Glycosaminoglycans of Cat Intervertebral Disc

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The glycosaminoglycan contents of samples from cat intervertebral discs were examined by using cetylpyridinium chloride salt elution techniques. The values obtained related to the region of the vertebral column from which they were derived, to the area of the disc, and to water content. In wet tissue there was a significant difference between regions of the vertebral column and between areas of the disc and findings agreed with previous histological reports. The greater part of the glycosaminoglycans present consisted of chondroitin sulphate and dermatan sulphate with smaller amounts of hyaluronic acid; little keratan sulphate was found. The maximum amounts of chondroitin sulphate and dermatan sulphate occurred in the 0.5M-magnesium chloride fractions usually, but moved towards higher molar concentrations in samples derived from some sites, particularly in the lumbar region. Mean values for the water content of the areas of the disc were: nucleus pulposus, 82.4%; inner anulus, 65.6%; outer anulus, 50.5%. The water content was directly related to the amounts of chondroitin sulphate and dermatan sulphate.

The present investigation was undertaken, despite the limitations set by low concentrations, to examine further the nature of the substances from cat discs, which have been tentatively identified as glycosaminoglycans by histological methods, and also to determine their distribution within the disc. Butler (1968) suggested from histological observations that the amount of stainable glycosaminoglycan in the anulus fibrosus of the cat (Plate 1) is related to later breakdown of the fibrous structure of the anulus, leading to prolapse of the nucleus pulposus (Plate 1).

It should be mentioned that in human discs the major fractions appear to be keratan sulphate and chondroitin sulphate, present in a 1:1 ratio (Lyons, Jones, Quinn & Sprunt, 1964). Cattle discs also contain keratan sulphate and dermatan sulphate in high ratio (Lowther & Baxter, 1966).

MATERIALS AND METHODS

Tissue from nucleus pulposus, inner ventral anulus and outer ventral anulus of 12 discs of a 4½-year-old cat and from two areas, nucleus pulposus and inner anulus, of six cervical discs of a 13-month-old cat was weighed in polythene cups in closed containers on a micro-balance, after it had been quickly dissected from the opened disc (Plate 1). The amount of tissue varied with disc and with area of the disc in the range 2-20 mg. After freeze-drying the dry weight was determined, and the dried tissue was stored in sealed containers at -20°C until required.

Digestion of the tissue was performed after placing the tissue contained in the polythene cups in a Perspex block

similar to that described by Antonopoulos, Gardell, Szirmai & de Tyssonsk (1964). About 0.1 ml of a suspension of 2.5 mg of crude papain (British Drug Houses Ltd., Poole, Dorset, U.K.) in 10 ml of 0.1M-sodium phosphate buffer, pH 6.5, containing 5 mM-EDTA and 5 mM-cysteine hydrochloride was added to the tissue and the whole was incubated at 65°C for 4 h in a water bath.

After incubation, the digest was transferred to a column (120 mm × 3 mm) containing a suspension in water of cellulose powder (Whatman standard grade) that had been packed at a pressure of about 500 mmHg and washed with 1% cetylpyridinium chloride. The following solutions were used to elute the glycosaminoglycans, which were collected in centrifuge tubes: 1% (w/v) cetylpyridinium chloride; 0.5M-NaCl; 0.25M-MgCl₂; 0.50M-MgCl₂; 0.75M-MgCl₂; 1.25M-MgCl₂; 1.50M-MgCl₂; all dissolved in each case in 0.05% (w/v) cetylpyridinium chloride; and 6M-HCl. A 2 ml portion of each solution was used and the eluate was collected in 2 ml fractions.

The glycosaminoglycans in the fractions from the oldest animal were precipitated with 1 ml of 1% cetylpyridinium chloride and 6 ml of water, left at room temperature for 30 min and centrifuged at 3000g for 1 h. The supernatants were discarded, and 1 ml of 6M-HCl was added to the precipitates, which were hydrolysed in sealed tubes for 8 h at 100°C. Acid and water were removed in a vacuum desiccator containing NaOH and connected to a 'cold-finger' trap; the dried material was stored at -20°C until required. Hexosamine was estimated by an Elson-Morgan procedure (Antonopoulos *et al.* 1964).

