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The Isolation, and Amino Acid and Carbohydrate Composition, of Polymeric Collagens Prepared from Various Human Tissues

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1. Insoluble polymeric collagens from various human tissues were prepared by the EDTA method. Almost all of the collagen from simple soft tissues such as dermis, tendon, submucosa, sclera and cornea could be extracted, whereas the more complex tissues such as intercostal cartilage and intervertebral disc yielded only small amounts of collagen. Amino acid and carbohydrate analysis indicated that most of the preparations were highly purified on the basis of their tyrosine, hexosamine, mannose, xylose and fucose contents. 2. Wide variation in the total hexose content was observed, the lowest being 8.5 residues/3000 amino acid residues for collagen from dermis and the highest being 42.1 residues/3000 in corneal collagen. The molar ratios of sugars also varied, submucosal collagen having a galactose/glucose ratio of 1.0 and corneal collagen having a ratio of 2.3. 3. The presence of glucosylgalactosylhydroxylysine was confirmed in submucosal collagen by compositional and chromatographic analysis of this component after its isolation from alkaline hydrolysates of the collagen. Evidence was also obtained for the presence of galactosylhydroxylysine. 4. Determination of the hydroxylysyl glycosides was carried out and it was observed that the amounts of these components varied widely from tissue to tissue. Corneal collagen contained 19.1 hydroxylysine-linked carbohydrate units/3000 amino acid residues, whereas tendon collagen contained only 4.1 units/3000. Variation in the ratio disaccharide unit/monosaccharide unit was also observed, the ratio being 1.2 in intercostal cartilage collagen and 4.1 in submucosal collagen. The proportion of the total hydroxylysine that was substituted by carbohydrate also varied from tissue to tissue.

Although much is now known about the chemistry of collagen, most studies have so far dealt with soluble collagen, which accounts for only a small portion of the total collagen in most tissues, and further, few studies have dealt with human collagens. It was therefore decided to prepare and analyse insoluble collagens from human tissues to determine the differences in the composition of collagens extracted from various tissues.

Insoluble polymeric collagens have usually been prepared by exhaustive extraction of tissues with various buffers in the hope that all non-collagen components are removed (Rubin *et al.* 1965). Steven has developed two techniques for the preparation of highly purified native insoluble collagen, which involve incubation of the tissue with crude bacterial α-amylase (Steven, 1964) or extraction with a chelating agent (Steven, 1967), and which result, in either case, in the insoluble collagen becoming dispersible in dilute acetic acid. The collagen may then be purified by its precipitation from dispersion by the addition of salt, by raising the pH to near neutrality, or by the addition of organic solvents such as acetone, and such procedures have been shown to produce collagen of higher purity than that obtained by exhaustive extraction (Grant & Jackson, 1968).

The demonstration of the presence of proteolytic activity in crude bacterial α-amylase (Michlik, 1969; Bailey & Etherington, 1970; Steven, Grant, Ayad, Weiss & Leibovich, 1970), however, has thrown some doubt on the mode of action of the amylase, in view of the ability of proteases to depolymerize insoluble collagen (Nishihara & Miyata, 1962; Drake, Davison, Bump & Schmitt, 1966), although Steven *et al.* (1970) showed that inhibition of the proteolytic activity did not prevent dispersion of the insoluble collagen. It is also possible, since the amylase also contains a variety of glycosidases (Muzzafar, 1968), that cleavage of carbohydrate covalently attached to collagen might occur, although Spiro (1969)

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showed that an α-glycosidase from *Aspergillus niger* would not cleave glucose from glucosylgalactosylhydroxylysine whether this is in the free state or in peptide linkage. The mode of action of chelating agents in facilitating the dispersion of insoluble collagen is also not known, but the procedure is not envisaged as leading to any type of degradation, and for this reason EDTA was used during the preparation of the human polymeric collagens.

