Collagens and associated glycoproteins in the human intervertebral disc

Variations in sugar and amino acid composition in relation to location and age

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There is good evidence that hexoses form an integral part of the collagen molecule. In mammalian collagens a number of the hydroxylysine residues are covalently linked to D-galactose and a variable fraction of the latter is bound to D-glucose (Butler and Cunningham, 1965, 1966; Cunningham and Ford, 1968). The galactose and glucose contents of soluble, insoluble, and 'polymeric' (Steven and Jackson, 1967) collagens from a variety of tissues have been compared (Spiro, 1969; Grant, Freeman, Schofield, and Jackson, 1969), but there is very little similar information on cartilaginous tissues. It is also very noticeable that possible changes occurring during maturation or ageing have not been explored, although Moczar and Moczar (1971) have briefly reported results relating to the embryonic and post-natal periods.

In previous work on the human intervertebral disc, we found that papain digests of certain portions of 'whole' tissue that were rich in collagen gave two clearly defined glycopeptide peaks when subjected to gel filtration on Sephadex G25 (Pearson, Happey, Naylor, and Turner, 1967). It is now clear that the second (retarded) peak contains the hydroxylysine-linked sugars derived from the collagen, whereas sugars originally bound to noncollagenous proteins are found in the excluded Peak 1. If the proteoglycans (protein-polysaccharides) are removed by extraction before digesting the insoluble residue with papain, Peak 1 represents the noncollagenous compounds most closely associated with the collagen. With this method it has now been possible to carry out a systematic quantitative age study of the two distinct types of glycopeptides in different regions of the disc. The noncollagenous components of insoluble fractions may also be removed by trypsin digestion (Pearson,

Happey, Shentall, Naylor, and Turner, 1969) which has allowed the amino acid composition of the collagens to be determined.

Materials and methods

Specimens of nucleus pulposus and annulus fibrosus were taken from lumbar intervertebral discs obtained at autopsy. In fairly young discs the nucleus pulposus was clearly defined by its gel nature. Only a small central region from older discs was designated as nucleus, usually distinguished by a more yellow appearance than the rest of the tissue. The specimens of annulus fibrosus were drawn almost entirely from the laminated ventral region; part of the dorsal annulus may have a very different composition (Gardell and Szirmai, 1967). Most of the tissue between the annulus and nucleus was taken as representing a boundary or intermediate zone, but its quantity and appearance varied greatly with different specimens. The specimens were stored at -20°C . For most age groups 3 to 6 g. of wet pooled tissue were employed, the samples being coded by age range and location (A = annulus, N = nucleus, B = intermediate zone).

High speed homogenization of the tissue, giving efficient extraction of soluble proteoglycans was carried out three times as previously described (Pearson and others, 1969), except that 0.9 per cent. w/v NaCl solution was employed instead of water. When sufficient quantity was available, part of the insoluble residue was retained. The rest was digested with papain (suspension of twice recrystallized grade, Sigma) in a 0.1M solution of sodium acetate-acetic acid, pH 6.0, containing 0.79 mg. cysteine hydrochloride and 1.86 mg. EDTA (disodium) per ml. The volume of buffer solution was kept small, usually not more than about 3 ml./g. of original weight of tissue being employed, together with 0.05 to 0.1 ml. of activated papain suspension. Digestion was carried out at 65°C . overnight.

Portions of a number of saline-insoluble residues were given two treatments with trypsin as described below and then digested with papain. Trypsin-soluble components were concentrated by rotary evaporation below 40°C .

adding cysteine, EDTA, and acetic acid to pH 6 before papain digestion.

