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The Organization of Tissues of the Eye by Different Collagen Types

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Summary. Bovine cornea, sclera, iris, ciliary body, choroid, zonular fibers, lens capsule, lens nucleus, vitreous body, and retina were investigated for collagen content and type.

Cornea, sclera, iris, ciliary body, choroid, lens capsule, and vitreous body contain hydroxyproline, whereas in zonular fibers, lens nucleus, and retina no hydroxyproline was detectable.

Preparative isolation of collagen was achieved by digestion of the different eye tissues with pepsin. The pepsin-solubilized collagen was separated by differential salt precipitation into different collagen types.

The polyacrylamide gel electrophoresis of the pepsin-solubilized collagens revealed type I collagen in cornea, sclera, iris, ciliary body, and choroid. As well as type I collagen, type III collagen was isolated from cornea, sclera, and uveal tissues. The identification of types I and III collagen was supported by the CNBr-derived peptides of these collagens. Lens capsule collagen consisted mainly of type IV collagen.

Zonular fibers contained no hydroxyproline but when examined by polyacrylamide gel electrophoresis, a band migrating in the α-position of collagen was observed. Polyacrylamide gel electrophoresis of both the pepsin-solubilized component and the CNBr-derived peptides of vitreous body protein showed no relation to any of the four common collagen types.

Zusammenfassung. Hornhaut, Lederhaut, Regenbogenhaut, Ziliarkörper, Aderhaut, Zonulafasern, Linsenkapsel, Linsenkern, Glaskörper und Netzhaut werden auf ihren Kollagengehalt untersucht. Auch soll festgestellt werden, welche Kollagentypen diese Gewebe aufbauen. Hornhaut, Lederhaut, Regenbogenhaut, Ziliarkörper, Aderhaut, Linsenkapsel und Glaskörper enthalten Hydroxyprolin. Hingegen ist in Zonulafasern, Linsenkern und Netzhaut kein Hydroxyprolin nachzuweisen. Aus den verschiedenen Augengeweben wurde mit Pepsin Kollagen gelöst, das durch Präzipitation mit NaCl-Lösungen verschiedener Molarität in verschiedene Kollagentypen getrennt werden kann.

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Die Polyacrylamidgel-Elektrophorese des pepsin-gelösten Kollagens zeigte Type I Kollagen in Hornhaut, Lederhaut, Regenbogenhaut, Ziliarkörper und Aderhaut. Neben Typ I Kollagen wurde aus Hornhaut, Lederhaut und den Geweben der Uvea Typ III Kollagen isoliert. Typ I und Typ III Kollagen wurden durch die Peptide, die man mit CNBr-Spaltung erhalten kann, identifiziert. Die Linsenkapsel besteht hauptsächlich aus Typ IV Kollagen.

Zonulafasern enthalten kein Hydroxyprolin, zeigen aber in der Polyacrylamidgel-Elektrophorese eine Farblinie, die im α-Bereich von Kollagen wandert.

Die Polyacrylamidgel-Elektrophorese des pepsin-gelösten Kollagens des Glaskörpers sowie die CNBr-Spaltprodukte dieses Kollagens zeigten keine Identität mit einem der vier bisher isolierten Kollagentypen.

Introduction

Collagen has been identified in many tissues of the eye. However, only a small portion of collagen is soluble in neutral salt solutions or diluted acids. Therefore, investigation has been difficult and little is known about the way in which the four common collagen types are involved in the different tissues of the eye. Solubilization with pepsin yields high amounts of soluble collagen and it is possible to separate different types of pepsin-solubilized collagen by differential salt precipitation from heterogeous collagen solutions (Trelstad et al., 1976). Therefore, different tissues of the eye were digested with pepsin and purified types isolated by differential salt precipitation.

The CNBr-derived peptides of the pepsin-solubilized collagen of different eye tissues were separated by polyacrylamide gel electrophoresis and were further characterized by comparing them with the CNBr-derived peptides of reference preparations of types I, II, III, and IV collagen.

