Discussion. When the lipid and protein components of lipofuscin granules are removed, a black insoluble residue remains which has the same chemical and spectral characteristics of known melanin isolated from other sources[6]. The quantity of melanin isolated from normal hearts and livers and those with brown atrophy correlated well with the microscopic counts of lipofuscin granules in these tissues. This suggests that lipofuscin pigment contains a melanin component.

Strehler et al.[10] observed by histochemical studies, that the number of lipofuscin granules in the heart increased with age. In our study, although the melanin pigment in control hearts and livers appeared to increase during the first 3 decades of life, the overall correlation of melanin pigment with age was not significant in either the heart or the liver. Melanin is absent in fetal liver and heart, decreased in fatty metamorphosis of the liver and increased in brown atrophy of the heart and liver. The absence of lipofuscin in fetal organs has been previously reported[11] and suggests that an adequate period of time is necessary for the synthesis of this pigment. In fatty metamorphosis there is liver cell destruction and increased cellular turnover. The newly formed cells may also not have had sufficient time to synthesize lipofuscin granules which could account for our observation of decreased melanin in livers with fatty metamorphosis. On the other hand, in brown atrophy, there is a marked increase in the lipofuscin granule count as well as melanin concentration. Large quantities of melanin pigment also have been found in the black livers obtained from mutant Corriedale sheep[12–14], Howler monkeys[15,16] and patients with Dubin-Johnson syndrome[12,14]. Further studies are needed to determine the pathogenesis and function of this visceral pigmentation.

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The effects of acetylation on the binding region of cartilage proteoglycans to hyaluronic acid[1]

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Summary. Proteoglycans in cartilage are found as aggregates and as monomers. Evidence has been obtained indicating that hyaluronic acid, normally present in this tissue, binds together monomers into large molecular weight aggregates. In this investigation, the interacting region of the protein backbone has been studied. The results unequivocally demonstrated that the epsilon amino groups of lysine are involved in hyaluronic acid binding to proteoglycans and that their blocking by acetylation either prevents reaggregation or disaggregates the high mol.w aggregates.

The chondroitinsulfate proteoglycans are characteristic components which occur in cartilage as aggregates of a mol.wt ranging from 30×10^6 to 100×10^6 dalton. These aggregates are made up by proteoglycan monomers of an average mol.wt of 2 to 2.5×10^6 dalton, by 2 link proteins[2] and by hyaluronic acid[3]. Recently, evidence has been obtained demonstrating that the main function of hyaluronic acid consists of binding together proteoglycan monomers into aggregates of large molecular size[3]. This interaction appears, therefore, to be of fundamental importance for aggregation and thus for the organization of proteoglycans in cartilage extracellular matrix. Certain features of this phenomenon are now known in some detail, e.g. the general position of the interacting side(s) in the polysaccharide-free region of the core protein of the monomer[4], the minimum size of a hyaluronic acid segment capable of interacting with the core protein[5], and the function of the link proteins which do not promote aggregate formation per se, but they seem to stabilize the whole molecular system, preventing its dissociation under the stress of ultracentrifugal forces[6].

The present experiments were devised to study the chemical characteristics of the hyaluronic acid-proteoglycan interaction, and attempts are made to identify the sites on the protein backbone of the proteoglycan involved in the linkage with hyaluronic acid.

Materials and methods. Viscosimetric and chromatographic analyses of the A_1 preparations demonstrated that the sample were high molecular weight compounds (fig. 1a) and that about 50% of the material was excluded from the gel suggesting that a large proportion was in an aggregated form (fig. 2-1). On acetylation, the drastic drop in viscosity of these samples, indicating a decrease in hydrodynamic size of the proteoglycans and their chromatographic behaviour on Sepharose 2B, demonstrates that they were all eluted in the included volume and provides evidence for their disaggregation (figs. 1b and 2-2. In this regard, it is interesting to note that the size of the acetylated samples corresponded to that of the A_1D_1 preparations (figs. 1c and 2–4) and was also similar to the A_1

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samples digested with Streptomyces hyaluronidase (fig. 2–3). This enzyme, by degrading HA, determined the disaggregation of the complex to proteoglycan subunits, the size of which was similar to the A_1D_1 preparations used in this study (fig. 2–4). This data suggested that the products obtained after acetylation were the expression of a specific chemical reaction.

Reaggregation of A_1D_1 occurred readily upon addition of HA as indicated by viscosity measurements and gel chromatography (figs. 1d and 2–5). However, when these subunits were first acetylated and then combined with HA, reaggregation did not take place (figs. 1f and 2–6). These findings strongly suggest that the region on the protein in the proteoglycan molecule responsible for hyaluronic acid binding is rich in lysine residues and that blocking of their positively-charged groups prevents aggregation. Although the lack of interaction between glucuronic carboxyls and the protonated amino side chains may suggest that Coulombic forces are involved no definite information is thus far available. The other possibility, that upon acetylation major conformational changes may lead either to disaggregation of the large molecular weight complex or prevent proteoglycan subunits to be bound into aggregates, remains to be demonstrated.