GEL FILTRATION OF PAPAIN DIGESTS

Columns of Sephadex G25 (fine grade, Pharmacia) were well washed with water before use. Papain digests containing about 2 mg. or less of nonamino sugars in not more than 3 ml., were added to 20 × 2 cm. columns and fractions of 3 ml. were collected. For larger quantities, up to 10 mg. in 10 ml., 40 × 2.5 cm. columns were used, collecting fractions of 5 to 6 ml. Elution was with water at room temperature at about 60 ml./hr. Small portions of each fraction were analysed by the orcinol method and the remaining effluent corresponding to Peak 1 or 2 was pooled, rejecting one or two fractions in the trough between the peaks. Further analyses were then performed and for greater accuracy the orcinol value was usually redetermined. Recoveries of orcinol-positive substances from the Sephadex columns were close to the theoretical.

COLORIMETRIC DETERMINATION OF SUGARS

One volume of 2.9 per cent. w/v of crystalline orcinol (Merck) was mixed with 7.5 volumes of 66 per cent. w/v H₂SO₄ (BDH Chemicals, A.R.) and 5 ml. of this reagent were added to 0.3 to 0.5 ml. of the test solution. The covered tubes were heated for 10 min. in a boiling water bath and the absorptions measured at 505 mμ (EEL Spectra, flow through cell). This method measures non-amino sugars. D-Galactose (Sigma, glucose-free grade) was employed as standard, and the relative colour yields of the other neutral sugars were determined. A modified carbazole method (Bitter and Muir, 1962) was employed for uronic acid, with glucuronolactone as standard. The colour yield of glucuronolactone is ten times that of hexose in this method, but only half of that given by galactose with orcinol, allowing neutral sugar (mainly hexoses and fucose) and corrected uronic acid contents to be calculated. Total hexosamine or individual hexosamines were determined by methods similar to those previously described (Pearson, 1963), after hydrolysis in 4N HCl for 16 hrs at 100°C.

GAS-LIQUID CHROMATOGRAPHY (GLC)

Neutral sugar ratios were determined by GLC (Sweeley, Bentley, Makita, and Wells, 1963). The monosaccharides were liberated by hydrolysis for 17 hrs with a cation exchange resin in the presence of 0.05N HCl, essentially as described by Grant and Jackson (1968), but Amberlite 120 (chromatographic grade) was employed (1 to 2 g/10 ml.).

The solution, together with the water wash from the resin, was neutralized with the bicarbonate form of Dowex 2 beads. After concentrating by rotary evaporation at 30–35°C., portions of the solution were evaporated to dryness under the same conditions. In order to obtain consistent anomer ratios, dry residues were equilibrated with 0.3 per cent. w/v of lithium perchlorate in dry pyridine (Bethge, Holmström, and Juhlin, 1966) for 2 hrs at 37°C. or overnight at room temperature.

Usually 0.1 ml. of TMS reagent, made up every few days from 2 volumes of hexamethyl-disilazane, 1 volume of trimethyl-chlorosilane (both from Koch-Light), and 2 volumes of dry pyridine, was added after equilibration. 10 min. standing was sufficient before injection. A Pye 104 gas chromatograph was employed with dual columns

and a flame ionization detector. The stationary phase was 3 per cent. E30 (Pye) on a Celite support in a glass column (1.5 m. × 4 mm.). The temperature programme was 140°C. for 10 min. then 1°C./min. rise to about 190°C. (Grant and others, 1969).

PURIFICATION OF THE COLLAGENS AND AMINO ACID ANALYSES

Insoluble residues remaining after three homogenizations in water were suspended in 0.067M phosphate buffer, pH 7.4. Porcine trypsin (three times recrystallized, Miles-Seravac) was added to give a concentration of 0.3 mg./ml. of suspension, and digestion was carried out for 1 hr. at 37°C. After removing the supernatant, digestion of the insoluble material was continued for at least 16 hrs in a fresh solution of the trypsin (0.3 mg./ml.). The insoluble collagens were then dispersed in 0.2M acetic acid, precipitated by the addition of NaCl or dialysis, washed with water, and freeze dried.

