The content and structural characteristics of the collagenous protein of rabbit lens capsules at different ages

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A collagenous protein containing 15 to 19 per cent (12.9 to 16.7 per cent residues) hydroxyproline is found in capsules of rabbit lenses. The amount of this protein increases from about 0.6 to about 5 per cent by wet weight between the ages of 4 weeks and 1 year. The capsule contains also sialofucoglycoproteins and an acid mucopolysaccharide. The amount of the two carbohydrate polymers increases between 4 weeks and 3 months of age, but later in contradistinction to collagen either remains constant or decreases. The unusually high content of hydroxyproline in the unorganized collagen of the lens capsule appears to be related to its origin from epithelial elements.

In 1953 Pirie[1] reported that beef lens capsules contain a protein which appeared to be collagen, as hydroxyproline was found as one of its amino acids. Electron microscopic studies on lens capsules of rats and mice did not show the characteristic appearance of typical collagen fibrils, but rather the presence of numerous lamellae, apparently connected by some cementlike layers.[2] In 1954[3] it was reported that it is possible to extract a great part of the proteins of beef lens capsule by heating it for 30 minutes with 0.25 N trichloroacetic acid (TCA) at 90°, a behavior characteristic of collagen found in other tissues. The extracted material contained about 10 per cent of a carbohydrate which was resistant to heating with alkali, and consisted only of two hexoses, galactose and glucose, in a ratio of about 1.25 to 1. When this trichloroacetic acid extract was neutralized and dialyzed, the dissolved protein partly precipitated and with it precipitated the characteristic carbohydrate. The ratio of the latter to the protein was nearly identical in this precipitate with that found in the solution before dialysis. It was, therefore, concluded that this carbohydrate is linked by firm chemical bonds to the collagenous protein. It was recently found in our laboratory that in addition to carbohydrate firmly linked to collagen lens capsules of rabbit and beef contain two other types of sugar polymers; one identified as a hexosamino hexuronide and the other containing, in addition to glucosamine, two hexoses—the methylpentose fucose and sialic acid.[4, 5] This finding indicated a fundamental similarity between the composition of the lens capsule and other various forms of connective tissue which were

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shown to contain, in addition to collagen with varying amounts of carbohydrate linked to it, a significant amount of a hexosamino hexuronide and sialofucoglycans in the ground substance between the collagen fibers.[6] This appeared remarkable because of the epithelial origin of the capsule. It had been reported already in 1954[3] that the collagen-linked carbohydrate of the beef lens capsule is unequally distributed in various parts of the lens as, in the polar region, the capsule contains per unit wet weight more protein with a lower content of carbohydrate than in the metabolically active equatorial region. It was already then suggested that this situation may be related to different biological activities of the different parts of the lens and that the concentration of protein and carbohydrate in the lens capsule might influence the diffusion rate through the lens capsule and its elasticity. Investigations, on the other hand, of the hexosamino glycans in various forms of connective tissue suggested that the glycans may play a role in the regulation of the maturation process of collagen.[6] As attempts were repeatedly made to relate the permeability of the lens capsule to the stability of the lens structure, it appeared of particular interest to investigate changes with age in the content in collagenous protein and the carbohydrate linked to it as well as in hexosamino glycans of lens capsules. This report concerns such experiments on the lenses of rabbits.

Experimental

Materials. Lenses from two groups of rabbits, one 8 to 12-weeks-old and the other 1-year-old or more, were obtained from the Pel-Freeze Company in Arkansas in batches of 200 to 400 and arrived in a frozen state by air in the laboratory. In addition, 4-week-old rabbits were killed in the laboratory and the fresh lenses used for the experiment. The capsules were stripped from lenses after they were thawed out superficially. The epithelium was not removed from the capsules, nor was it possible to prevent the contamination by small amounts of lens fibers. The contaminations, however, could be shown not to have any significant effect on the results of the analysis of the capsule. The capsules were weighed, washed repeatedly with saline, and then coagulated and washed with 4 per cent TCA.

Experimental procedures.

Extraction of various constituents of lens capsule. For as accurate as possible determination of collagen, noncollagenous protein (residual), and various types of sugars, it proves necessary to use a fractionated extraction which separates most of the collagen from the residual protein and permits an as accurate as possible determination of hexuronic acids. The following procedure was finally adopted as most convenient: The lens capsule, washed first in saline and then with 4 per cent TCA, was extracted with the latter first at 90° C. for 30 minutes and then for an additional 90 minutes. In some experiments this second time interval was divided in two of 30 and 60 minutes, respectively. During the first time interval 93 per cent of the total hydroxyproline, and that means collagen, was extracted and 75 to 80 per cent of the total hexuronic acid. On the other hand, no more than 6 per cent of the residual protein went into solution. This was found by comparing the amount of hydroxyproline and N extracted during the first, the second 30 minute, and the following extraction periods.