Materials and Methods

Reference Preparations of Pepsin-Solubilized Collagen

Type I. Bovine achilles tendon was cut into small pieces and treated twice with 10 ml 0.5 M sodium acetate/g wet weight at 4° C and after washing with water the acid-soluble collagen was extracted with two changes of 10 ml/g wet weight of 0.5 M citrate buffer (pH 3.7). Each extraction lasted at least 24 h.

After centrifugation the supernatant was discarded and the residue solubilized with 5 mg/g wet weight pepsin (2500 U/mg; Boehringer/Mannheim Nr. 108 057) at 4° C for 24 h. After centrifugation the pepsin-solubilized collagen was precipitated from the supernatant by the addition of solid NaCl to a final concentration of 0.9 M. The precipitate was collected by centrifugation (10 min, 4° C, 3000 g), redissolved in 0.05 M Tris-HCl (pH 7.5) containing 1.0 M NaCl, kept for 4 days at 4° C to inactivate the pepsin, dialyzed exhaustively against 0.167 M acetic acid, precipitated with acetone and dried in air.

Type II. Bovine articular cartilage was treated twice with sodium acetate and citrate buffer respectively, as described above. From the residue, type II collagen was prepared as described by Miller (1972).

Type III. Type III collagen was prepared from calf skin according to the method of Fujii and Kühn (1975) with minor modifications as described by Schmut (1977).

Type IV. Bovine lens capsules were extracted with two changes of sodium acetate and citrate buffer respectively and solubilization with pepsin was performed as described for type I collagen.

Preparation of Different Tissues of the Eye

The tissues of the bovine eyes were isolated immediately after slaughtering. The eyes were freed of muscle and adhering tissues. After the removal of the cornea the iris could be cut at the root.

A piece of the anterior lens capsule measuring 6 × 6 mm was cut out and the epithelium was abraded with a razor-blade. In order to isolate the lens nucleus, the lens capsule and zonular fibers were carefully removed.

The sclera was dissected by an equatorial cut and the posterior vitreous body was isolated with a spatula. Adhering retina was removed and the vitreous body was homogenized. Vitreous body homogenate was treated with acetone in a volume ratio of 1:1 and a precipitate of a mixture of collagen and hyaluronic acid could be obtained. Hyaluronic acid was removed to a great extent by treating the precipitate with 1 M NaCl for 5 h. The residue which was isolated by centrifugation (10 min, 3000 g) consisted mainly of collagen. After removing the vitreous body, retina and choroid could be isolated.

The remaining sclera was freed of adhering tissues and pigments, and then cut into small pieces. Ciliary body was prepared paying special attention to the removal of zonular fibers.

To isolate the zonular fibers, eyeballs without cornea and iris were treated with acetone for 24 h. The sclera was then dissected with an equatorial cut about 0.5 cm from the limbus, the vitreous body was carefully removed and the remaining anterior part of the eye was treated with fresh acetone for another 24 h. Zonular fibers were then clearly visible and it was possible to isolate the fibers with the aid of a stereomicroscope.

Pepsin-Solubilized Collagen of Eye Tissues

The isolated tissues of the eye were extracted with sodium acetate and citrate buffer respectively and treated with pepsin as described for type I collagen. To precipitate the pepsin-solubilized collagen with solid NaCl the final concentration was 0.9 M the case of cornea, sclera, iris, ciliary body, and choroid, and 2.58 M in the case of lens capsule, zonular fibers, vitreous body, and retina. Collagen was collected by centrifugations (10 min, 4° C, 3000 g), redissolved in 0.05 M Tris-HCl (pH 7.5) containing 1.0 M NaCl, kept for 4 days at 4° C, dialyzed against 0.167 M acetic acid, precipitated with acetone and dried in air.

Differential Salt Precipitation of Pepsin-Solubilized Collagen

Differential salt precipitation was performed according to the method of Chung and Miller (1974) with minor modifications as described by Schmut (1977).

Digestion with CNBr

Samples of 50 mg pepsin-solubilized collagen were chemically cleaved with 5 mM CNBr in 10 ml 15 M formic acid under N_2 at 24° C for 4 h. After the incubation period the solution was dried under reduced pressure, redissolved in H_2O and dried under reduced pressure again.