A 1-hr. trypsin digest of infant annulus fibrosus was treated as before (Pearson and others, 1969), but dialysis was for a longer period, a total of 4 days. After removing a precipitate, the non-diffusible material was isolated for amino acid analysis by adding 6 volumes of ethanol and potassium acetate. A Technicon Autoanalyser was employed with gradient elution and a modified ninhydrin reagent (Technicon Research Bulletin No. 20). No corrections were made for losses of amino acids on hydrolysis, which was carried out in 6N HCl at 100°C. for 24 or 36 hrs. The hydroxyproline contents of papain digests were determined by the method of Stegemann and Stalder (1967) after a similar hydrolysis for 24 hrs.

Results

GENERAL SUGAR COMPOSITION OF PEAKS 1 AND 2

Table I shows that, with one exception, Peak 1 had a considerably higher neutral sugar:uronic acid ratio than the corresponding NaCl extract. In addition to galactose, significant amounts of mannose and fucose were present, but usually less glucose. Traces of xylose were also found in Peak 1. Mannose and fucose were rarely detected in Peak 2 which contained very small quantities of hexosamines (5 per cent. or less of the neutral sugars) and little if any uronic acid.

GLC results obtained using mannitol as internal standard suggested that 80 to 100 per cent. of the orcinol value of Peak 2 was accounted for as galactose and glucose. In a few analyses of Peak 2 and more frequently with Peak 1, exceptionally high recoveries were found with the internal standard method which were not explained. Determinations of galactose and glucose with enzymic-colorimetric techniques, employing galactose and glucose oxidases, confirmed the reliability of the orcinol method for measuring the sum of these hexoses in Peak 2. However, glucose:galactose ratios found with the oxidases were lower than those given by GLC which are recorded in Table I. No significant age trend in the ratio was found with either method, but a tendency towards higher values in the nucleus than in the annulus was apparent.

Table I Sugar composition of collagen glycopeptides (Peak 2) and noncollagenous components

The specimens marked with an asterisk were treated with trypsin before papain digestion

Age (yrs)	Peak 2		Peak 1		Per cent. of NS					NaCl extract	
	NS	GI/Ga	NS	Ga	M	F	Gl	u	NS/u	NS/u.	NS/u.
3-5 N	1.8	0.81	0.8	74	9	?	18	0.4	2.1	0.9	
5 N	4.9	—	—	—	—	—	—	—	—	—	
6½ N	4.5	—	—	—	—	—	—	—	—	—	
7-10 N	4.1	0.78	2.0	53	17	20	10	0.9	2.3	0.7	
14-17 N	3.7	0.99	2.0	57	16	20	7	1.0	2.0	1.0	
21-41 N*	4.5	0.78	—	—	—	—	—	—	—	—	
26-40 N	4.2	1.1	2.8	57	20	18	4	0.9	3.0	1.4	
50-54 N	4.2	—	—	—	—	—	—	—	—	—	
54-72 N*	4.0	—	—	—	—	—	—	—	—	—	
65-68 N	3.6	1.2	7.8	53	24	17	6	1.3	6.0	2.2	
65-68 N*	4.0	0.81	—	—	—	—	—	—	—	—	
3-5 A	2.6	0.78	0.83	50	17	15	19	0.3	2.5	1.3	
3-5 A*	2.7	0.64	—	—	—	—	—	—	—	—	
5 A	2.6	—	—	—	—	—	—	—	—	—	
7 A	2.0	0.58	0.7	52	12	21	15	0.6	1.2	1.5	
14-17 A	2.0	1.0	0.5	47	18	24	10	0.2	2.3	1.4	
28-31 A*	2.0	—	—	—	—	—	—	—	—	—	
31-40 A	1.8	0.95	0.8	51	19	19	10	0.3	2.5	1.9	
65-68 A	1.7	0.81	1.8	46	28	18	8	0.4	4.9	1.7	
65-68 A*	1.6	1.2	—	—	—	—	—	—	—	—	
7-9 B	1.9	0.87	0.4	55	17	19	9	0.2	1.6	1.0	
51-62 B	3.2	0.58	1.1	46	21	20	13	0.7	1.7	—	
51-62 B*	3.2	0.71	—	—	—	—	—	—	—	—	