The comparable chromatographic fractions obtained from the six samples of nucleus pulposus of the 13-month-old animal were pooled for each eluting solvent and precipitated with the reagents used above. The glycosaminoglycans were separated by centrifugation and suspended in 10 ml of a 5% (w/v) solution of NaI in

ethanol by shaking for 30 min. These suspensions were centrifuged at room temperature for 15 min at 5000g and the supernatants decanted. The precipitates were dissolved in 0.2 ml volumes of water, and the solutions were dialysed against 0.1 M-zinc acetate for 18 h. The dialysis residues were examined by means of differential Alcian Blue staining of 5 μ l samples on Whatman no. 1 filter paper (Scott & Dorling, 1965) and by electrophoresis on strips (2.5 cm $\times$ 18 cm) of cellulose acetate (Oxoid). Electrophoresis was performed on 5 μ l samples with a voltage gradient of 13 V/cm for 4 h and a buffer solution of 0.1 M-zinc acetate, pH 4.8. The chromatographic papers were stained with 1% (w/v) Alcian Blue in 10% (v/v) acetic acid, washed in 10% acetic acid and running water, dried at room temperature, and estimated visually. Samples of inner anulus were treated similarly.

Hyaluronic acid (British Drug Houses Ltd.), chondroitin sulphate (bovine trachea; British Drug Houses Ltd.), dermatan sulphate (pig skin; from Dr A. G. Lloyd of the University of Cardiff) and keratan sulphate (bovine cornea; from Dr A. G. Lloyd) were used as standards in experiments in which the amounts of glycosaminoglycans from the various fractions were determined. They were tested for purity by electrophoresis and each was found to give a single band except for the chondroitin sulphate, which gave a weak second band corresponding to the position of hyaluronic acid. This impurity represented about 2% of the total glycosaminoglycan present as measured on an EEL Scanner.

Inner anulus fibrosus tissue from 26 discs of a 10-month-old cat was pooled to give enough material for some chemical characterization of the fractions. Nucleus pulposus was treated in a similar manner and both inner anulus and nucleus pulposus were subjected to the following procedures (Hardingham & Phelps, 1970). The tissue was extracted with acetone for 2 h in a Soxhlet apparatus and defatted with chloroform-methanol (2:1, v/v). The residue was freed of solvents by vacuum desiccation and suspended in 5 ml of 0.1 M-sodium acetate buffer, pH 5.5, which contained also 5 mM-cysteine and 5 mM-EDTA as well as papain (0.6 mg/100 mg of dry tissue). The papain was preactivated by incubation in the buffer for 30 min at 60°C before addition of the tissue. Digestion was allowed to proceed overnight at 60°C. The digest was cooled to 4°C and centrifuged at 5000g for 10 min. Further incubation did not decrease the very small amount of undigested material which was resuspended in 0.1 M-sodium acetate and recentrifuged. Supernatant and washings were combined and 30% (w/v) trichloroacetic acid was added to give a final concentration of 5% (w/v). The preparation was kept at 4°C for 2 h, and the precipitate was removed by centrifuging at 5000g for 10 min and washed with 5% (w/v) trichloroacetic acid. The pH of the combined supernatant and washings was adjusted to 6.8 with 4 M-NaOH and the solution was dialysed against water for about 16 h. Then 4 M-NaOH was added to give a final concentration of 0.5 M and the digest was shaken for 16 h at 4°C. The pH of the solution was adjusted to 6.5 with 4 M-HCl and the preparation was dialysed for 48 h against several changes of water. It was applied to a column (10 cm $\times$ 1.5 cm) of Zeo-Karb 225 (H⁺ form), which was eluted with water. The pH of the eluate was adjusted to 6.5 with 1 M-NaOH and the solution was freeze-dried.

