munological mechanism. If antibody is assumed, our results would suggest that tolerance is a host response to an antigen-antibody interaction, a circumstance which has not been demonstrated for any endotoxic activity.

Summary. Rabbits given volumes of endotoxin-tolerant donor plasma too small to modify significantly the course of fever produced by a first test dose of endotoxin injected shortly thereafter, display a remarkable degree of tolerance to a larger dose of endotoxin given 24 hours later, compared to identically endotoxin-treated controls. The results are inconsistent with hypotheses invoking any direct effect of the humoral mediator upon the endotoxin.

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Protein-Polysaccharide Complexes of Normal and Herniated Human Intervertebral Discs.* (28984)

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Changes in the chemical composition of the intervertebral disc with aging or prolapse have been studied by a number of investigators(1,2,3,4). They reported the following changes with increasing age: a decrease in total polysaccharide content, an increase in collagen content and an increase in ratio of keratosulfate to chondroitin sulfate-C. It has also been shown that, in rabbits, growth hormones, androgens and estrogens are able to alter the composition of mature tissue toward that representative of a younger age.

Very little work has been published on the chemical and physical properties of the mucopolysaccharide-protein complexes from either the nucleus pulposus or annulus fibrosus of the human intervertebral disc, despite the fact that this is very probably the form in which these compounds exist *in vivo*. This paper presents results of a study of some of the chemical and physical properties of these complexes and their changes with increasing age and in disc prolapse.

Methods. Material for the "normal" specimens was obtained from subjects of ages 15 to 88 years, who were autopsied within 6 hours after death. In each case a large section of the vertebral column was removed and immediately frozen at the temperature of dry ice. When frozen solid, the bone was removed from the disc with an electric band saw, the material from the disc placed in a polyethylene bag and stored over dry ice until ready for extraction. Samples of herniated disc were obtained at surgery and frozen immediately in dry ice until ready for extraction.

The annulus fibrosus and nucleus pulposus were separated and extracted separately. Thin sections were freeze-dried and weighed. The freeze-dried tissue was minced and extracted at 0 to 5°C with water for 1.5 hours in a Virtis "23" homogenizer running at half speed. Soluble collagen was eliminated by precipitation with 2 volumes of ethanol in the

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presence of Celite as described by Partridge *et al*(5). The complex was then precipitated by addition of 3.5 g of sodium acetate per 100 ml of solution and the precipitate was washed with 80% ethanol. This precipitate, referred to below as "crude complex" was sometimes isolated by dissolving in the minimum amount of water, centrifuging to remove insolubles, dialyzing 4 hours against running water and freeze drying. In other cases, a "purified complex" was obtained by dissolving the crude complex in a minimum amount of 2 M NaCl solution containing an excess of cetyl trimethylammonium bromide (CTB), centrifuging to remove insolubles, followed by 8-fold dilution with water to precipitate the CTB-polyanion complex. The precipitated complex was washed with water and then dissolved in 3 M NaCl from which the protein-polysaccharide complex was precipitated as the sodium salt by addition of 3 volumes of methanol. After suitable washing with 80% methanol, the complex was dissolved in water, dialyzed 4 hours against running water and then freeze dried.

The Limiting Viscosity Number (LVN) (ml/g) at zero shear rate was determined at 25.0°C. A Cannon-Ubbelohde semi-micro 4 bulb variable rate of shear dilution viscometer having shear rates for water of approximately 1200, 800, 400 and 200 sec^{-1} was used. All solutions were made up in a pH 7.0 phosphate buffer which was adjusted to an ionic strength of 0.4 with sodium chloride. The viscosity of at least 4 concentrations for each sample at the 4 rates of shear was determined. Extrapolation to zero concentration and zero shear rate was done by the procedure of Mathews and Lozaityte(6).