NS = neutral sugars expressed as g./14g. hydroxyproline.
u = uronic acid expressed as g./14g. hydroxyproline.Ga = galactose.
Gl = glucose.M = mannose.
F = fucose.**SUGAR COMPOSITION OF TRYPSIN-SOLUBLE FRACTIONS**

Table II shows that the higher molecular weight com-

ponents liberated by a short digestion with trypsin have a general resemblance to Peak 1. The main differences are the higher proportions of uronic acid and

Table II Hexosamine and other sugar components of noncollagenous fractionsThe following fractions were analysed: excluded peaks from Sephadex G25 obtained from papain digests of either the whole of an insoluble residue (Peak 1) or of 1-hr. trypsin-soluble fractions (T.Sol_E); nondiffusible components remaining after dialysis of 1-hr. trypsin-soluble fractions (T.Sol_D) GNH₂ = glucosamine; GaNH₂ = galactosamine. Other symbols as in Table I.

Fraction	Age (yrs)	Loca- tion	Molar ratios					Per cent. of NS	
			GNH ₂	GaNH ₂	u	NS	Ga	M + F	
Water Sol. PPL*	4½	N	1.0	2.5	—	—	—	—	
Water Sol. PPL*	4½	A	1.0	1.5	—	—	—	—	
Peak 1	3-5	N	1.0	0.85	0.85	1.8	1.3	?	
T.Sol _E	3-5	A	1.0	0.50	—	—	—	40	
T.Sol _D	1-10	A	1.0	1.5	1.4	4.9	2.5	21	(+26 per cent. Gl)
T.Sol _D	54-72	N	1.0	0.41	0.44	2.2	—	—	
T.Sol _D	50-63	N	1.0	0.56	0.73	1.9	—	—	
T.Sol _E	59-62	N	1.0	0.47	0.48	2.1	—	—	
T.Sol _D	59-66	B	1.0	0.54	0.40	1.7	0.85	33†	
Peak 1	65-68	N	1.0	0.38	0.23	1.3	0.71	41	
Peak 1	65-68	A	1.0	0.43	0.31	1.4	0.61	46	
T.Sol _E	65-68	A	1.0	0.33	—	—	—	—	
Water Sol.	50-63	N	1.0	1.1	0.86	1.6	1.3	15†	
Water Sol.	59-66	B	1.0	1.2	0.63	1.3	1.0	18†	

Data of Gower and Pedrini (1969).

† Hydrolysed in 0.5 N HCl (no resin), 16 hrs, 100°C.

neutral sugars in the trypsin-soluble fractions. Probably a distinct fraction exists with a limited resistance to trypsin. However, after two treatments, including overnight digestion, an excluded peak is only just detectable in the papain digest of the insoluble residue (Fig. 1a). Except for the trypsin-soluble fraction of 1-10 A (which was also anomalous in amino acid composition), a much lower galactosamine: glucosamine ratio clearly distinguished Peak 1 or the trypsin-soluble components from water-soluble proteoglycans derived from tissue of similar location and age (Table II).

FIGURE 1 Gel filtration of papain digests on Sephadex G 25. Fractions analysed by the orcinol method

Trypsin-soluble fractions removed in a short treatment and afterwards digested with papain do not usually show a clearly defined retarded sugar peak. Specimen 3-5 A was exceptional (Fig. 1b). The substances solubilized in the second, longer trypsin digestion more often gave a retarded peak, and it represented a higher proportion of the total sugars liberated from 3-5 A (Fig. 1b). Analysis with GLC of two similar retarded peaks from overnight trypsin digests of specimens 65-68 A and 51-62 B showed that only galactose and glucose were present. When expressed on a hydroxyproline basis, g./14 g. of hydroxyproline in the trypsin-soluble fractions, total hexose values of 1.8 (65-68 A) and 4.4 were obtained (compare with Table I). There seemed little doubt that these hexose fractions, unlike the greater part of the sugars released by trypsin, were derived from

dissolved collagen. The latter was a very small proportion of the total, 2 to 5 per cent. of the total hydroxyproline of a variety of specimens being found in 1-hr. trypsin digests and 6 to 11 per cent. in 1 hr. plus 16 hr. digests.