Polyacrylamide Gel Electrophoresis in Sodium Dodecylsulphate

Polyacrylamide gel electrophoresis was performed according to Furthmayr and Timpl (1971). The samples were dissolved at a concentration of 2–3 mg/ml in a solution of 0.01 M phosphate buffer (pH 7.2) containing 6.95 mM SDS and 2 M urea. Analyzing type III collagen, the solvent was 0.128 M with mercaptoethanol. For pepsin-solubilized collagen and CNBr-peptides 7.5 % polyacrylamide gels were used. Electrophoresis was performed at 6 mA per tube for 4 h for collagen and for 3 h for the CNBr-peptides. The gels were stained with 0.2 ml Coomassie Brillant Blue R 250 (0.3 % in ethanol) in 10 ml 0.615 M trichloroacetic acid for 3 h and destained in H_2O for at least 2 days. The gels were scanned with a Beckman Scanning Densitometer R-112.

Determination of Hydroxyproline

The purified tissues of the eye were treated three times with acetone and then dried in vacuo over P_2O_5. Samples of 3–4 mg were hydrolyzed under nitrogen in 6 M HCl at 105 ° C. Hydroxyproline was determined by the method of Stegemann (1958).

Table 1. Collagen concentrations (% dry weight) of different eye tissues. Vitreous body collagen content was determined from the acetone precipitate of vitreous body homogenate and calculated for vitreous body homogenate

Cornea	54±5
Sclera	78±4
Iris	49±9
Ciliary body	41±8
Choroid	49±6
Lens capsule	69±8
Vitreous body	0.006
Lens nucleus	0
Zonular fibers	0
Retina	0

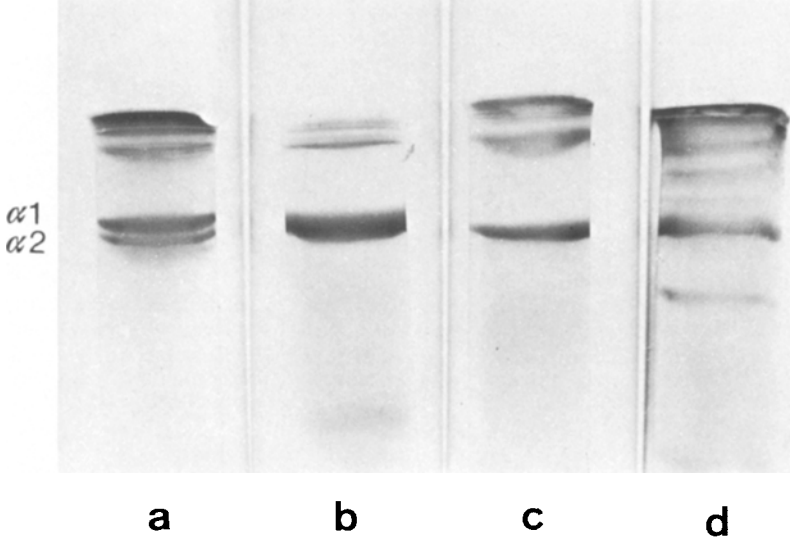


Fig. 1. Polyacrylamide gel electrophoretic patterns of collagen type I (*a*), type II (*b*), type III (*c*), and type IV (*d*)

Results

Total Collagen Content

For the conversion to collagen content, a hydroxyproline content of 13.4% in collagen was assumed (Adelmann et al., 1966).

The collagen values obtained for the different eye tissues are summarized in Table 1.

Solubilization with Pepsin

Collagen of the different eye tissues was solubilized with pepsin. Polyacrylamide gel electrophoresis of the pepsin-solubilized collagen of cornea, sclera, iris, ciliary body, and choroid shows the α1 (I) and the α2 band of type I collagen as compared with type I collagen from bovine achilles tendon (Fig. 1 and 2). By differential salt

