in earlier studies from this laboratory (Sandson *et al.*, 1966). PP-L fraction gave at least two precipitin lines with anti-(glycoprotein link fraction) serum (Fig. 4); the line near the antibody well fused with the line formed against proteoglycan subunit and the other fused with lines against proteoglycan complex and glycoprotein link fraction.

Immunoelectrophoresis of subfraction PPL5, subfraction PPL3 and crude proteoglycan complex with anti-(crude proteoglycan complex) serum gave a rapidly migrating arc near the anode in each case; two additional precipitin arcs of slower mobility were noted with crude proteoglycan complex (Fig. 5*a*). Immunoelectrophoresis with anti-(glycoprotein link fraction) serum gave a rapidly migrating arc near the anode with both proteoglycan subunit and glycoprotein link fraction and a second more slowly migrating arc with glycoprotein link fraction only (Fig. 5*b*).

Bovine cartilage proteoglycan fractions were subjected to polyacrylamide-gel electrophoresis followed by staining with Coomassie Blue or Toluidine Blue. After electrophoresis of proteoglycan complex, proteoglycan subunit, glycoprotein link fraction, subfraction PPL3 and subfraction PPL5 in basic 5% polyacrylamide gels and staining with Coomassie

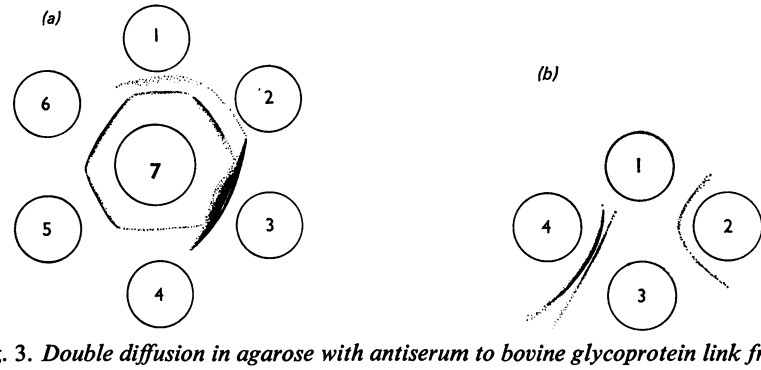

Fig. 3. *Double diffusion in agarose with antiserum to bovine glycoprotein link fraction*

(*a*) The centre well (7) contained antiserum to glycoprotein link fraction derived from bovine nasal cartilage. The circumferential wells contained the indicated hyaluronidase-digested proteoglycan fractions (see Table 1). 1, Crude proteoglycan complex; 2, proteoglycan complex; 3, glycoprotein link fraction; 4, subfraction PPL5; 5, subfraction PPL3; 6, proteoglycan subunit. (*b*) Well no. 1 contained hyaluronidase-digested proteoglycan subunit fraction and well no. 3 contained hyaluronidase-digested glycoprotein link fraction. Well no. 2 contained anti-(subfraction PPL3) serum and well no. 4 contained anti-(glycoprotein link fraction) serum that had been absorbed with proteoglycan subunit fraction (5mg/ml).

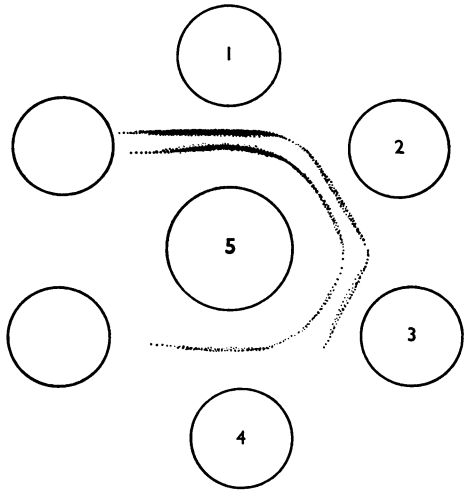

Fig. 4. *Double diffusion in agarose with antiserum to bovine glycoprotein link fraction*

The centre well (5) contained antiserum to link fraction and the circumferential wells contained the indicated hyaluronidase-digested antigens (see Table 1). 1, Proteoglycan complex; 2, PP-L fraction; 3, glycoprotein link fraction; 4, proteoglycan subunit.