It has been suggested that there may be some relationship between fibril diameter and carbohydrate content of collagens (Robert & Robert, 1967; Rudall, 1968; Grant, Freeman, Schofield & Jackson, 1969; Spiro, 1969), and a further object of this study was to examine this aspect more fully. This was done by determining the total neutral sugar content and the hydroxylysyl glycoside content of the collagens obtained from various tissues, and comparing these values with known fibril diameters (Harkness, 1961). The presence of glucosylgalactosylhydroxylysine and galactosylhydroxylysine, which have been found in various non-human collagens (Butler & Cunningham, 1966; Cunningham & Ford, 1968; Spiro, 1969) and recently in human collagens (Segrest & Cunningham, 1970), was also confirmed.

METHODS

Preparation of collagens. Human tissues were obtained *post mortem* from subjects having no record of connective tissue diseases. All operations were carried out at 4°C. Achilles tendons were dissected free of muscle, fat and tendon sheath; the epidermis and subcutaneous fat were removed from abdominal skin to leave the dermis; sclera were dissected free of muscle, optic nerve, ciliary muscle and cornea and the retina and choroid were scraped away; corneas were dissected out as described by Freeman, Steven & Jackson (1968); submucosae were prepared as described by Steven, Jackson, Schofield & Bard (1969); perichondrium was removed from intercostal cartilage; whole lumbar intervertebral discs were used without subdivision and heart valves from a single heart were pooled. The tissues were cut up finely, frozen in liquid N_2 and disintegrated in a stainless-steel hammer mill (Walser & Bodenloss, 1951). The powdered tissues were defatted by soaking in acetone and, after re-equilibration with water, they were extracted with 1.0M-NaCl, 0.07M-Na_2HPO_4 and 0.15M-sodium citrate, pH3.7, successively to remove soluble collagen, glycoproteins and proteoglycans.

After removal of the salts by dialysis against water, the tissues were incubated at 4°C for 18h with 4% (w/v) EDTA, pH7.4, and the collagen was subsequently dispersed in dilute acetic acid as described by Steven (1967). The acetic acid extract was then centrifuged at 300*g* for 20min and the dispersed collagen in the supernatant was precipitated by addition of saturated NaCl to give a final concentration of approx. 5% (w/v). The white fibrous precipitate was re-dispersed in acetic acid, centrifuged at 300*g* for 20min and re-precipitated by raising the pH to about 6.0 by addition of 1.0M-NaOH. The re-dispersion and re-precipitation were carried out a further four times by using salt and alkali alternately to precipitate the collagen. The collagen was dialysed exhaustively against water and finally freeze-dried.

Amino acid analysis. Determinations of amino acids were made with a Technicon amino acid analyser as described by Steven & Jackson (1967), after hydrolysis of samples in 6M-HCl in sealed Pyrex tubes, under N_2 at 105°C for 24h at a concentration of 0.5mg of collagen/ml of acid. Hydrolysates were filtered through glass wool and then dried on a rotary evaporator at 50°C. Traces were integrated by using a Technicon integrater–calculator and no corrections were applied for hydrolytic losses. Minor components were determined by chromatographing five times the normal amount of hydrolysate.

Carbohydrate analysis. Collagen samples were hydrolysed and total neutral sugar and total hexosamine determined spectrophotometrically as described by Grant & Jackson (1968). G.l.c. of the neutral sugars was carried out as described by Grant *et al.* (1969).