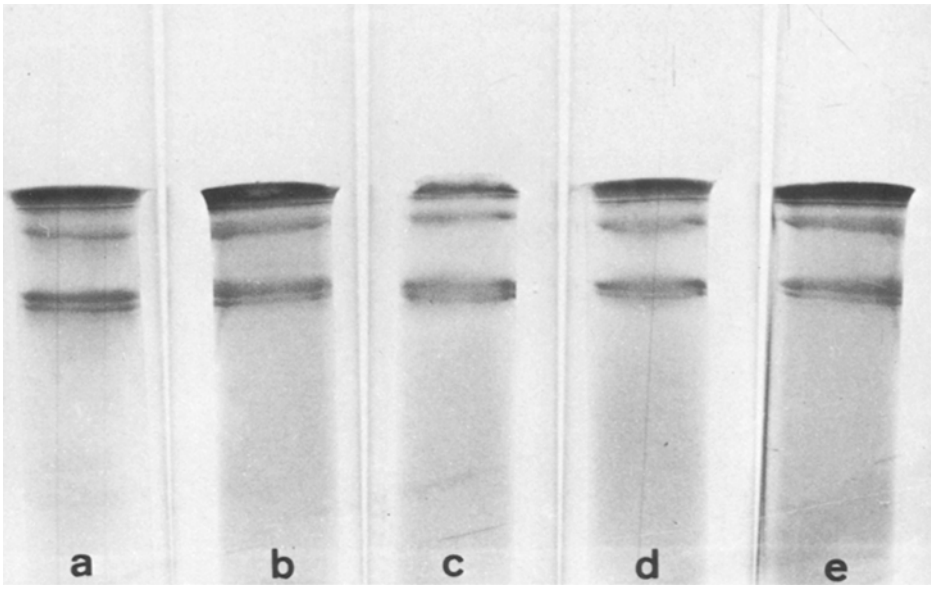
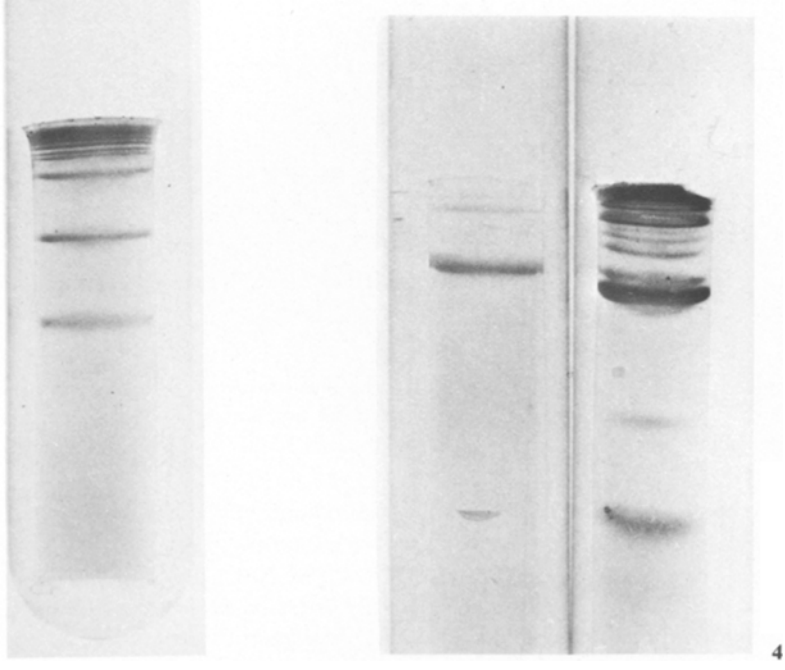


Fig. 2. Polyacrylamide gel electrophoretic patterns of pepsin-solubilized collagen from cornea (*a*), sclera (*b*), iris (*c*), ciliary body (*d*), and chroid (*e*)



3 4

Fig. 3. Polyacrylamide gel electrophoretic pattern of pepsin-solubilized collagen type III from iris

Fig. 4. Polyacrylamide gel electrophoretic patterns of pepsin-solubilized protein from zonular fibers (*left*) and vitreous body (*right*)