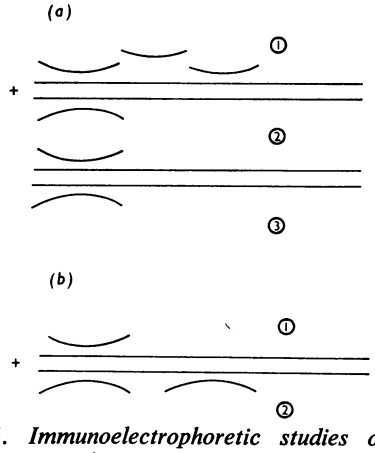

Fig. 5. *Immunoelectrophoretic studies of bovine cartilage proteoglycan fractions*

(*a*) Both troughs contained antiserum to crude bovine proteoglycan complex and the wells contained hyaluronidase-digested proteoglycan fractions (see Table 1). 1, Crude proteoglycan complex; 2, subfraction PPL5; 3, subfraction PPL3. (*b*) The trough contained antiserum to bovine glycoprotein link fraction and the wells contained hyaluronidase-digested proteoglycan fractions (see Table 1). 1, Proteoglycan subunit; 2, glycoprotein link fraction.

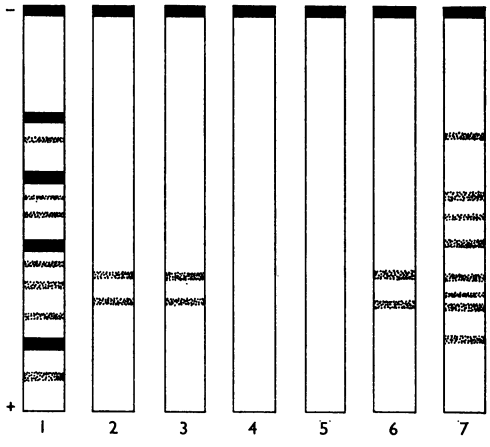

Fig. 6. *Composite diagram of gel-electrophoresis studies of bovine cartilage proteoglycan fractions*

The indicated proteoglycan fractions (see Table 1) were subjected to electrophoresis in 5% (w/v) polyacrylamide gels at neutral pH with sodium dodecyl sulphate and the gels were stained for protein with Coomassie Blue. The origin (cathode) is shown at the top and the location of the protein bands depicted has been corrected to account for the distance travelled by the tracking dye (Bromophenol Blue) in that gel. 1, Glycoprotein-I; 2, proteoglycan complex; 3, glycoprotein link fraction; 4, proteoglycan subunit; 5, subfraction PPL3; 6, subfraction PPL5; 7, PP-L fraction.

Blue, protein was demonstrated only at the gel origin. The gel origin was also stained metachromatically by Toluidine Blue. Electrophoresis in neutral 5% polyacrylamide gels with sodium dodecyl sulphate followed by staining with Coomassie Blue yielded a protein band at the gel origin with all the proteoglycan preparations tested. Glycoprotein link fraction, proteoglycan complex and subfraction PPL5, but not proteoglycan subunit or subfraction PPL3, showed two additional protein bands within the sodium dodecyl sulphate gels, at about two-thirds and three-quarters the distance travelled by the tracking dye (Fig. 6). These bands did not stain with Toluidine Blue although the material that remained at the origin stained metachromatically. Glycoprotein-I, the fraction containing material excluded from proteoglycan complex by the initial CsCl density gradient, and PP-L fraction both gave numerous protein bands on polyacrylamide-gel electrophoresis in sodium dodecyl sulphate (Fig. 6). Two of the bands found with PP-L fraction corresponded in mobility to the two bands found with glycoprotein link fraction, proteoglycan complex and subfraction PPL5.

Discussion

Less than a decade ago the anionic polysaccharides of connective tissue were considered to be non-antigenic (Boake & Muir, 1955). Once it became clear that these anionic polysaccharides were not free in tissue but were firmly bound to non-collagenous protein (Malawista & Schubert, 1958), the question of their antigenicity was restudied. The immunogenicity of intact proteoglycan was then demonstrated in several laboratories (Saunders *et al.*, 1962; White *et al.*, 1963; Di Ferrante, 1963; Loewi & Muir, 1965). Chondroitin sulphate free of protein was found to be non-antigenic and digestion of proteoglycan with testicular hyaluronidase to remove much of its chondroitin sulphate was shown to facilitate the demonstration of its antigenic determinants by immunodiffusion and haemagglutination (Sandson *et al.*, 1966; Loewi & Muir, 1965).