Partial characterization of hydroxylysyl glycosides. Submucosal collagen (500mg) was hydrolysed with 2M-NaOH under N_2 for 24h at 105°C at a concentration of 15mg/ml in polypropylene bottles sealed into large Pyrex tubes. The hydrolysate was acidified with 2M-HCl to pH2.5 and desalted as described by Spiro (1967). The desalted hydrolysate was then applied to a column (5.5cm×100cm) of Sephadex G-25 (fine grade), to separate the hydroxylysyl glycosides from the amino acids, and elution was carried out with 0.1M-pyridine acetate, pH5.0, at a flow rate of 20ml/h; 10ml fractions were collected. Amino acids were detected by the method of Moore & Stein (1948) and carbohydrate was detected by the method of Fuller & Northcote (1956) and the carbohydrate-containing fractions were pooled and freeze-dried. The material was dissolved in water and applied as a streak to the midline of a piece of Whatman no. 3 paper (110cm×40cm) at a concentration equivalent to approx. 2.5mg of collagen/cm. Separation of the carbohydrate-containing components was achieved by electrophoresis at a potential of 45V/cm in pyridine–acetic acid–water (5.0:0.1:975, by vol.) buffer, pH6.5, in a Savant electrophoresis tank. Guide strips were stained for amino acids with 1% (w/v) ninhydrin in acetone and for carbohydrate with a periodate–2,4-pentanedione reagent (Weiss & Smith, 1967). The carbohydrate-staining bands were cut out and eluted with water, and any neutral carbohydrate eluted from the paper was removed by adsorbing the hydroxylysyl glycosides on to Dowex 50 (X8; 200–400 mesh; H^+ form) and eluting them with aq. 1.5M-NH_3 soln. After removal of the NH_3 by rotary evaporation the material was hydrolysed with a suspension of Dowex 50 resin in 0.05M-HCl (Grant & Jackson, 1968) and the neutral sugars were identified and determined by g.l.c. Amino acids were identified by paper chromatography on Whatman no. 1 paper by using butanol–acetic acid–water (4:1:5, by vol.) as solvent, and they were determined by the method of Moore & Stein (1948). The material in the bands was also analysed chromatographically with the amino acid analyser.

Determination of hydroxylysyl glycosides. Collagens were hydrolysed in 2M-NaOH and the acidified hydrolysates desalted as described above. Glucosylgalactosylhydroxy-

lysine was found to co-chromatograph on our amino acid analyser with isoleucine. It was therefore necessary to remove the neutral amino acids and this was achieved either by high-voltage electrophoresis, as described above, or by paper chromatography (Spiro, 1969). The basic amino acids were eluted from the paper and applied to the amino acid analyser.

RESULTS AND DISCUSSION

Preparation of collagens. Treatment of tendon, dermis, submucosa, sclera and cornea with EDTA resulted in almost complete dispersion of the insoluble collagen in dilute acetic acid. Very little material sedimented on centrifugation of the acetic acid dispersion at 300*g*, although the actual recoveries were not determined. On the other hand, very little collagen was obtained from intercostal cartilage, intervertebral disc and heart valve, since most of the tissue sedimented on centrifugation. Low yields of dispersed collagen have also been observed by using the α-amylase method on the more complex tissues (Steven, 1970), which may be indicative either of a different type of collagen–proteoglycan interaction, or of a greater extent of interaction in these tissues compared with the simpler soft tissues such as dermis or tendon.

The mechanism by which EDTA facilities dispersion is not understood, but removal of bivalent metal ions seems to be involved since the insoluble collagen cannot be dispersed if Ca^{2+} is dialysed back into the tissue after incubation with EDTA (Steven, 1967). The concept of metal ions participating in the stabilization of connective tissues also received support from the work of Spichtin & Verzar (1967), who found that removal of Ca^{2+} from connective tissue led to a decrease in the stability of the tissue to heat denaturation. Veis, Bhatnagar, Shuttleworth & Mussel (1970), however, considered that the Ca^{2+} content of bovine dermis (6 μg/g of collagen) was too low to account for the action of chelating agents in facilitating dispersion of the insoluble collagen. However, if one calculates this amount of Ca^{2+} on a molar basis, there is approx. 1 mol of Ca^{2+}/20 mol of collagen, which could conceivably be significant in interactions either between collagen fibrils or between collagen fibrils and interfibrillar-matrix components.

EDTA can also solubilize proteoglycans and glycoproteins (Herring, 1968), which might, by their interactions with collagen, prevent its dispersion. It is also possible that EDTA treatment might lead to depolymerization of proteoglycans by analogy with its effect on hyaluronic acid (Ogston & Sherman, 1959), which is thought to be due to production of free radicals. Experiments involving incubation of bovine tendon with EDTA in the presence of the free-radical 'scavenger' dimethylsulphoxide, however, showed that the presence of this compound had no effect on the amount of collagen dispersed (J. D. Schofield, unpublished work). Of the three explanations of the effect of EDTA, the one concerning removal of metal ions would appear to be the most likely.