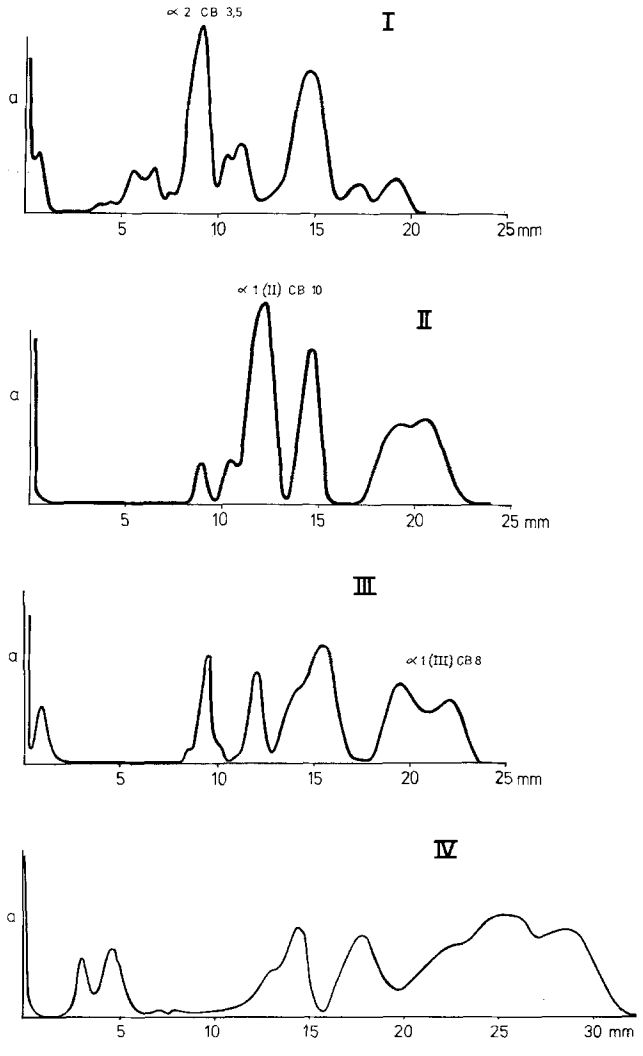


Fig. 5. Polyacrylamide gel electrophoresis of the CNBr-derived peptides of pepsin-solubilized collagen type I (*a*), type II (*b*), type III (*c*), and type IV (*d*). The stained gels were scanned at 570 nm

precipitation of pepsin-solubilized collagen, type III collagen can be isolated from bovine cornea and sclera. Also the tissues of the uvea contain type III collagen. The polyacrylamide gel electrophoretic patterns of the 1.5 M NaCl precipitate of the pepsin-solubilized collagen of uveal tissue show only one α-band (Fig. 3). This is in analogy with the α1 (III)-band of reference type III collagen of calf skin (Fig. 1).

Vitreous body protein reveals one fraction of differential salt precipitation only, which precipitated at a NaCl concentration similar to type II collagen. This protein shows only one α-band in polyacrylamide gel electrophoresis. But, in contrast to the four common collagen types (Fig. 1), between the α- and the β-component some protein-bands are detectable (Fig. 4).

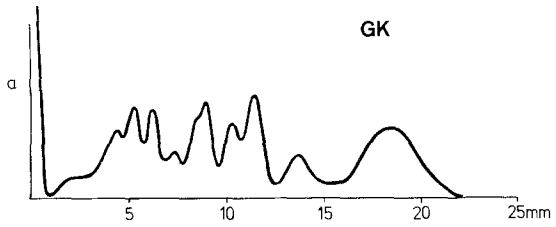


Fig. 6. Polyacrylamide gel electrophoresis of the CNBr-derived peptides of pepsin-solubilized collagen from vitreous body. The gel was scanned at 570 nm

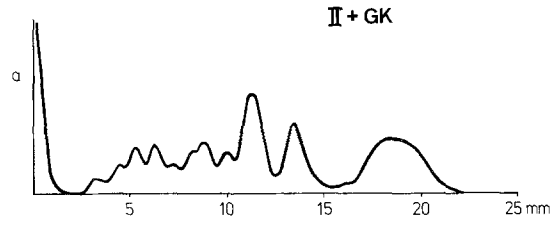


Fig. 7. Polyacrylamide gel electrophoresis of a mixture of the CNBr-derived peptides of pepsin-solubilized collagen type II and vitreous body collagen. The gel was scanned at 570 nm

The pepsin-solubilized protein of zonular fibers shows a solubility similar to collagen, and the polyacrylamide gel shows a band migrating in the α-position of collagen (Fig. 4). Retina and lens nucleus yield no pepsin-solubilized collagen.