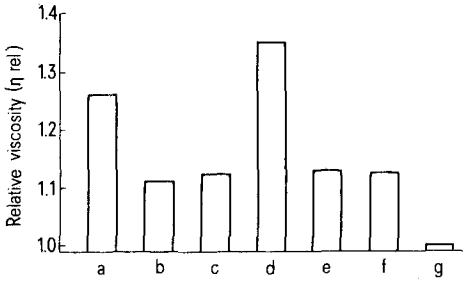

Fig. 1. Viscosimetric analyses of bovine A_1 and A_1D_1 preparations. Samples (1.0 mg/ml) were dissolved in 0.05 M Na-acetate, pH 5.8 containing 0.5 M GuHCl. The variations of relative viscosity were recorded at 23 °C.
a A_1 (aggregate), *b* acetylated A_1, *c* A_1D_1 (subunit), *d* A_1D_1 mixed with 0.6% (w/w) of hyaluronic acid, *e* acetylated A_1D_1, *f* acetylated A_1D_1 and then mixed with 0.6% (w/w) of hyaluronic acid, *g* relative viscosity of the solvent.

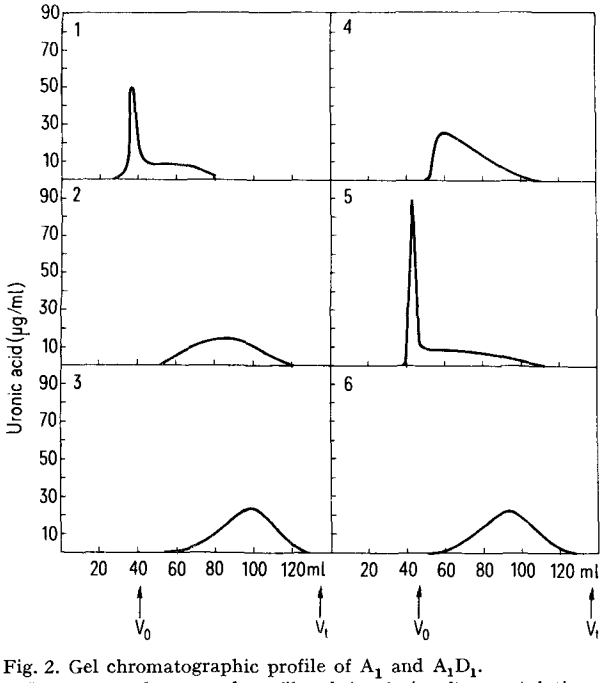

Fig. 2. Gel chromatographic profile of A_1 and A_1D_1.
1 Represents the normal profile of A_1, *2* A_1 after acetylation, *3* A_1 preparation after digestion with Streptomyces hyaluronidase, *4* A_1D_1, *5* A_1D_1 mixed with 0.6% HA (w/w), *6* acetylated A_1D_1 with 0.6% HA (w/w).

Results and discussion. Aggregates (A_1)[7] and monomers (A_1D_1) were extracted from bovine nasal cartilage while hyaluronic acid (HA) was a sample obtained from a human mesothelioma.

The hexuronic content of these preparations, calculated as glucuronate, was 24% for the A_1 30.6% for the A_1D_1 and 34% for HA. Streptomyces hyaluronidase, obtained from Amano Pharmaceutical Co., was used as described previously[8]. In order to study the sites on the protein backbone of the monomer, the epsilon-amino side chains of A_1 and A_1D_1 were acetylated. 20 mg of these preparations were dissolved in a cold, half-saturated solution of Na-acetate (pH ca. 7.5), acetic anhydride was added to a final concentration of 3.1 μl/mg and acetylation was carried out at 4 °C under continuous stirring. The anhydride was added to the solution every 20 min over a period of 1 h, the pH was controlled throughout the reaction with 2.0 N NaOH. Controls were treated like the experimental except that they were not acetylated. The samples were then dialyzed against 0.05 M Na-acetate, pH 5.8 containing 0.5 M GuHcl (Eastman Organic Chemicals).

Reaggregation of proteoglycan monomers was obtained using A_1D_1 (1.5 mg/ml) dissolved in 0.05 M Na-acetate buffer pH 5.8 containing 0.5 M GuHcl. This preparation was combined with 0.6% (w/w) of hyaluronic acid and mixed under constant stirring in the cold. Viscosity measurements were carried out on the control on acetylated A_1 and A_1D_1 preparations and on A_1D_1 combined with hyaluronic acid. Relative viscosity was measured in a Cannon Ubbelohde dilution viscosimeter and was established from the outflow times and from the density of the solutions with the relationship:

$$\text{rel}\,\eta = \frac{\eta}{\eta_0} = \frac{t \cdot \varrho}{t_0 \varrho_0}$$

where η is the viscosity of the solute, η_0 the viscosity of the solvent, t the outflow time of the solution and t_0 the outflow of the solvent, ϱ indicates the density of the solute and ϱ_0 the density of the solvent. The loading concentration of the samples was 1.5 mg/ml, which were filtered into the viscosimeter and viscosity taken after equilibration of the solutions at 23 °C. In a separate experiment, the aggregate was digested with Streptomyces hyaluronidase and 3 mg of this preparation were dissolved in 0.05 M Na-acetate buffer, pH 5.0, and digested for 6 h at 38 °C, using an enzyme to substrate ratio of 3 TRU/mg. Boiled enzyme preparations were made as controls prior to incubation of the mixtures. Column chromatography on normal samples, hyaluronidase-digested preparations, and control and acetylated A_1 and A_1D_1 was also carried out. Samples were dissolved in 0.05 M Na-acetate buffer pH 6.8 at a concentration of 1 mg/ml, and 3 ml were eluted in the cold through Sepharose 2 B column (68 × 1.6 cm). The flow rate was 4 ml per h, 2 ml fractions were collected and their content analyzed for hexuronic acid by the carbazol method of Bitter and Muir[9].

7 Abbreviations. A_1 proteoglycan aggregates; A_1D_1, proteoglycan subunits or monomers; HA, hyaluronic acid; GuHcl, guanidinium hydrochloride; TRU, turbidimetric units.

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