The dried material was taken up in 6 ml of water over a period of 3 days at 4°C. The undissolved material, which contained negligible amounts of uronic acid, was removed by centrifugation (5000g for 10 min). Samples of 1.0 ml of material from outer anulus and nucleus pulposus and 2.5 ml of material from inner anulus were applied to cellulose columns (7.5 cm $\times$ 1 cm) prepared in a manner similar to that described above. The columns were eluted at 30°C with 20 ml of each of the solutions described above and collected as single fractions.

The acid glycosaminoglycans were isolated by the method described by Hardingham & Phelps (1970).

Original samples and eluates were assayed for uronic acid by the methods of Bitter & Muir (1962) and Khym & Doherty (1952), with glucuronolactone as standard.

RESULTS AND DISCUSSION

About 80% of the hexosamine present in glycosaminoglycans was recovered from standards when the methods used in the present investigation were applied (Table 1).

The total amount of hexosamine in discs of a 4½-year-old cat (Table 2) showed significant differences between regions ($P < 0.05$). Highest values were found in the thoracic region and lowest in the lumbar, and these values confirm and extend histological findings by Butler (1968). Comparison of areas showed that highest values were seen in the inner anulus and lowest in the outer anulus, agreeing with tissue basophilia and with metachromasia and Alcian Blue staining.

Table 1. *Recovery of hexosamine after chromatography of known glycosaminoglycans*

Glycosaminoglycans were dissolved in water to give 1.296 mg of hyaluronic acid (British Drug Houses Ltd.)/ml and 2.001 mg of chondroitin sulphate (British Drug Houses Ltd.)/ml, a total of 3.297 mg/ml. Portions (10, 20 and 40 μ l) were applied to each of three cellulose columns (120 mm $\times$ 3 mm) and eluted in the usual manner (A). Similar portions were hydrolysed and measured without passage through columns (direct determinations, B).

Solvent	Recovery of hexosamine (μ g)		
	Column no. ... 1	2	3
A 1% Cetylpyridinium chloride	0.6	1.0	0.8
0.5 M-NaCl	2.6	6.6	12.0
0.25 M-MgCl ₂	1.0	1.4	4.4
0.50 M-MgCl ₂	2.0	4.9	9.4
0.75 M-MgCl ₂	1.0	1.8	4.2
1.25 M-MgCl ₂	0.6	0.4	1.0
1.50 M-MgCl ₂	0.6	0.4	0.4
6 M-HCl	0.4	0.6	0.6
Totals	8.8	17.1	32.8
B Direct determination	11.4	21.0	39.8
C Recovery	77%	81%	82%

EXPLANATION OF PLATE I

Sagittal section of a cat intervertebral disc showing the areas from which samples were taken. Nch, notochordal cells; PNch, perinotochordal matrix. The 1 mm thick section was cut free-hand after decalcification of the adjacent vertebrae.

Table 2. *Water and total hexosamine contents of the intervertebral disc tissues of a 4½-year-old cat*

Tissue was weighed, dried and reweighed and the water content was calculated as a percentage of the fresh weight. Total hexosamine was derived by addition of the results for eluting solvents (see Table 3). See the text for experimental details.

Region	Disc*	Water content (%)			Total hexosamine (μg/mg of fresh tissue)			Mean
		Nucleus pulposus	Inner anulus	Outer anulus	Nucleus pulposus	Inner anulus	Outer anulus	
Cervical	C2-3	81	62	55	15.4	18.6	5.3	18.20
	C3-4	77	67	48	20.4	25.3	18.3	
	C4-5	80	72	58	18.3	13.2	20.3	
	C5-6	64	54	54	17.9	23.4	22.0	
Thoracic	T1-2	93	79	63	22.0	21.3	11.8	20.17
	T2-3	96	73	47	10.4	17.7	24.7	
	T8-9	91	69	49	25.3	40.1	12.5	
	T9-10	78	65	29	14.8	25.4	16.0	
Lumbar	L3-4	79	61	51	20.2	7.5	8.0	10.83
	L4-5	81	63	49	15.0	18.2	5.8	
	L5-6	74	56	55	9.2	10.0	4.1	
	L6-7	84	64	48	13.2	11.9	6.8	
Mean		82.4	65.6	50.5	16.84	19.38	12.97	16.40

* C, cervical; T, thoracic; L, lumbar. The numbers refer to the vertebrae between which the disc is found.