Extraction of hexosamino glycans. The TCA insoluble residue remaining after the first extraction contained about 20 per cent of the total hexuronides of the lens capsule and about 90 per cent of the sialohexosamino glycans. This residue was, therefore, washed with 4 per cent TCA, resuspended in this solution, and re-extracted either directly for 90 minutes or in two stages for 30 minutes and 60 minutes each at 90° in the same volume as was used with the first extraction. After that the extraction residue was washed with a 10 per cent TCA solution and extracted with this solvent during two intervals of 4 and 12 hours at 90°, respectively. As heating at 90° slowly degrades TCA, an appropriate amount of this acid found in control experiments was added every 2 hours to bring up its concentration to 10 per cent in the extraction mixture.

Analytical procedures. Hydroxyproline was determined in 30 minute TCA extracts of the capsule after 6 hour hydrolysis with 5.5 N HCl, according to the method of Neumann and Logan.[7] The amount of collagen present in the capsule was calculated by first determining the nitrogen of the TCA extract by micro-Kjeldahl. Control experiments in which capsules had been extracted during a second 30 minute period indicated that, in the first 30 minute extraction, 93 per cent of the total hydroxyproline and thus of collagen went into solution. The concentration of collagen in the capsule was calculated in the following way: Nitrogen (N) and hydroxyproline (OH Pr) were extracted from the capsule during two successive 30 minute periods with 4 per cent TCA. N 1, OH

Pr 1, and C 1 designate nitrogen, hydroxyproline, and collagen concentrations in the first, and N 11, OH Pr 11, and C 11 in the second extract. The amount of the extracted noncollagen protein (P) is assumed to be equal in the two extracts. This assumption appears valid if the amount of P extracted in 30 minutes is only a small fraction of total P. If we assume that the content of N is 18 per cent in collagen and 16 per cent in P, then the amount of C 1 and C 11 can be calculated from the following equations:

$$(1)\ \frac{\text{C 1}}{\text{C 11}} = \frac{\text{OH Pr 1}}{\text{OH Pr 11}}$$

$$\text{N 1} = 0.18\ \text{C 1} + 0.16\ \text{P}$$
$$\text{N 11} = 0.18\ \text{C 11} + 0.16\ \text{P}$$

$$(2)\ \text{C 1} - \text{C 11} = \frac{\text{N 1} - \text{N 11}}{0.18}$$

$$(3)\ \text{C 1} = \frac{\text{N 1} - \text{N 11}}{0.18} \times \frac{\text{OH Pr 1}}{\text{OH Pr 1} - \text{OH Pr 11}}$$

$$(4)\ \text{C 11} = \frac{\text{N 1} - \text{N 11}}{0.18} \times \frac{\text{OH Pr 11}}{\text{OH Pr 1} - \text{OH Pr 11}}$$

The noncollagen residual protein was determined by nitrogen determinations in the combined 2, 6, and 18 hour extracts and a solution of the extraction residue in 0.5 NaOH. The carbohydrate linked to collagen was obtained by determining the hexoses in the 30 minute extracts by the primary and secondary cysteine reactions of hexoses[8] and calculating the total hexose under the assumption that this carbohydrate consists only of galactose and glucose. The content was then expressed in terms of anhydroresidues of these sugars. Hexosamine was determined in the combined 30 minute, 2, 6, and 18 hour extracts by the indole HCl reaction.[9] The extracts, in addition to the glycoproteins, contain certain amounts of acid mucopolysaccharide. This mucopolysaccharide has been identified in experiments on bovine lens capsule as identical or closely related to heparitin sulfate.[10]

The amount of hexuronic acid present in the 30 minute, 2, 6, and 18 hour TCA extracts was, therefore, determined by the carbozole reaction,[11] and from this the amount of hexosamine linked to this hexuronic acid was calculated. The yield of hexosamine from different mucopolysaccharides in the indole HCl is not completely identical. The calculation was based on the assumption that all hexuronic acid is present in the form of heparitin sulfate, which yields 67 per cent of the theoretical value in our determination. Neuraminic acid could not be accurately determined directly in the 30 minute TCA extract of the capsule because of the large amounts of collagen, hexuronic acids, and hexoses. For this determination, therefore, the 30 minute TCA extract was neutralized with solid $NaHCO_3$ and dialyzed for 24 hours against an equal volume of distilled water. Neuraminic acid was then determined in the dialysate by the Svennerholm[12] method, and its amount calculated under the assumption that it distributes evenly between the dialysant and dialysate and is present as the N-acetyl derivative (sialic acid).