Amino acid composition. The amino acid compositions of the polymeric collagens from various tissues are presented in Table 1. In general the results show good agreement with each other,

Table 1. *Amino acid compositions of EDTA-prepared human polymeric collagens*

Compositions are expressed as residues/1000 amino acid residues.

	Tendon	Dermis	Sub-mucosa	Sclera	Cornea	Heart valve	Disc	Cartilage
Hyp	85.9	92.1	96.3	88.6	82.9	98.6	84.2	99.8
Asp	45.0	42.1	47.5	45.5	48.8	42.1	43.9	47.8
Thr	16.8	15.6	20.2	17.0	18.8	16.5	19.5	18.5
Ser	33.8	32.1	38.4	31.8	31.8	29.7	31.8	29.4
Glu	78.0	76.4	81.4	74.7	81.7	72.9	81.5	77.5
Pro	120.0	128.4	95.9	116.2	102.6	117.2	111.5	109.0
Gly	334.2	334.6	321.2	332.9	332.2	324.2	317.4	330.5
Ala	114.1	111.0	104.8	112.4	106.2	113.6	119.3	106.9
Val	23.5	24.7	25.1	27.7	25.5	26.6	25.1	25.8
Met*	5.5	6.7	6.7	5.5	5.9	7.4	6.6	5.6
Ile	10.0	11.3	15.2	13.4	14.0	12.0	11.8	11.1
Leu	24.8	23.1	31.9	27.7	30.6	30.2	29.9	26.6
Tyr	3.6	3.6	6.9	3.5	3.9	3.4	3.0	3.7
Phe	13.8	10.0	12.7	14.4	13.2	11.6	13.7	14.0
Hyl	8.7	3.6	6.2	6.4	8.7	7.9	10.0	5.4
Lys	24.4	27.6	29.4	27.1	31.8	24.9	24.9	24.6
His	5.9	4.5	6.9	5.7	5.4	5.7	6.6	6.1
Arg	52.9	50.0	53.2	49.5	49.6	51.0	54.5	53.0

* Methionine + methionine sulphoxides.

and in particular the analysis for tendon collagen agrees well with an analysis of a gelatin from the same tissue (Eastoe, 1955) and one of an α-amylase-prepared collagen from that tissue (Steven & Jackson, 1967), and the analysis of the dermal collagen agrees well with an analysis of a gelatin from human dermis (Fleischmajer & Fishman, 1965). The agreement of the analyses for these EDTA preparations with those of gelatins from the same tissues suggests that the collagens are highly purified, and this is also suggested by the very low tyrosine contents with the exception of that of the submucosal preparation.

The hydroxylysine values ranged from as low as 3.6 residues/1000 amino acid residues in dermal collagen to 10.0 residues/1000 in disc collagen, whereas tendon and corneal collagen both had 8.7 residues/1000. Variability of the hydroxylysine content of collagens extracted from various tissues of the rat was also observed by Piez & Likins (1957).

A component chromatographing in the position of ornithine was observed in trace amounts in all collagens examined, an observation that has also been made by Jackson, Leach & Jacobs (1958), Steven & Jackson (1967) and Steven (1967). Although the component was not characterized in the present work, the work of Bensusan (1969) indicates that ornithine is present in collagen preparations, although an explanation of the way in which it arises has not been forthcoming.

3-Hydroxyproline was also observed in some collagens, but was present only in trace amounts, and was sometimes obscured by methionine sulphoxides.

Carbohydrate composition. The total neutral sugar and total hexosamine contents, as determined spectrophotometrically, and the identity and relative concentrations of the individual neutral sugars, as determined by g.l.c., associated with the various collagens are as indicated in Table 2. The values were calculated in terms of the hydroxyproline present in the 'acid' eluate after hydrolysis on resin, assuming 94 residues of hydroxyproline/1000 amino acid residues as described by Grant & Jackson (1968), and the results for these multi-sugar solutions must be interpreted with some caution, as pointed out by these authors. Insufficient collagen was available from cartilage and heart valve for these determinations.