AMINO ACID RESULTS

The amino acid compositions of collagens and non-diffusible components of 1-hr. trypsin digests are shown in Table III. Judging by hydroxyproline contents, the annulus fibrosus from 50 to 70-year-old specimens yielded the purest collagen. Its general composition closely resembled tendon collagen, but it contained more hydroxylysine. The collagen from the older nucleus pulposus did not differ significantly in hydroxylysine and like the other preparations it contained little tyrosine, but the lower glycine and other variations, as well as the low hydroxyproline content, indicated the presence of non-collagenous proteins. Some of the differences between the young collagens and 50-70 N were larger than expected from the very similar hydroxyproline contents, possibly because the composition of the residual noncollagenous compounds was not identical.

The rather high proline content of 1-10 A collagen was interesting in view of the exceptionally high proline in the corresponding trypsin-soluble fractions. This had a very unusual composition, most of the values, including those of tyrosine and hydroxylysine, indicating fairly equal proportions of collagen and noncollagenous proteins. However, the results obtained for hydroxyproline (and lysine), suggesting a very high collagen content, conflicted with the low alanine value. No evidence was found for the presence of the unknown ninhydrin-positive compound (Pearson and others, 1969; Shentall, Happey, Pearson, Naylor, and Turner, 1969; Kao, Leslie, Hitt, and McGavack, 1970) which appears to be characteristic of trypsin-soluble components of older discs. The dialysis precipitate from such a fraction contained the highest proportion of the unknown so far encountered (Table III). Not surprisingly, the precipitate was slightly enriched in collagen (compare with the unprecipitated fraction of 59-66 B) but the noncollagenous character clearly predominates.

Discussion

AMINO ACID COMPOSITION: PURIFICATION OF INSOLUBLE COLLAGEN

Significant errors in the determination of some amino acids, including hydroxylysine, must be considered when collagens from different tissues are compared. However, there is little doubt that hydroxylysine shows real variations. A fundamental change in biosynthesis is not involved, because hydroxylation

Table III Amino acid composition of collagens and trypsin-soluble components

Amino acid residues/1,000 residues	Collagens					Trypsin-soluble (released in 1 hr at 37°C)		
	1-10	7		50-70		1-10	59-66	54-72*
Age (yrs)								
Location	A	N	N	A	Tendon†	A	B	N
Period of hydrolysis (hrs)	24	24	36	36	24	24	24	24
Hydroxyproline	76	75	73	88	85	68	3	6
Aspartic acid	42	38	49	59	46	74	99	92
Threonine	18	20	24	18	17	41	71	52
Serine	37	42	42	37	33	57	69	69
Glutamic acid	74	87	99	74	77	94	117	120
Proline	131	120	113	114	120	232	69	48
Glycine	338	338	313	329	337	180	113	180
Alanine	102	106	108	111	113	61	63	82
Valine	21	15	20	20	21	33	63	41
Cystine	—	—	—	—	—	—	24	3
Methionine	—	—	Tr	Tr	Tr	5	14	1
Isoleucine	11	10	13	11	12	19	44	32
Leucine	21	27	33	25	25	41	73	81
Tyrosine	4	3	2	3	3	13	36	27
Phenylalanine	27	20	19	14	14	19	35	33
Hydroxylysine	15	16	14	14	10	7	—	—
Unknown‡	—	—	—	—	—	—	18	25
Ornithine	Tr	7	Tr	1	5	1	Tr	Tr
Lysine	22	23	21	22	25	19	41	41
Histidine	5	6	5	5	6	12	21	13
Arginine	56	47	54	50	51	24	25	52

Tr = Trace.