Results

Changes in the concentration of collagen in the lens capsule with the age of the animal. As can be seen in Table I, the capsules of lenses weighing about 500 mg. each from animals one year old or older contained 3.3 to 5.3 per cent collagen based on wet weight. The values were not corrected for small amounts of noncollagen proteins which, however, as can be seen in the table, could not add more than a few per cent to the value for collagen. In lenses of animals between 8 and 12 weeks of age, and with a weight of the lens varying between 200 and 240 mg., the concentration of collagen was only between

Table I. Content of rabbit lens capsules of different ages in collagen, noncollagen (residual) protein, in per cent, and of hexosamine, hexuronic acid, and sialic acid in milligrams per cent of wet weight

	Wet weight (mg.)						
Exper. No.	*Lens*	*Capsule*	*Collagen*	*Residual protein*	*Hexosamine*	*Hexuronic acid*	*Sialic acid*
1	240	4.5	2.59		169.0	108.9	29.2
2	530	11.4	4.85		136.0	87.8	32.4
3	200	6.5	2.07	4.6	143.8	127.4	32.4
4	500	13.0	4.12	5.4	146.6	78.5	32.5
5	500	13.0	3.32		125.8	86.2	28.4
6	500	11.8	5.34		163.9	91.6	39.1
7	100	4.5	0.60	3.8	61.2	44.0	15.5

2 and 2.6 per cent. When the neutralized 30 minute extracts were subjected to extensive dialysis, the concentration of collagen went down by about 20 per cent in lens extracts from young animals, and about 15 per cent in old animals. In lens capsules of 4-week-old animals in which determinations were carried out only after dialysis, the concentration of collagen was not more than 0.6 per cent.

On the other hand, there were no comparable changes with age as far as the residual protein is concerned. In this fraction, however, contamination with lens fiber proteins may have played an ill-defined role.

Concentration of hexosamino glycans in the lens capsule of rabbits of different ages. A very different picture appears when we consider the concentration in lens capsules of sugars which are representative constituents of acid mucopolysaccharides and sialofucoglycans of glycoproteins, namely, hexosamine, hexuronic acid, and sialic acid, of the same animals in which collagen had been determined. In the lens capsules of older rabbits the concentration of hexosamine varies between 126 and 164 mg. per 100 Gm. capsule, of hexuronic acid between 78 and 92 mg., and for sialic acid between 28.4 and 39.1 mg. For the young 8- to 12-week-old rabbits, the same values range between 136 and 169 mg., 109 and 127 mg., and 29.9 and 30.4 mg., respectively. The values for hexosamine, therefore, overlap completely for those with older rabbits. The values for hexuronic acids appear even significantly higher in younger rabbits. In 4-week-old rabbits the values for hexosamine, hexuronic acid, and sialic acid were significantly lower than those for older animals.

The ratios between the concentrations of hexosamino glycans and of collagen in lens capsules of rabbits of different ages. As can be seen in Table II and Fig. 1, the difference in the age distribution of collagen on the one hand and hexosamino glycans on the other hand is particularly well illustrated when the amount of hexosamine, hexuronic acid, and sialic acid is related to the amount of collagen in the lens capsule. In mature animals the amount of hexosamine,

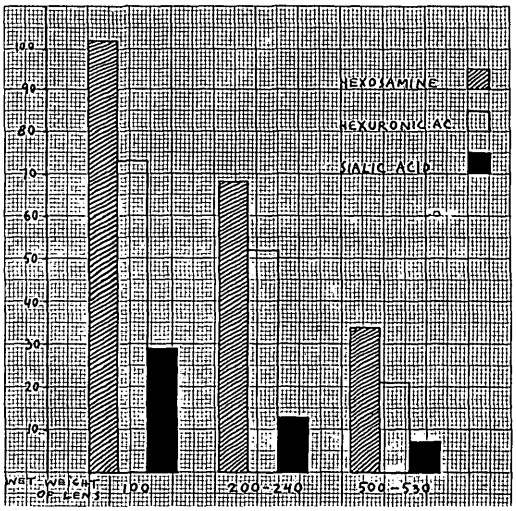

Fig. 1. Hexosamine, hexuronic acid, and sialic acid of the lens capsule at different ages in milligrams per gram collagen.