Hexosamines were determined by the method of Elson and Morgan(7) modified by Boas(8). The identity of the isolated hexosamines was determined by the ninhydrin degradation method of Stoffyn and Jeanloz(9). Uronic acids were determined by both the carbazole(10) and orcinol(11) procedures using glucuronolactone as a standard. Glucosamine and galactosamine were quantitatively determined in mixtures by the Gardell(12) procedure. Hydroxyproline was determined by the method of Newman and Logan(13) and nitrogen by the micro Kjeldahl method. Paper electrophoresis and zone electrophoresis were carried out by the procedure of Dyrbe, Kirk and Wang(14) and Hamerman and Sandson(15) respectively.

Results. Crude Complex-Samples of "crude complex" obtained from both the nucleus pulposi and annulus fibrosi taken at 15 autopsies of individuals ranging in age from 32 to 80 years were analyzed. The nitrogen content showed a gradual increase with age, ranging from 5.5% at age 32 to 11.3% at age 80. The uronic acid (carbazole method) content ranged from 5.1% to 12.1% while hexosamine content ranged from 11.2% to 25.7%. The ratio of uronic acid to hexosamine in the individual samples ranged from 1.0:1.9 to 1.0:2.9 showing a general increase with increasing age, indicative of the increasing ratio of keratosulfate to chondroitin sulfate-C with age as reported by others(3). This is further indicated by the fact that when a number of the samples were analyzed for individual hexosamine content, a ratio of galactosamine to glucosamine was obtained which corresponded to the ratio of one mole of galactosamine per mole of uronic acid with the excess hexosamine being accounted for by glucosamine. The maximum hydroxyproline content in any of these preparations was less than 0.3%, indicating the absence of all but very small amounts of collagen. The yield of "crude complex" ranged from 22.5% to 48.3% based on dry weight of nucleus pulposus or annulus fibrosus. Qualitatively similar results were obtained for the crude complex derived from the nucleus or annulus of each specimen.

Purefied Complex-Analytical results on 13 samples of purified complex from nucleus pulposi derived from "normal" autopsies on subjects of various ages are given in Table I. The ratio of uronic acid to hexosamine is quite constant at a value of approximately 1.0:2.0 which would indicate a one-to-one ratio of chondroitin sulfate-C to keratosulfate. This is further substantiated by a 1.0:1.0 ratio of glucosamine to galactosamine which is found in 6 random samples which

TABLE I. Analytical Data—Purified Complex—Na Salt—"Normal" Specimens.

Nucleus

Age	Sex	% yield*	% uronic acid		% hexosamine	% nitrogen
			Carbazole	Orcinol		
15	♂	35.5	13.8	14.0	27.7	5.3
21	♂	16.0	9.3	9.1	19.0	4.8
32	♂	18.8	10.9	10.8	20.1	6.5
39	♀	48.3	7.0	7.0	13.7	7.0
45	♂	20.9	11.4	11.8	22.4	7.0
62	♂	34.4	10.5	10.6	21.3	5.6
63	♀	33.1	7.7	7.8	15.3	8.0
64	♀	18.7	10.1	10.1	20.6	7.1
73	♀	30.8	6.2	6.3	12.7	6.9
74	♀	31.1	8.5	8.5	16.9	5.6
85	♂	20.2	10.4	10.7	20.4	6.8
86	♂	16.0	12.3	12.4	25.0	6.1
88	♂	29.1	12.8	12.6	25.9	8.0

* Based on dry weight of nucleus.

were analyzed, and the failure of paper chromatographic examination to reveal any other sugar derivative than galactose. Hydroxyproline contents of these complexes were all less than 0.2% indicating minimum collagen contamination. Qualitatively similar analytical results were obtained on purified complexes derived from the annulus fibrosi of the same samples.

A number of experiments were performed to determine whether the purified complex was in reality a mixture of keratosulfate-protein complexes with chondroitin sulfate-C protein complexes and to study the stability of the protein-polysaccharide linkage. It was found that after prior splitting of the complex with strong base or proteolytic enzymes, both keratosulfate (KS) and chondroitin sulfate-C (CS-C) could be eluted separately from a cellulose column saturated with 80% ethanol containing 0.3% barium acetate(16). The KS was eluted with 40% ethanol while the CS-C was eluted with 10% ethanol. The original unsplit complex had no material eluted with 40% ethanol while 10% ethanol eluted the complex with a uronic acid to hexosamine ratio of 1.0:2.0.