* Precipitate obtained on dialysis of trypsin-soluble fraction.

† Results obtained by Steven and Jackson (1967) on tendon collagen purified by alpha-amylase treatment.

‡ x_n , overlapping with hydroxylysine (Shentall and others, 1969).

occurs after the lysine is incorporated into the peptide chains. In the present work 14 to 16 residues of hydroxylysine/1,000 residues were found in collagen of the nucleus pulposus or annulus fibrosus, significantly higher contents than in many mammalian collagens (Eastoe, 1967). These results agree fairly well with values of 12 to 15 residues found by Steven, Jackson, and Broady (1968) in polymeric collagen and gelatins derived from the disc. The collagen was purified by a treatment with crude bacterial alpha amylase. We have found this method to be ineffective against nucleus pulposus, but this is not necessarily true of the annulus fibrosus. The above workers used complete intervertebral discs but the yield of polymeric collagen was low.

The water-insoluble residue of mature (but not usually aged) annulus fibrosus may contain up to 12.6 per cent. hydroxyproline of the dry weight (equivalent to 88 residues/1,000, compare with Table III) before further purification, whereas values of 7 to 11 per cent. are more usual for analogous fractions of the nucleus or intermediate zones (Pearson, Dickson, Happey, Naylor, and Turner, 1966). It is quite clear that the removal of the insoluble non-collagenous proteins which accumulate on ageing is very difficult without affecting the collagen.

Purification of the collagen by proteolysis is unlikely to satisfy the purists, but earlier claims (e.g. Neuman and Tytell, 1950) concerning the resistance

of native collagens to trypsin have been mainly vindicated. The attack of the enzyme is confined to non-helical regions, of which the best characterized are the telopeptides at the N-terminal end of the molecule (Drake, Davison, Bump, and Schmitt, 1966). Effects on the physical properties of insoluble or even soluble collagens (Kühn, Schuppler, and Kühn, 1965) are minimal. Milder conditions of digestion than used here may sometimes be more appropriate, but the technique is likely to be most needed in studying cartilaginous and other intractable tissues. More information on the less accessible collagens is certainly required.

Purification appears to be less difficult with young tissue, especially of species other than human. Furthermore, if lathyrism is induced, a high yield of soluble collagen (lacking covalent cross-linkages) may be obtained. With such an approach, Trelstad, Kang, Igarashi, and Gross (1970) have recently isolated purified alpha chains from lathyrus chicken xiphoid cartilage. The most important conclusion from this work and that of Miller and Matukas (1969) is that most if not all of the collagen in the cartilage matrix is quite different from that in skin, tendon, or bone and a different structural gene is indicated. The cartilage collagen was deficient in alpha 2 chains, apparently consisting of three alpha 1 chains which were not identical with the alpha 1 of the other collagens.

A comparison with insoluble collagens is complicated by many factors, but it should be emphasized that the purified alpha 1 chains from 2½-week-old chickens (Trelstad and others, 1970) contained about 20 residues of hydroxylysine/1,000 *i.e.* even higher than found here or by Steven and others (1968). A reduction in hydroxylysine in early life and a corresponding increase in lysine has been observed with bone collagen (Miller, Martin, Piez, and Powers, 1967). There now seems to be the possibility of more fundamental changes during maturation or ageing, possibly leading to the replacement of one type of collagen with another. Species differences may be significant (Click and Bornstein, 1970), so that the problems of purifying aged human collagen demand attention. The present results suggest that a small part of the associated noncollagenous protein resists an extended trypsin digestion, confirming earlier indications (Pearson and others, 1966). Impressive data on the firmness of the association with the collagen was also obtained by Steven and others (1968). It is tempting to believe that covalent linkages are involved, but positive evidence is lacking.