Cleavage with CNBr

The polyacrylamide gel electrophoretic patterns of the CNBr-derived peptides of collagen types I, II, III, and IV which were prepared as a reference are shown in Figure 5a–d.

Analysis of the CNBr-derived peptides of the pepsin-solubilized collagen of cornea, sclera, iris, ciliary body, and choroid, reveals the presence of type I collagen in these tissues. This can be proved by the occurrence of the α2 CB3,5 peptide which is characteristic of type I collagen (not shown).

Pepsin-solubilized collagen which can be isolated by differential salt precipitation at a concentration of 1.5 M NaCl from cornea, sclera, and tissues of the uvea, yields CNBr-derived peptides which contain type III collagen-specific peptide α1 (III) CB8 (not shown).

The CNBr-derived peptides of pepsin-solubilized vitreous body protein show in polyacrylamide gel electrophoresis a pattern which can not be attributed to any of the hitherto known collagen types (Fig. 6).

Cochromatography with the CNBr-derived peptides of type II collagen confirms partial connection between pepsin-solubilized vitreous body collagen and type II collagen (Fig. 7). If one assumes the presence of type II collagen in vitreous body collagen, a concentration of about 24 % of type II can be calculated from the cochromatography.

Shortage of material prevented the analysis of the CNBr-derived peptides of the pepsin-solubilized protein of zonular fibers.

Discussion

An abundance of hydroxyproline is present in collagen. Therefore, it is possible to evaluate the collagen content of a tissue by determining the hydroxyproline concentration. By this method it could be established that the main protein of cornea, sclera, the tissues of the uvea, and lens capsule, is collagen. Polatnick et al. (1957) and Smits (1957) found a collagen concentration of 70.2% and 53% in cornea and a concentration of 74.5% and 76% in sclera. These results and our findings indicate that cornea contains less collagen than sclera.

The tissues of the uvea have not been well studied for their collagen content until now. We found that collagen constitutes 49 ± 9% of the dry weight of the whole iris. However, it is not equally distributed in all parts of the iris. We found that the root of the iris contains 58% collagen, whilst the pupillary part is only 11% collagen. Krause and Chan (1933) found that in the whole iris the level of collagen amounts to 38.62% of the dry weight.

Our findings showed a lower collagen concentration in the uveal tissues than in cornea and sclera. We determined collagen in ciliary body 41 ± 8% and in choroid 49 ± 6% of the tissues dry weight. Krause and Chan (1933) found 27.93% collagen in choroid. Whilst in lens capsule the collagen content was very high (69 ± 8%), in lens nucleus no collagen was detectable by the method we used. Also in zonular fibers and retina, no hydroxyproline was measurable.

Solubilization of collagen can be achieved by different methods. Relatively low amounts of collagen can be extracted from different tissues by using neutral salts or diluted acids. Enzymic solution with pepsin yields large quantities of soluble collagen which virtually consists entirely of intact molecules (Rubin et al., 1963; Drake et al., 1966). By pepsin digestion we were able to obtain sufficient amounts of soluble collagen. By differential salt precipitation we separated type I from type III collagen in different tissues of the bovine eye.

The α-chains of collagen can be cleaved at methionyl bonds by cyanogen bromide (Gross and Witkop, 1961). As the α-chains of collagen contain relatively few methionyl bonds, a defined number of CNBr-derived peptides can be obtained upon cleavage with CNBr (Bornstein and Piez, 1965). In polyacrylamide gel electrophoresis these collagen peptides can be separated and each collagen type yields a characteristic peptide pattern in the polyacrylamide gels.

Cornea and Sclera

Type I collagen has been identified in bovine cornea and sclera by amino acid analysis, column chromatography, and polyacrylamide gel electrophoresis (Freeman et al., 1968; Katzman et al., 1974; Schmut et al., 1975).