Zone electrophoresis was carried out at a variety of pH values and ionic strengths on a polyvinyl chloride bed followed by elution of various segments of the bed with water following the general procedure of Hamerman and Sandson(15). Analysis of the eluted material for uronic acids and hexosamines at pH values ranging from 4.5 to 8.6 and at ionic strengths between 0.0375 and 0.15 showed a uniform ratio of approximately 1.0:2.0 in all segments, although the complex migrated as a broad band, probably indicating considerable polydispersity. When the complex had been split with 0.5 N NaOH at room temperature for 40 hours almost complete separation of the KS and the CS-C could be obtained. On paper electrophoresis (14) using a pH 8.6 veronal buffer unsplit complex gave a diffuse metachromatic staining band with toluidine blue while complex split with NaOH as above gave a sharp metachromatic staining band which was largely CS-C followed by a KS band which did not stain but was detected by elution of the paper strips and subsequent analysis.

Purified complexes were prepared from nuclei pulposi of 8 specimens of herniated disc removed at surgery. Analytical data for these complexes are shown in Table II. Although the ratio of uronic acid to hexosamine is still about 1.0:2.0, the nitrogen content markedly elevated when compared to normal samples of the same age group and in general the polysaccharide content of the herniated samples is lower than the normal samples.

The values obtained for the limiting viscosity number of the purified complexes derived from both normal and herniated specimens of nuclei pulposi are shown as a function of age in Fig. 1. A similar plot of LVN *vs* age for complex derived from annulus of normal subjects was obtained. There appears

TABLE II. Analytical Data—Purified Complex—Na Salt—Herniated Specimens.

Nucleus

Age	Sex	% yield*	% uronic acid		% hexosamine	% nitrogen
			Carbazole	Orcinol		
25	♀	17.5	6.8	6.7	14.3	11.7
27	♂	10.2	8.0	9.6	16.0	11.1
37	♂	7.7	9.2	9.7	18.8	10.1
38	♂	—	6.4	7.4	12.9	8.7
43	♀	9.9	12.0	13.0	24.4	10.6
48	♀	12.7	6.5	6.7	13.1	12.0
55	♂	6.8	4.7	4.8	9.6	12.0
59	♂	5.7	4.6	4.8	9.5	12.0

* Based on dry weight of nucleus.

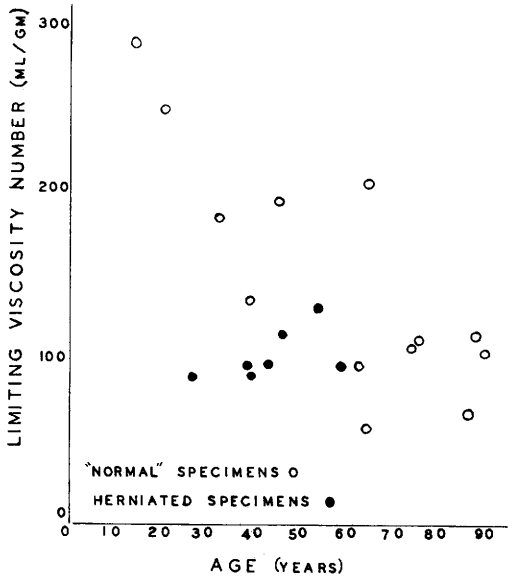

FIG. 1. Variation of limiting viscosity number of purified complex from nucleus pulposus with age.

to be a definite decrease in viscosity with increasing age as part of the normal aging pattern. There is a marked decrease in LVN for complex from herniated discs from subjects under the age of 50 as compared to LVN values for complexes derived from normal discs of the same age group.

Insoluble complexes. In normal subjects of age 60 or greater some insoluble material which carried along throughout the entire purification procedure but which would not dissolve in water in the final step in the process, was isolated. This material was absent in all normal subjects below the age of 60. The same type of insoluble material was found in all samples of herniated disc examined from patients in the 25 to 60 age group. Analytical values for this insoluble material are given in Tables III and IV. The presence of appreciable amounts of chondroitin sulfate-B (CS-B) is suggested by the discrepancy in the orcinol and carbazole values for the uronic acid content since iduronic acid is known to give low carbazole yields.