COLLAGEN-BOUND SUGARS

Difficulties in purification have contributed to the uncertainties relating to the sugar components of collagen. Fortunately, it is now clear that the problem may be circumvented, because of the different sizes of the glycopeptides derived from collagen and other proteins. A separation on Sephadex G25 was easily accomplished and appeared to be almost complete, as was found by Cunningham and Ford (1968) and Spiro (1969), using collagenase followed by trypsin or pronase instead of papain alone. Completely different results were reported for collagenase-pronase digests of corneal collagen, more hexosamine occurring in the second peak than in the first (Bosmann and Jackson, 1968). It may be significant that both glycopeptide peaks were eluted much earlier than found here (taking into account different column dimensions) and that recoveries were much lower. Contamination with glucose derived from the Sephadex is a hazard. However, column blanks (orcinol) were always low and the glucose:galactose ratio found for a Peak 2 separated on a polyacrylamide gel (Biogel P10) agreed very well with the result obtained using Sephadex G25.

The quantity of hexose bound to the collagen of the nucleus pulposus was very high, an average of 4.2 g./14 g. hypro being obtained (ignoring the anomalous result for 3-5N). This method of expression gives values approximating to percentages for a highly purified collagen, but as long as a ratio is employed less pure collagens may be compared. The present value was nearly ten times that of bovine skin collagen (0.45 g./14 g. hypro) which we determined in the same way. A high degree of glycosylation

of the hydroxylysine is indicated, up to about 80 per cent. *i.e.* about 12 of the 14-16 residues. The purified alpha 1 chains from chicken cartilage referred to above contained 5.5 per cent. of hexose (galactose:glucose, 2:1) so 85 per cent. of the correspondingly larger quantity of hydroxylysine (22 residues/1,000) was glycosylated. The differences in sugar and hydroxylysine content compared with other types of collagen were more marked than differences in general amino acid composition, important though the latter undoubtedly are. In different regions of the disc the insoluble collagens seem to differ chemically mainly in the degree of glycosylation, at all ages the collagen in the annulus containing only about half the hexose of that in the nucleus pulposus. Further work on the individual alpha chains may reveal other differences.

Trypsin had an insignificant effect on the values, from which it was deduced that the hexoses are not concentrated in the more accessible regions of the molecule. It was demonstrated that collagen hexose was in fact liberated, especially in an overnight treatment, but the total quantity was very small in relation to that in the insoluble residue. Hexose in rat skin collagen has been located in a small peptide, approximately 10 per cent. of the total length of the alpha 1 chain from the N-terminal end (Butler, 1970), but an additional glycopeptide with a different amino acid sequence occurs in human skin collagen (Morgan, Jacobs, Segrest, and Cunningham, 1970). The positions of the glycopeptides in the cartilage type of collagen have not been determined. It will be interesting to discover whether high hexose collagens contain unique sequences essential to the specificity requirements of the enzymes responsible for hydroxylation and glycosylation of lysine. The possible occurrence of other types of glycopeptides, not involving hydroxylysine, must not be dismissed.

Without more analyses and statistical examination, it cannot be said whether the variations observed in the hexose content of nucleus collagen are related to age. A safe conclusion is that any such changes occurring after the first decade are small. On the other hand a downward trend with age in the annulus collagen Peak 2 was fairly definite, the difference between the two extremes being large. We have not found any evidence so far of a reduction brought about by degradative enzymes in the disc. Another possibility is an inhibition of the glucosyl- or galactosyltransferases referred to above.

A number of authors have speculated on the possible effects of the bound hexose on fibril formation (see Morgan and others, 1970; Bailey, Peach, and Fowler, 1970, for recent discussions of the problem). One of the most important considerations is that the hexoses occur at or near the sites of potential intermolecular cross-linkages (Bailey and others, 1970). The formation of such linkages may therefore be sterically hindered, restricting any increase in fibril

diameter. The fibrils are thicker in the annulus than in the nucleus, supporting the idea of an inverse relationship with hexose content (Grant and others, 1969). If the fall in the hexose level in the annulus with age allows cross-linking to increase further, this may become detrimental, because flexibility as well as strength is needed. In the young nucleus, a random arrangement of very flexible fibrils seems to be essential and even a moderate cross-linking may oppose the correct functioning of the gel.