It is evident that all the collagens, apart from the disc collagen, are reasonably pure as judged by their hexosamine, mannose, fucose and xylose contents. These sugars, which are thought to be associated with proteoglycan or glycoprotein contaminants, are virtually absent in highly purified collagens (Grant & Jackson, 1968) and are present at low concentrations in most of the collagens, and especially in the dermis and tendon collagens. Spiro (1969) has suggested that the mannose, fucose and hexosamines associated with collagen preparations might be bound covalently to the collagen as some type of heteropolysaccharide unit. The virtual absence of these sugars from highly purified soluble collagen (Grant & Jackson, 1968) and the polymeric dermis collagen (Table 2), and their variable molar ratios in other collagens (Table 2; see also Grant *et al.* 1969), indicates that not all collagen molecules can possess such a unit and that the composition of the units must vary from tissue to tissue. Although no evidence has been obtained to show whether the sugars are covalently attached to collagen or not, their presence in non-collagenous contaminants seems more likely.

The intervertebral disc collagen, on the basis of its hexosamine content, is grossly impure, suggesting that the collagen ground-substance interactions are much stronger in this tissue than in the simple soft tissues such as dermis and tendon. In view of the high hexosamine content, the neutral-sugar content was determined after passing the water eluate, after hydrolysis on resin, through an anion-exchange resin (Bio-Rad AG2: 200–400 mesh; HCO_3^- form) to remove uronic acids, which interfere

Table 2. *Carbohydrate composition of EDTA-prepared human polymeric collagens*

Compositions are expressed as residues/3000 amino acid residues, since the tropocollagen molecule has been shown to consist of three α-chains, each of which contains approx. 1000 amino acids (see Piez, 1967). The results were calculated assuming a hydroxyproline content of 94 residues/1000 amino acid residues (see text). Abbreviation: Tr, trace.

	Total hexose	Total hexosamine	Molar proportions of sugars Gal : Glc : Man : Fuc : Xyl	Gal/Glc ratio
Dermis	8.5	0.5	4.9 : 3.9 : Tr : Tr : 0	1.3
Tendon	8.7	0.9	5.0 : 3.1 : 0.6 : Tr : Tr	1.6
Submucosa	17.9	1.9	8.0 : 8.0 : 1.9 : Tr : 0	1.0
Cornea	42.1	2.8	28.4 : 12.1 : 1.6 : Tr : Tr	2.3
Sclera	16.8	1.0	7.5 : 8.0 : 1.3 : Tr : Tr	0.9
Disc	9.8	110.0	5.6 : 3.3 : 0.8 : Tr : Tr	1.7

with determination of the neutral sugars. It might have been expected that the xylose, fucose and mannose contents would also have been abnormally high, but this was not observed, making interpretation of the nature of the contaminant difficult. The high degree of contamination of the disc collagen was not borne out by its amino acid analysis, which suggested that the collagen was as pure as the other preparations. The tyrosine content of the submucosal preparation suggested an abnormally high degree of contamination, but the carbohydrate composition was not indicative of such contamination. It is evident that amino acid analysis alone is not an adequate criterion of purity of a collagen preparation, and that other parameters, such as carbohydrate composition, should also be taken into account.