Table II. Hexosamine, hexuronic acid, and sialic acid of the lens capsule at different ages in milligrams per gram collagen

	Wet weight (mg.)				
Exper. No.	*Lens*	*Capsule*	*Hexosamine*	*Hexuronic acid*	*Sialic acid*
1	240	4.5	64.8	42.0	11.30
2	530	11.4	26.4	17.0	5.87
3	200	6.5	69.5	61.5	15.65
4	500	13.0	35.5	19.0	9.1
5	500	13.0	41.9	28.7	9.45
6	500	11.8	31.2	17.0	7.1
7	100	4.5	102	73.3	28.8

hexuronic acid, and sialic acid per gram collagen varies between 26.4 and 35.5 mg., between 17 and 28.7 mg., and between 5.9 and 9.45 mg., respectively; the corresponding values for 8- to 12-week-old animals are 34.8 to 68 mg., 42 to 68.5 mg., and 11.3 to 15.65 mg., respectively. In relation to collagen, the concentration of hexosamino glycans is much higher in capsules of younger rabbits, and the differences are still much more pronounced in the 4-week-old rabbits where the three values are 72.2, 102, and 28.8 mg., respectively, and very significantly higher than the ratios in the 8- to 12-week-old animals.

Content of collagen in hydroxyproline and the collagen-linked carbohydrate in lens capsules at different ages. As can be seen in Table III, the collagen of the rabbit lens capsule contains very high amounts of hydroxyproline. In three batches of lens capsule from mature rabbits, the content in hydroxyproline varied between 17 and 19.4 per cent of the collagen and in a fourth batch it was still 14.9 per cent. In one batch of capsules of younger animals it was 16.7 per cent, within the range found in older animals. When the neutralized TCA extracts of capsular collagen were dialyzed for 24 hours at 4° against an equal volume of distilled water, only traces of hydroxyproline could be detected in the dialysate. The concentration of the carbohydrate linked to collagen extracted by TCA varied between 15.7 and 19.3 per cent of the collagen in the mature animals, and was not significantly different, namely, 16.7 in the young animal of Experiment 1.

Discussion

The investigation of changes in composition of the rabbit lens capsule covered two distinct life periods of the animal, namely, the period before sexual maturation and a period comprising a short time before sexual maturation, and the young adult life up to about one year. The determinations of the constituents of the lens capsule were, therefore, carried out at three different stages of the life of the animal: in the sexually immature rabbit of 4 weeks of age, in rabbits of 2 to 3 months of age shortly before sexual maturity, and the adult rabbits of about one year of age. Our results show a striking difference between changes with age of the rate of accumulation of the collagen protein of the capsule and the carbohydrate linked to it on the one hand, and those of the noncollagenous protein, the glycoprotein, and acid mucopolysaccharide of the capsule on the other hand. The collagenous protein in 4-week-old animals represents less than 1 per cent of the wet weight of the lens capsule and increases by about 200 to 400 per cent during the period of maturation to 2.5 per cent in the 3-month-old rabbits. It about doubles again during the subsequent period of early adult life in which the weight of the lens increases from 200 to 240 mg. to about 500 mg. Two other components of the capsule, namely, the glycoprotein and

Table III. Effect of dialysis of TCA extracts of collagen of the capsule on their content in hydroxyproline and carbohydrate

Exper. No.	Wet weight (mg.)		Collagen (% of wet weight)	Hydroxyproline		Carbohydrate of collagen (% of collagen)
	Lens	Capsule		Wet weight (%)	Collagen (%)	
1	240	4.5	2.59	0.470	18.1	16.7
2	530	11.4	4.85	0.985	18.1	19.0
3	200	6.5	2.07	0.230		
4	200	13.0	4.12	0.800	19.4	15.1
5	500	11.8	3.32	0.680	19.3	19.3
6	500	13.0	5.34	0.795	14.9	
7	100	4.5	0.6*			

*Determined after dialysis.

mucopolysaccharide, show a completely different pattern of development. They are much lower in the 4-week-old than in mature rabbits, but increase so rapidly in the following 1 to 2 months that they reach the levels found in mature animals already in this short time interval. From the age of about 3 months, the three constituents of the lens capsule remain almost constant, with variations which cannot be considered significant, or even decrease with age. The data for sialic acid suggest near constancy of the level of glycoproteins between 3 months and 1 year, whereas those for hexuronic acids indicate a decrease during the same period.