A number of complicating features have yet to be resolved. Leibovich and Weiss (1970) have postulated that the fibril diameter-limiting mechanism is governed by the B end (C-terminal) of the molecule, which has been much less studied from the point of view of cross-linkages than the A end (N-terminal). We have found appreciable fluctuations in fibril diameter at different ages in both regions of the disc, apparently unrelated to collagen hexose content (Pearson, Happey, Palframan, Render, Naylor, and Turner, 1971). Other factors, particularly the association with noncollagenous compounds, probably affect the ultimate physical characteristics of the fibrils.

THE NON-COLLAGENOUS SUGAR COMPONENTS

An accurate measurement of noncollagenous components of insoluble tissue fractions is difficult, but they contain sugars which act as valuable markers. The excluded Peak 1 obtained here was significant at all ages in the nucleus and it rose dramatically after the fourth decade. Peak 1 had a similar composition in the annulus but the total quantities were much smaller. Nevertheless, a significant increase occurred in the oldest specimen.

About 40 per cent. of the neutral sugars consisted of mannose and fucose, strongly suggesting that glycoproteins were important components. The significance of the glucose is difficult to assess at present. It has rarely been found in well-characterized non-collagenous glycoproteins, although it has been claimed to occur in a relevant fraction of cartilage (Dunstone and Franek, 1967). Contamination with glycogen was possible, but it should be noted that the quantity of glucose increased in the oldest nucleus pulposus, even though it represented a smaller proportion of the total neutral sugars.

Peak 1 and the trypsin-soluble fractions provide good starting points for the further fractionation of the glycopeptides. The uronic acid is effectively removed by precipitation with cetyl pyridinium chlor-

ide, but keratan sulphate, a very probable component, is more difficult to separate. It seems likely that glycoproteins (distinct from keratan sulphate) also occur in the water-soluble fraction, but there is scant information on them. The proportion of mannose and fucose found in unpurified water extracts was appreciable (Table II) although lower than in fractions of the insoluble residue.

The reasons for the affinity for the collagen require further investigation. Hallén (1970), who was considering various proteoglycans, suggested that a lower molecular weight (shorter polysaccharide chains) and a higher protein content favour affinity for collagen. The second factor applies with greater force to glycoproteins of the type encountered here and the apparently similar compounds studied by Robert and his colleagues (Robert and Robert, 1968; Moczar, Moczar, and Robert, 1967). Trypsin-soluble fractions from old tissue contain 70 per cent. or more protein and usually less than 20 per cent. of sugars.

Summary

The amino acid compositions of intervertebral disc insoluble collagens, purified by digestion with porcine trypsin, were fairly normal except for elevated hydroxylysine contents (14-16 residues/1,000 residues). Trypsin removed, not always completely, saline-insoluble noncollagenous glycoproteins which were present in highest amount in older nucleus pulposus and which were recognized by their significant mannose and fucose contents. Other differences from the sugar composition of the saline-soluble proteoglycans were noted. After papain digestion a simple gel filtration technique separated the non-collagenous sugars from the collagen glycopeptides (Peak 2). The latter contained only galactose and glucose, the proportions of which were determined by gas chromatography. Peak 2 hexose content of the collagen in the annulus fibrosus was lower than in the nucleus pulposus and unlike the latter it appeared to decrease with age.

The implications of these results in relation to the hydroxylysine contents and the physical forms of the fibrils are discussed.

Mr. D. McCormick gave technical assistance. Financial support was received from the Medical Research Council, the Leeds Regional Hospital Board, and the Laming Evans Fund of the Royal College of Surgeons of England.

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