Table 3. *Hexosamine content of fractions eluted from the intervertebral-disc tissues of a 4½-year-old cat*

Tissue was digested with papain, applied to cellulose columns and eluted with the solvents shown. Hexosamine was estimated by an Elson-Morgan procedure. See the text for experimental details.

Disc ... Solvent	Hexosamine content (μg/mg of fresh tissue)									
	C4-5	C5-6	T1-2	T2-3	T8-9	T9-10	L3-4	L4-5	L5-6	L6-7
Nucleus pulposus										
1% Cetylpyridinium chloride	2.1	1.6	2.3	1.2	2.5	1.0	7.6	6.2	1.4	7.3
0.5 M-NaCl	1.5	2.4	3.1	1.7	3.8	1.9	1.6	1.6	1.5	1.0
0.25 M-MgCl ₂	0.9	0.9	2.5	1.0	5.3	1.4	0.7	1.3	0.2	0.4
0.5 M-MgCl ₂	7.4	7.4	4.0	2.1	3.7	6.3	1.3	1.4	1.2	0.5
0.75 M-MgCl ₂	4.0	3.1	2.7	1.3	2.4	1.6	4.6	2.6	3.3	2.4
1.25 M-MgCl ₂	0.8	0.8	2.8	1.1	2.2	0.9	4.2	1.7	1.4	1.4
1.50 M-MgCl ₂	0.8	1.0	2.3	1.1	2.6	0.9	0.1	0.1	0.1	0.1
6 M-HCl	0.8	0.7	2.3	0.9	2.8	0.8	0.1	0.1	0.1	0.1
Inner anulus										
1% Cetylpyridinium chloride	2.1	2.4	2.2	1.8	3.6	2.2	0.6	4.0	0.7	7.6
0.5 M-NaCl	1.9	5.2	3.5	3.0	6.3	4.0	1.7	6.4	3.5	0.8
0.25 M-MgCl ₂	1.4	2.3	2.1	1.4	5.7	2.9	0.9	1.4	0.1	0.4
0.5 M-MgCl ₂	3.7	6.4	4.6	4.1	7.0	4.5	1.1	0.8	0.7	0.4
0.75 M-MgCl ₂	1.5	3.0	2.4	1.9	3.9	3.7	2.2	3.4	0.7	1.1
1.25 M-MgCl ₂	1.1	1.0	2.3	1.7	4.9	2.7	0.8	2.0	3.9	1.4
1.50 M-MgCl ₂	0.9	1.5	2.3	2.2	4.7	3.0	0.1	0.1	0.3	0.1
6 M-HCl	0.6	1.6	1.9	1.6	4.0	2.4	0.1	0.1	0.1	0.1
Outer anulus										
1% Cetylpyridinium chloride	5.4	3.7	1.1	3.2	0.8	1.4	1.9	1.3	0.6	0.5
0.5 M-NaCl	1.9	3.1	2.0	3.7	1.9	2.4	3.8	1.8	1.3	2.0
0.25 M-MgCl ₂	1.8	2.0	1.0	2.6	1.8	1.9	0	0.9	0.4	0.4
0.5 M-MgCl ₂	2.3	4.1	2.7	3.4	2.6	3.4	1.7	0	0.4	0.5
0.75 M-MgCl ₂	2.6	2.7	2.0	3.4	1.5	2.0	0.3	0.7	0.4	1.0
1.25 M-MgCl ₂	2.4	2.4	1.1	3.0	1.3	1.7	0.2	1.0	0.8	2.1
1.50 M-MgCl ₂	2.8	1.6	1.0	2.9	1.4	1.7	0	0	0.1	0.2
6 M-HCl	1.1	2.4	0.9	2.5	1.2	1.5	0.1	0.1	0.1	0.1

Drying of the tissue altered some of the relationships (particularly between areas) as a result of the variation in water content (Table 2), and highest hexosamine concentration was now found in the nucleus pulposus.