As can be seen in Table I, the wet weight of individual capsules in the two respective age groups for which several samples were available varied up to about 50 per cent. These variations do not parallel differences in age. In Experiment 3, for instance, the average wet weight of the lens was lower than that in Experiment 1, but the wet weight of the capsule was almost 50 per cent higher in the first than in the last experiment. These variations cannot be due to contamination with different amounts of lens fibers, as the protein content of lens fibers can be assumed to be around 25 per cent and the content in residual protein found in the lens capsules varies between one-fifth to one-tenth of this value. A difference of 10 per cent contamination by lens fibers in heavier capsules from young animals would result in a 100 per cent increase of the residual protein, whereas, in reality, the residual protein between capsules of different ages show no significant difference. The variations in the wet weight of the lens can be due either to a more or less complete removal of the capsules from the lens in different preparations or to different degrees of dehydration during the storage and transport of the lens. In the first case, the calculated values per wet weight of the capsule would not be affected. In the second case, we would expect higher values of collagen, glycoproteins, and hexuronic acids in the lenses with lower wet weight. This does not appear to be the case. It appears, therefore, not unjustified to take the wet weight as reference point for the consideration of changes in the concentration. of various constituents of the lens capsules. Furthermore, the conclusions which result from calculations of concentrations of these constituents, on the basis of wet weight, appear in good agreement with those derived from observed changes in relative concentrations of collagen on the one and hexosamino glycans on the other hand. These relative concentrations cannot be affected by hydration or dehydration of the capsule.

This pattern of accumulation suggests that the glycoprotein and acid mucopolysaccharide of the lens capsule, which are built up more rapidly than collagen during the period of maturation, may play a role in the synthesis of collagen by the lens epithelium. This situation appears analogous to that observed in connective tissue when the synthesis of at least acid mucopolysaccharides has been shown to precede the synthesis of collagen in animals in which the inhibition of this synthesis, for instance by a scorbutic diet, was experimentally removed. The low level of collagen in the capsule of younger animals suggests that the capsule possesses a much looser texture in younger lenses, particularly in those of sexually immature animals and, therefore, may well be more permeable for various metabolically important substances like nutrients or waste products and thus regulate their access or the exit from the epithelium of the lens. It now appears of particular interest to determine whether the increase in the concentration of the collagenous protein of the lens capsule continues with progressing age of the rabbit beyond one year or becomes stationary at this age. If the concentration of this collagenous protein in the capsule affects the permeability of the lens capsule, such an increase might well represent at least one of the pathogenic factors in senile cataracts.

Another finding in our investigation which appears of interest concerns struc-

tural properties of the collagenous protein of the capsule related to its content in hydroxyproline and carbohydrate. The collagen of the lens capsule contains, according to our analysis (Table III), between 14.9 and 19.4 hydroxyproline, if it is assumed that its nitrogen content is 18 per cent as in other kinds of collagen. This is very significantly higher than the content in hydroxyproline in any other vertebrate or invertebrate collagen which so far has been investigated and which yielded in vertebrates values between 11.8 and 14.2 per cent. Even if we assume that the composition of the capsular collagen is such that its nitrogen content is only 16 per cent (which would mean that it would not be as rich in glycine and basic amino acids as other kinds of collagen), the content in hydroxyproline in three out of four of our experiments would still be higher than that found in other mammalian tissues. As the collagenous protein in the lens capsule appears to be unorganized, the possibility had to be considered that the high content in hydroxyproline is characteristic for this type of collagen. It must be noted, however, that the analysis of the collagenous fibrils from the center of the bovine vitreous, which are very thin with a diameter of no more than 250 Å and are not striated, showed in our laboratory no more than 11.8 per cent hydroxyproline. Apparently the maturation of the collagen does not increase its content in hydroxyproline. The possibility, therefore, must be considered that the high content in hydroxyproline is characteristic for the epithelial collagen in contrast with the collagen of mesodermal origin. It is noteworthy in this connection that the other type of epithelial collagen which has so far been investigated, namely, that of the cuticle of certain annelides, also shows a significantly higher content in hydroxyproline than the collagen of other invertebrate tissues. It is noteworthy that the content in hydroxyproline in collagen is directly correlated to the contraction temperature of the collagen, and the latter again decreases with the age of the animal. It seems possible, therefore, that the high content in hydroxyproline in the capsular collagen represents a protective factor as far as the aging of the lens is concerned.

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