Recently, type III collagen has been identified by differential salt precipitation and polyacrylamide gel electrophoresis in bovine and calf cornea and in sclera (Schmut, 1977). In the present study, type III collagen of cornea and sclera was further characterized by the polyacrylamide gel electrophoretic patterns of the CNBr-derived peptides.

The presence of type III collagen in cornea and sclera seems to be important for the healing of both corneal wounds and corneal transplants because it has been

proved that type III collagen was preferentially synthesized in dermal wound tissue (Bailey et al., 1975; Barnes et al., 1976).

Uvea

Until now type I collagen has been identified in bovine iris, ciliary body, and choroid by polyacrylamide gel electrophoresis of acid-soluble collagen (Schmut et al., 1975). Results presented in this paper gave evidence to show that type III collagen is present in the tissues of the uvea.

Type III collagen could be identified by characteristic solubility in differential salt precipitation, by electrophoretic mobility in polyacrylamide gel electrophoresis and the significant pattern of the CNBr-derived peptides of pepsin-solubilized collagen.

Vitreous Body

The presence of collagen in vitreous body has not been generally accepted until recently. Matoltsy (1952) called the insoluble protein of vitreous body vitrosin. In 1955, Gross et al. found a similarity between vitrosin and collagen, and Olsen (1965) showed by electron microscopy that the fibrils of vitreous body contain collagen.

Nevertheless, it has not until now been clear which type of collagen the vitreous fibers consist of. Swann et al. (1972) found a similarity between vitreous body collagen and type II collagen, whilst Swann, et al. (1975) suggested that the fibrils of the rabbit vitreous body contain type IV collagen. On the other hand, Swann et al. (1976) concluded that vitreous body collagen is composed of either a distinct type or a mixture of α-chains. Smith et al. (1976) found that the vitreous body collagen produced by chick neural retina tissue contains type II collagen.

In our investigations we found no similarity between the polyacrylamide gel electrophoretic patterns of pepsin-solubilized collagen and those of the four common collagen types. Also the CNBr-derived peptides of pepsin-solubilized vitreous body collagen revealed no identity with type I, II, III, and IV collagen. Cochromatography of type II collagen showed that vitreous body collagen yielded more CNBr-derived peptides than type II collagen.

Lens Capsule

Kefalides and Denduchis (1969) found that lens capsule contains a distinct collagen which they designated type IV collagen. This type of collagen is characteristic of basement membranes and has also been isolated from Descemet's membrane.

Zonular Fibers

There are many opinions about which kind of protein the zonular fibers consist of. Propst and Hofmann (1960) and Gärtner (1970) suggested that the zonular fibers contain collagen. Gloor (1974) supposed the zonular fibers to be fibers of an embryonic type of collagen. Raviola (1971) suggested the presence of elastic fibers and Wollensak (1965) glia fibers. Electron microscopic investigations showed a

great similarity between zonular fibers and collagen. On the other hand, there was no identity between the amino acid analysis of zonular and collagen fibers.In this study we found no hydroxyproline in zonular fibers, as did Wollensak (1965).

A protein migrating in the α-position of collagen in polyacrylamide gels was dissolved from zonular fibers with 0.05 M acetic acid (Schmut et al., 1975). Also solubilization with pepsin yielded a protein which showed a protein-band similar to α-chains of collagen. The possibility of contamination with vitreous body collagen could be excluded, because in the preparation of zonular fibers no hydroxyproline was detected. It still remains to be investigated whether or not zonular fibers contain a hydroxyproline free form of collagen.

In many connective tissue diseases the tissues of the eye are involved. In Ehlers-Danlos syndrome, Marfan's syndrome, or osteogenesis imperfecta opaque corneas, keratokonus, thin, blue scleras, ectopia lentis, and chorioretinitis can be observed. Disturbances of the collagen metabolism might be involved in pathogenesis of these connective tissue disorders. Defects were found in the chemical structure of collagen, in fiber formation, and in the rate at which collagen is deposited in or removed from tissues. High deficiency or abundance of type III collagen in relation to normal tissues may play an important role in some of these disorders (for review see Uitto and Prockop, 1975). Our studies show that collagen occurs in most tissues of the eye in relatively high concentrations and therefore it may be possible to explain why the eye is involved in connective tissue diseases.

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