The mean water content of the nucleus pulposus of all regions of the oldest animal (82.4%; Table 2) and the water content of pooled nucleus pulposus of the youngest (82%) agree with the estimate given by Sylvén (1951) of 83% for human and calf nucleus pulposus, despite the fact that they differ histologically from the cat nucleus pulposus. Hansen (1956) found that there is a fall of moisture in the ox nucleus pulposus from 85.3% at birth to 62.8% at 12 years of age. In man the water content of the nucleus pulposus falls from 88% at birth to 69% at 77 years of age (Naylor & Horton, 1955) or 87% at 4½ years to 72% at 76 years of age (Gower & Pedrini, 1969). Age changes in moisture content of cat discs are not yet known, but from histological considerations it would seem that the fall in moisture content of the nucleus pulposus by 4½ years of age might be much less.

Water content of human anulus fibrosus falls from 75% at 4½ years to 70% at 76 years (Gower & Pedrini, 1969). In the cat disc the anulus fibrosus appears to have much lower water content.

Table 3 shows the distribution of fresh-tissue

hexosamine concentrations in the various fractions. Only ten discs were included, since some solvents were omitted for discs C2-3 and C3-4: the totals (Table 1) are valid, however, because 6M-hydrochloric acid was used to remove any undissolved residue (Antonopoulos *et al.* 1964). These fractions can tentatively be identified (after Gardell & Szirmai, 1967; Szirmai, de Tyssensk & Gardell, 1967) as follows: 1%-cetylpyridinium chloride fraction, keratan sulphate and glycoprotein; 0.50M-sodium chloride fraction, hyaluronic acid; 0.25M-, 0.50M-, 0.75M-, 1.25M- and 1.50M-magnesium chloride fractions, dermatan sulphate and/or chondroitin sulphate of increasing molecular weight and/or degree of sulphation; 6M-hydrochloric acid fraction, uneluted residue. It was not possible to characterize the disc fractions any further because only minute amounts of material were available.

Results of uronic acid and hexosamine determinations (Table 4) on material from the 10-month-old animal suggest that, contrary to findings in other species, keratan sulphate is not an important part of the 1%-cetylpyridinium chloride fraction in the nucleus pulposus but may be present in the inner anulus in addition to some uronic acid containing component. Because of lack of material further examination could not be made, but it is noteworthy that Hardingham & Phelps (1970) also

Table 4. *Hexosamine and uronic acid content of pooled tissue from inner anulus and nucleus pulposus of a 10-month-old cat*

Tissue was digested with papain, applied to cellulose columns and eluted with the solvents shown. See the text for experimental details.

Experimental conditions		Hexosamine (nmol/mg of fresh tissue)	Uronic acid (nmol/mg fresh of tissue)	
Solvent			Orcinol method	Carbazole method
Inner anulus				
1% Cetylpyridinium chloride		6.4	2.4	4.3
0.5 M-NaCl		0.8	1.5	1.2
0.25 M-MgCl ₂		0.1	0	0.2
0.50 M-MgCl ₂		10.2	3.2	2.8
0.75 M-MgCl ₂		0	1.0	0
1.25 M-MgCl ₂		0	1.0	0
1.50 M-MgCl ₂		0	1.2	1.2
6 M-HCl		0	0	0.5
Total		17.5	10.3	10.2
		(approx. 45% recovery)		
Nucleus pulposus				
1% Cetylpyridinium chloride		4.8	5.1	7.9
0.5 M-NaCl		4.0	6.4	0.6
0.25 M-MgCl ₂		0.8	3.6	1.3
0.50 M-MgCl ₂		22.4	18.6	11.6
0.75 M-MgCl ₂		0.4	2.0	3.2
1.25 M-MgCl ₂		0	0.1	0
1.50 M-MgCl ₂		0	0.1	0.2
6 M-HCl		0.2	2.0	0
Total		32.6	37.9	24.8
		(approx. 82% recovery)		