In an earlier report from this laboratory, PP-L fraction, derived from cartilage homogenates by ultracentrifugation, was shown to have at least two antigenic determinants; one was common to all species studied and had a rapid mobility on immunoelectrophoresis, whereas the second was species-specific and had a slower immunoelectrophoretic mobility (Sandson *et al.*, 1966). Further studies with doublet chains derived from cartilage proteoglycan by exhaustive digestion with trypsin and chymotrypsin (Mathews, 1968), suggested that the common antigenic determinant was associated with a site near the juncture of the polysaccharide chains to the core protein (Sandson *et al.*, 1970).

The only previously published immunological study of proteoglycan components derived from cartilage by the method of Hascall & Sajdera (1969) is the preliminary report by Di Ferrante *et al.* (1970). These investigators coated glutaraldehyde-fixed erythrocytes with bovine proteoglycan subunit and glycoprotein link fraction and used antisera to disruptively prepared bovine and human PP-L fraction in haemagglutination and haemagglutination-inhibition assays. On the basis of their results they could conclude only that the glycoprotein link fraction contained most of the species-specific antigenic determinants whereas the proteoglycan subunits contained most of the cross-reacting ones.

The results of the present investigation serve to clarify these previous reports and to correlate the immunological properties of proteoglycan fractions prepared by various methods with the recently proposed structural model (Rosenberg *et al.*, 1970*b*). Precipitating antibodies were obtained by immunization of rabbits with bovine cartilage proteoglycan aggregate, subunit and link fractions. By means of these antisera it was possible to identify an antigenic component in bovine subunit preparations with rapid mobility on immunoelectrophoresis which was also found in human, porcine, chicken and rat proteoglycan. Bovine proteoglycan aggregate and link fractions were shown to have antigenic components in addition to the subunit-associated antigenic component which characteristically formed the precipitin line nearest the antibody well on immunodiffusion. These bovine link-associated antigens had slower mobility on immunoelectrophoresis and were not identified in proteoglycan preparations from the four other species tested. The subunit- or core-protein-associated antigen appears to be identical with the previously described fast determinant of PP-L fraction, whereas the slow species-specific determinant of PP-L fraction appears to be associated with the link fraction (Sandson *et al.*, 1966).

Polyacrylamide-gel electrophoresis with sodium dodecyl sulphate provided further evidence for the presence of proteins in proteoglycan aggregates and in link fraction which are not present in subunits. The anionic detergent sodium dodecyl sulphate is capable of dissociating a wide variety of non-covalently linked complexes and in combination with polyacrylamide-gel electrophoresis it forms a powerful tool for the separation and identification of components of macromolecular aggregates (Maizel, 1969). By using this system, cartilage proteoglycan aggregate and link fractions were shown to contain two proteins that entered sodium dodecyl sulphate gels, in addition to the metachromatically staining material found at the origin with all proteoglycan fractions. It is likely that one or both of these proteins is related to the proposed link protein (Hascall & Sajdera, 1969; Rosenberg *et al.*, 1970*b*), but isolation and characterization of these proteins is necessary to establish this point.

The results of polyacrylamide-gel electrophoresis also indicate that the proteoglycan fractions prepared by the CsCl density-gradient method are much more pure than those produced by previous techniques. Purified proteoglycan complex and glycoprotein link fraction gave only two protein bands on polyacrylamide-gel electrophoresis with sodium dodecyl sulphate whereas glycoprotein-I, the excluded top fraction of the initial density gradient used to prepare proteoglycan complex, gave at least 11 bands, and PP-L fraction, prepared from cartilage homogenate by an earlier ultracentrifugation procedure (Gerber *et al.*, 1960), gave at least eight bands.

The finding of material that stained metachromatically at the polyacrylamide-gel origin and the demonstration of the subunit-associated antigen on immunodiffusion and immunoelectrophoresis provide evidence for the presence of proteoglycan subunit in glycoprotein link fraction. It is not clear whether the presence of subunit in the glycoprotein link fraction represents contamination due to overloading of the CsCl density gradient or whether a relatively small number of proteoglycan subunits

remain attached to the link protein even in 4M-guanidine.

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