Partial characterization of hydroxylysyl glycosides. Fractionation of an alkaline hydrolysate of submucosal collagen on Sephadex G-25 resulted in separation of a carbohydrate-containing peak from the bulk of the amino acids. The material in the carbohydrate-containing fractions was further fractionated by high-voltage electrophoresis, which resulted in the observation of three ninhydrin- and periodate–2,4-pentanedione-positive bands having R_{Hyl} values of 0.70, 0.72 and 0.65. Analysis of the neutral sugars and amino acids, after hydrolysis on resin of the slowest-moving component, revealed the presence of glucose, galactose and hydroxylysine in the molar proportions 1.1:0.9:0.9. Inadequate amounts of sugars were isolated after resin hydrolysis of the faster-moving components to permit either their identification or determination by g.l.c., but hydroxylysine was identified as the only amino acid present. Amino acid analysis of the unhydrolysed material in the bands revealed that the material in both faster-moving components chromatographed as a double peak just after phenylalanine, whereas the material in the slower-moving band chromatographed as a double peak in the position of isoleucine. It was concluded from the compositional results and by comparison of the chromatographic positions reported by Spiro (1969), that the faster-moving components were galactosylhydroxylysine and the slower-moving component was glucosylgalactosylhydroxylysine. The reason why two bands were observed for galactosylhydroxylysine is not known. Cunningham, Ford & Segrest (1967) and Cunningham & Ford (1968) observed two peaks containing galactose and hydroxylysine when alkaline hydrolysates of glycopeptides from collagen were chromatographed on Dowex 50 and they attributed the occurrence of two peaks as being due to alkali-catalysed racemization at the α-carbon atom of the amino acid. It may be that this type of racemization can explain, the occurrence of the two bands containing galactose and hydroxylysine, although the material from both bands also chromatographed as a double peak on the amino acid analyser. Although the mode of linkage of the sugars to each other and to hydroxylysine was not investigated, it seems likely that the structures of the two glycosides are the same as those determined by Spiro (1969) in ichthyocol.

The positions in which the two glycosides were eluted from the amino acid analyser were somewhat different from those reported by Spiro (1967, 1969), but he has also found some variation in the elution positions, apparently depending on the batch of resin used in the column, although the reason for this variability is not known.

Determination of the hydroxylysyl glycosides in various collagens. The emergence of glucosylgalactosylhydroxylysine in the position of isoleucine on the amino acid analyser made direct amino acid analysis of alkaline hydrolysates impossible, and a preliminary separation of the basic residues from the neutral and acidic residues was effected either by high-voltage electrophoresis or by paper chromatography, virtually identical results being obtained by either method. The results obtained for the determinations of hydroxylysyl glycosides in the various collagens are presented in Table 3.

All collagens examined contained both glucosylgalactosylhydroxylysine and galactosylhydroxylysine, although wide variation in the amounts of these components occurred from one tissue to another, ranging from 4.1 units/3000 amino acid residues in the tendon collagen to 19.1 units/3000 residues in the corneal collagen. The collagens from cornea and heart valve were the most highly glycosylated, 73% and 60% respectively of the total hydroxylysine being glycosylated, whereas only 16% of the hydroxylysine in tendon was glycosylated. This confirmed that variation in the total neutral-sugar content (Table 2) was in fact due to variation in the amount of sugar covalently bound to collagen. Spiro (1969) has also observed variation in the degree of glycosylation of collagens from various tissues of non-human species.

In all the collagens examined in the present work the disaccharide/monosaccharide ratio was greater than 1 (Table 3), the range being from 1.2 in cartilage collagen to 4.1 in submucosal collagen. Segrest & Cunningham (1970) showed that bone collagen has a disaccharide/monosaccharide ratio of 0.15, but whether this difference between bone and soft-tissue collagens has any significance in relation to function is not known.

The amount of hexose contained in hydroxylysyl glycosides (Table 3) compares quite well with the total hexose content determined spectrophotometrically for dermis, tendon, submucosa and sclera, most of the hexose being accounted for as hydroxylysyl glycosides. The agreement in cornea

Table 3. *Determination of the hydroxylysyl glycosides in EDTA-prepared human polymeric collagens*

Compositions are expressed as residues/3000 amino acid residues. Total hydroxylysine was determined by amino acid analysis of acid hydrolysates, and Glc-Gal-Hyl, Gal-Hyl and 'free' hydroxylysine were determined by amino acid analysis of alkaline hydrolysates. Total hexose, and the contents of galactose and glucose and their molar ratios, were calculated from the Glc-Gal-Hyl and Gal-Hyl contents. Fibril diameters are taken from Harkness (1961).