Table 5. *Results of electrophoresis and of Alcian Blue staining of samples of intervertebral disc from a 13-month-old cat*

Electrophoresis of samples of standard glycosaminoglycans was carried out under a gradient of 13 V/cm; other samples of standards were applied to filter paper and stained with Alcian Blue at pH 5.6 in the presence of MgCl_2 . Disc tissue was digested with papain, eluted with the solvents shown and treated in a similar manner to the standards. See the text for experimental details. Staining intensity is given in arbitrary units of 0–6, T indicating a trace of stain. Standard heparin was not used, since no mast cells have been identified in the tissue.

Electrophoresis on cellulose acetate strips followed by staining in Alcian Blue				Intensity of standard spots stained with Alcian Blue in		
Standards	Band width (cm from origin)	Remarks		0.0M- MgCl_2	0.3M- MgCl_2	0.7M- MgCl_2
Hyaluronic acid	3.9–4.1	—		4	T	0
Chondroitin sulphate	5.9–6.1	Additional very faint band at 4 cm		4	5	0
Dermatan sulphate	4.5–5.3	—		4	5	0
Keratan sulphate	4.7–6.9	Covers same range as chondroitin sulphate and dermatan sulphate		4	5	2

Inner-anulus sample fractions	Band width (cm from origin)	Alcian Blue staining intensity	Possible presence of	Intensity of sample spots stained similarly		
1% Cetylpyridinium chloride	3.95–4.05	$\frac{1}{2}$	Hyaluronic acid	$\frac{1}{2}$	0	0
0.5M-NaCl	3.95–4.05 and 4.9–5.3	$\frac{1}{2}$ and 1	Hyaluronic acid and dermatan sulphate	1	0	0
0.25M- MgCl_2	4.8–5.4	2	Dermatan sulphate	$\frac{1}{2}$	0	0
0.50M- MgCl_2	5.0–5.5	5	Dermatan sulphate	4	4	0
0.75M- MgCl_2	None	0		0	0	0
1.25M- MgCl_2	None	0		0	0	0
1.50M- MgCl_2	None	0		0	0	0
6M-HCl	None	0		0	0	0
Nucleus-pulposus sample fractions						
1% Cetylpyridinium chloride	4.9–5.1	1	Keratan sulphate or dermatan sulphate	1	0	0
0.5M-NaCl	4.0–4.2 and 5.0–5.5	$\frac{1}{2}$ and 4	Hyaluronic acid and dermatan sulphate	4	T	0
0.25M- MgCl_2	5.0–5.5	1	Dermatan sulphate	2	0	0
0.50M- MgCl_2	5.3–6.1	6	Dermatan sulphate or chondroitin sulphate	6	4	0
0.75M- MgCl_2	5.4–5.9	1	Dermatan sulphate or chondroitin sulphate	2	1	0
1.25M- MgCl_2	None	0		0	0	0
1.50M- MgCl_2	5.5–6.0	$\frac{1}{2}$	Chondroitin sulphate	1	1	0
6M-HCl	None	0		0	0	0

found uronic acid in the 1%-cetylpyridinium chloride fraction from rat skin.

Electrophoresis and Alcian Blue staining of spots (Table 5) also suggests the presence of polymers that contain uronic acid in the 1%-cetylpyridinium chloride fraction, i.e. dermatan sulphate in nucleus pulposus and hyaluronic acid in inner anulus.

In two animals the 1%-cetylpyridinium chloride fraction is often present in large amounts (Tables 3 and 4) and varies with region, and with area of disc, being a prominent feature in the nucleus

pulposus and inner anulus of the lumbar discs and the outer anulus of cervical discs.