Discussion. The electrophoretic and chromatographic experiments indicate that the bonding between the polysaccharide and protein components of the complex is quite stable and apparently of the same covalent nature as in an analogous complex from cartilage. The constancy of the 1:1 ratio of CS-C and KS in the purified complex at all ages is quite surprising in view of the fact that the complex from cartilage contains only small amounts of KS, and the KS:CS-C ratio increases with age in crude preparations. The possibility of both KS and CS-C chains being substituted on the same protein core has been suggested by Partridge(5) for a complex derived from bovine cartilage, and the experimental results on the purified complex would be concordant with such a structure.

Evidence has been presented which indicates that herniation of the intervertebral disc may be a pathological acceleration of the normal aging pattern of this tissue. One of the characteristics of the aging of both nucleus pulposus and annulus fibrosus has

TABLE III. Analytical Data — "Insolubles" — "Normal" Specimens.

Nucleus

Age	Sex	% yield*	% uronic acid: Carbazole	% uronic acid: Orcinol	% hexosamine	% nitrogen
15	♂	No insoluble material				
21	♂	"	"	"		
32	♂	"	"	"		
39	♀	Trace insoluble material				
45	♂	"	"	"		
62	♂	—	3.4	5.7	10.5	9.5
63	♀	6.8	2.9	3.4	9.9	11.9
64	♀	—	6.8	8.2	17.8	9.2
73	♀	—	3.1	4.0	7.7	11.6
74	♀	13.7	3.0	4.3	8.6	10.7
85	♂	—	4.3	6.9	9.6	12.0
86	♂	9.4	3.3	4.5	7.5	12.8
88	♂	11.1	4.9	7.1	13.4	12.6

* Based on dry weight of nucleus.

TABLE IV. Analytical Data — "Insolubles" — Herinated Specimens.

Nucleus

Age	Sex	% yield*	% uronic acid: Carbazole	% uronic acid: Orcinol	% hexosamine	% nitrogen
25	♀	—	4.3	4.8	8.7	12.1
27	♂	3.1	5.1	5.8	12.3	12.1
37	♂	2.0	8.4	9.7	18.8	—
38	♂	2.0	4.0	4.9	7.6	9.0
43	♀	3.6	4.6	6.0	12.0	12.0
48	♀	—	2.8	3.8	7.5	12.1
55	♂	1.1	3.4	5.8	11.7	12.0
59	♂	1.6	2.4	4.6	9.0	12.1

* Based on dry weight of nucleus.

been shown to be a decrease in LVN of the purified complex with age. This decrease could be explained by a change in degree of branching although it is felt that a more likely explanation would be a decrease in molecular weight. The LVN of herniated discs from subjects below the age of 50 is markedly lower than normal for the age group.

Another characteristic of aging appears to be the presence of insoluble complex containing appreciable quantities of CS-B beginning at about age 60. This complex was found in significant amounts in all 8 of the herniated discs from subjects in the 25-59 age group which were studied, and was not found in measurable amounts in any normal sample below the age of 60. The presence of CS-B in herniated discs has been previously reported by Davidson and Woodhall(1). The suggestion that disc herniation is an example of pathological aging of tissue has also been made by Blakely *et al*(17). They reported the presence of a clearly defined ring of 4.7 A^0 spacing in X-ray diffraction patterns of nuclei pulposi of aged specimens which they postulated as being due to the unfolding of soluble protein molecules to form a hydrogen bonded lattice. This 4.7 A^0 ring was found in greater amounts in herniated than in normal discs.

Summary. A protein-polysaccharide complex containing a 1:1 ratio of chondroitin sulfate-C and keratosulfate has been found in both nucleus pulposus and annulus fibrosus of human intervertebral discs. The limiting viscosity number of this complex decreases gradually with age in normal subjects. Complex prepared from herniated discs has a much lower limiting viscosity number than normal. An insoluble complex having considerable amounts of chondroitin sulfate-B has been found in normal aged discs and in all herniated discs.

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