	Total Hyl	Glc-Gal-Hyl	Gal-Hyl	Free Hyl	Total Hyl / 'free Hyl' ratio	Total hexose	Gal:Glc	$\frac{\text{Gal}}{\text{Glc}}$ ratio	Fibril diam. (Å)
Dermis	10.8	3.1	1.2	6.6	1.6	7.4	4.3 : 3.1	1.4	600–1100
Tendon	25.1	2.5	1.6	21.0	1.3	6.6	4.1 : 2.5	1.6	300–1300
Submucosa	18.6	6.5	1.6	10.5	1.8	14.6	8.1 : 6.5	1.1	—
Cornea	26.1	13.9	5.3	6.9	3.8	33.1	19.2 : 13.9	1.4	200–400
Sclera	19.2	3.6	1.5	14.1	1.4	8.7	5.1 : 3.6	1.4	280–2800
Heart valve	23.7	10.0	4.3	9.4	2.5	24.3	14.3 : 10.0	1.4	200–300
Disc	30.0	8.8	6.0	15.2	2.0	23.6	14.8 : 8.8	1.7	—
Cartilage	16.2	3.1	2.6	10.5	1.5	8.8	5.7 : 3.1	1.8	—

and disc is not as good, however. Similarly, the molar ratios of galactose to glucose as determined by g.l.c. (Table 2) agree quite well with these values calculated from the hydroxylysyl glycoside contents (Table 3) for dermis, tendon, submucosa and disc, whereas the ratios for cornea and sclera show fairly wide variation. For the corneal collagen it is possible that the discrepancy between the total hexose content and the value calculated in Table 3 may be due to the presence of contaminating glycoprotein, since the hexosamine content of this collagen is fairly high. Corneal collagen prepared by the α-amylase method has been shown to have a hexose content of 32.1 residues/3000 amino acid residues, and also a much lower hexosamine content than that prepared by the EDTA method (I. L. Freeman, unpublished work), and this hexose content would agree very well with the calculated value in Table 3.

Attention has been drawn to the apparent relationship between the morphology of the collagen fibril and its carbohydrate content (Robert & Robert, 1967; Rudall, 1968; Grant *et al.* 1969; Spiro, 1969). The results for the carbohydrate analyses presented in Tables 2 and 3 and comparison of the results with known fibril diameters (Harkness, 1961) again confirm that the collagens with low carbohydrate content, such as dermis, tendon and sclera, have a high mean diameter, whereas collagens with a high carbohydrate content, such as in cornea and heart valve, have a low mean value. It is evident, however, that not only the mean fibril diameter varies, but that also the distribution of fibril diameters varies from tissue to tissue. Thus in cornea and heart valve, which have high carbohydrate contents and a low mean fibril diameter, the range of fibril diameters is also very narrow, whereas in the collagens with low carbohydrate contents and high mean fibril diameters, such as dermis, tendon and sclera, the range of fibril diameters is very wide. The results neither prove nor disprove the hypothesis that carbohydrates play a role in determining the morphology of the fibril. It is possible that carbohydrates may be blocking potential cross-linking sites involving hydroxylysine (Bailey, Peach & Fowler, 1970), although no evidence for this has yet been presented. Whether blocking of cross-link formation would affect fibril diameter is also not known. Leibovich & Weiss (1970) have suggested that a sequence of amino acids at the *C*-terminal end of the molecule may have some function in controlling fibril diameter, but whether carbohydrates were involved is again unknown. In discussing differences in fibril diameters from tissue to tissue, it should also be borne in mind that the non-collagen components in the tissues may also have some effect, and that variations in the amount and type of such components in different tissues may be a contributory factor in determining fibril morphology. An entirely different function for carbohydrates in glycoproteins was suggested by Eylar (1965), which was that they in some way facilitated extrusion of the protein from the cell, although experimental proof of this hypothesis has not been forthcoming. Indeed, it has been shown that a soluble form of elastin, another extracellular protein, contains no carbohydrate (Grant, Steven, Jackson & Sandberg, 1971) and this finding may not be in accord with this hypothesis.

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