The elution profile of pooled material from the 10-month-old animal (Table 4) agrees in general with values for the $4\frac{1}{2}$ -year-old (Table 3) and with the intensity of Alcian Blue staining of material from the 13-month-old (Table 5). The 0.5M-sodium chloride fraction contains uronic acid and hexosamine (Table 4). In the inner anulus these are in approximately equimolar proportions and might be hyaluronic acid: electrophoresis and spot

staining, however, suggest small amounts of hyaluronic acid and larger amounts of dermatan sulphate (Table 5). In the nucleus pulposus this fraction contains large amounts of hexosamine and uronic acid. Since, however, the ratio of the values given by the carbazole and orcinol reactions is low the fraction may contain dermatan sulphate, and electrophoresis (on material from another animal) also suggests mainly dermatan sulphate (Table 5).

The magnesium chloride fractions show a peak in 0.5M concentration in the two young animals (Tables 4 and 5) and in 0.5M, 0.75M or higher concentration in the older animal (Table 3). The 0.5M-magnesium chloride fraction (Table 4) shows the ratio 0.62 for the values given by the carbazole and orcinol reactions in the nucleus pulposus, suggesting that this fraction contains dermatan sulphate. Electrophoretic mobilities seem to agree with this (Table 5). The electrophoretic mobility of the material eluted increases with salt concentration and reaches the mobility of the standard chondroitin sulphate (Table 5). Since the chondroitin sulphate and dermatan sulphate peak value (Table 5) varies in the older animal from the 0.5M-magnesium chloride fraction (in the nucleus pulposus and inner anulus of cervical and thoracic regions) to the 0.75M-magnesium chloride fraction or higher (in the outer anulus of the cervical and thoracic regions and the whole of the lumbar-region discs) it could mean that the discs of the lumbar region contain chondroitin sulphate and dermatan sulphate of higher average molecular weight and/or degree of sulphation than the others, i.e. are perhaps more mature (Davidson & Woodhall, 1959; Kaplan & Meyer, 1959) and are perhaps more stable than the other regions. If this is so it might help to explain the distribution of ruptures in cat discs.

Results (Table 3) show that no significant difference exists between discs for the nucleus pulposus, as one would expect from histological findings; but that they do, however, exist ($P < 0.001$) between discs for the outer anulus and inner anulus, with the highest amounts of hexosamine being found in the cervical and thoracic regions and lowest in the lumbar (Table 3), thus again agreeing with the histology of the cat disc.

Butler (1968) interpreted the variation in glycosaminoglycan staining in cat discs as being due either to differences in the quantity present or to differences in the degree of blockage by, for example, proteins (French & Benditt, 1953). The present study suggests that variation in quantity does occur, although it does not refute the other alternative.

The mean concentration of hexosamine in dry tissue of the oldest animal (74.2 µg/mg) is similar to the mean found by Antonopoulos *et al.* (1964) in one lumbar rabbit disc (95 µg/mg); further, these

authors found highest amounts in the nucleus pulposus and lowest in the outer anulus, and the 1%-cetylpyridinium chloride fraction was particularly large in the nucleus pulposus of the rabbit. Similarly, in a disc from *Macacus cynomolgus* (*Macaca ira*, crab-eating macaque) Gardell & Szirmai (1967) showed a large 1%-cetylpyridinium chloride fraction, with total nucleus pulposus hexosamine of 66 µg/mg of dry tissue and ventral anulus hexosamine of 57 µg/mg. In the 10-month-old cat the concentrations were approximately half that in the 4½-year-old cat.

The relationship of water content of the tissue and the concentration of glycosaminoglycans (as hexosamine) was tested by regression analysis. All relationships tested were positive for dry tissue and regression of water content on the peak chondroitin sulphate and dermatan sulphate concentration showed a significant positive relationship ($P < 0.025$). No significant difference between nucleus pulposus, inner anulus and outer anulus exists. Thus it seems likely that the water content in the cat disc is related to the concentration of chondroitin sulphate and dermatan sulphate in the dry tissue.

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