Embryonic Avian Cornea Contains Layers of Collagen with Greater than Average Stability

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Abstract. A unique morphological feature of the embryonic avian cornea is the uniformity of its complement of striated collagen fibrils, each of which has a diameter of 25 nm. We have asked whether this apparent morphological uniformity also reflects an inherent uniformity of the structural and physical properties of these fibrils. For this we have examined the in situ thermal stability of the type I collagen within these fibrils. Corneal tissue sections were reacted at progressively higher temperatures with conformation-dependent monoclonal antibodies directed against the triple-helical domain of the type I collagen molecule. These studies show that the cornea contains layers of collagen fibrils with greater than average stability. The two most prominent of these extend uninterrupted across the entire width of the cornea, and then appear to insert into thick bundles of scleral collagen, which in turn appear to insert into the scleral ossicles, a ring of bony plates which circumscribe the sclera of the avian eye. Once formed, the bands may act to stabilize the shape of the cornea or, conversely, to alter it during accommodation.

THE mature avian corneal stroma consists of layers of small diameter, striated collagen fibrils. Throughout the entire thickness of the corneal stroma, there are no obvious morphological differences in the fibrils; instead, the most striking characteristic of this extracellular matrix is the uniformity of its fibrils (25-nm diameter) (3, 9, 11). Conversely, in most other connective tissues such as sclera and dermis, the collagen fibrils are morphologically heterogeneous, occurring in a wide variety of diameters, most of which are considerably greater than those within the cornea (6, 11, 19). The corneal stroma also appears to react uniformly in immunofluorescence analyses with anti-collagen type-specific monoclonal antibodies against the interstitial collagen types I, V, and VI (2, 7, 13, 18). The types I and V are probably co-assembled within the 25-nm stromal fibrils (2, 7, 13), while the type VI exists in thin filaments (Linsenmayer, T. F., A. Mentzer, R. Bruns, and R. Mayne, manuscript submitted for publication).

The major structural unit within the corneal stroma is the collagen triple helix. Each stromal collagen fibril consists chiefly of an assemblage of collagen molecules, and each collagen molecule consists predominantly of a triple-helical domain (for review see reference 12). We (14, 15, 16, 21) have previously demonstrated that anti-collagen monoclonal antibodies directed against conformation-dependent epitopes within the triple-helical domain of a collagen can be used as sensitive probes for detecting the native conformation of this structure in solution and in situ. In solution, the relative or absolute concentration of native molecules present is determined by antibody binding, as evaluated by a competition enzyme-linked immunosorbent assay (ELISA)[1] (14, 15, 21). In situ, antibody binding to native collagens within tissues (15, 16) is evaluated by fluorescence immunohistochemistry.

These antibody-binding assays can also be used to compare the thermal stability of collagen molecules within different environments. For a collagen in neutral solution, thermal denaturation data can be obtained by examining the loss of antibody binding to samples of collagen that have been raised to progressively higher temperatures. In general, under these conditions we have observed good agreement between the temperature at which antibody binding is lost and that at which the helical structure of the collagen is destroyed, as physically measured by circular dichroism spectroscopy (see, for example, references 14 and 21). To examine the thermal stability within tissues in situ, we have devised a procedure in which unfixed cryostat tissue sections are preheated in culture medium or physiological saline to progressively higher temperatures, reacted with monoclonal antibody at the same elevated temperature, and then assayed for antibody binding by indirect immunofluorescence histochemistry (15, 16). The denaturation temperature is operationally defined as the temperature at which a detectable immunofluorescence signal, indicative of antibody binding, is lost.

In the present investigation, the assay has been used to establish whether the morphological uniformity of the striated

1. *Abbreviation used in this paper*: ELISA, enzyme-linked immunosorbent assay.

collagen fibrils within the cornea reflects structural uniformity. For this, we examined the thermal stability of type I collagen in embryonic chick cornea. Sclera and dermis were also examined. We have observed that the cornea contains layers of collagen with greater than average stability. Two of these extend uninterrupted across the entire width of the cornea and then appear to insert into thick bundles of scleral collagen. The scleral bundles, in turn, appear to insert into the scleral ossicles, a ring of bony plates which circumscribes the sclera of the avian eye.

Materials and Methods

Antibody-containing medium, harvested from hybridoma cultures, was used undiluted as the source of anti-type I collagen antibody. The description of one of the two antibodies used (I-I_1B6) has been previously reported (17); the other (I-BA1) has more recently been characterized (see Results).

Inhibition ELISAs were performed as previously described (14). Aliquots of the inhibitor collagen solution at a concentration capable of effecting complete inhibition were raised to elevated temperatures for 30 min, rapidly cooled to 0°C to prevent reformation of native molecules by renaturation, and added to antibody-containing medium before testing in the ELISA.

Corneas, with surrounding sclera unless otherwise stated, were dissected from normal 18–19-d chick embryos. 8-μm sections were cut on a cryostat and duplicate sections were air dried onto albuminized glass slides. The slides were stored at −20°C until used. In certain experiments, corneas from 8–14-d normal embryos or 17–18-d lathyritic embryos were used. The lathyritic embryos were produced by in ovo administration of B-aminopropionitrile (0.5 mg/egg on day 12, 1 mg/egg on day 14, and 1 mg/egg on day 16) (2, 7). The corneas were removed on day 17–18.

Some sections of 18–19-d tissues were predigested with testicular hyaluronidase (Sigma Chemical Co., St. Louis, MO; type I-S, 5 mg/ml in PBS) for 30 min at 37°C; keratanase (Miles Scientific Div., 1 U/ml in 50 mM Tris, pH 7.4, plus the protease inhibitors 1 mM *N*-ethylmaleimide, 50 μg/ml phenylmethylsulfonyl fluoride, 5 mM EDTA) for 2 h at 37°C; or trypsin (Sigma Chemical Co.; type III, 0.1 mg/ml in Hanks' balanced salt solution) for 10 min at 37°C followed by soybean trypsin inhibitor (Sigma Chemical Co.; 0.1 mg/ml in Hanks' balanced salt solution) for 4 min at room temperature. Other sections were pretreated with 0.1 M HAc for 15 min at room temperature (2, 14).

For the in situ thermal denaturation experiments, flat-bottomed 50-ml tubes containing either 15 ml of complete medium (Dulbecco's modified Eagle's medium plus 10% fetal calf serum) plus protease inhibitors (50 μg/ml phenylmethylsulfonyl fluoride, 50 μg/ml *p*-mercuribenzoate, 1 mM *N*-ethylmaleimide, 25 mM EDTA) or 15 ml of monoclonal antibody-containing medium with the same protease inhibitors were heated in a circulating water bath to the desired temperature. The temperature of the medium in the tubes was monitored with an immersed thermocouple attached to an electronic thermometer (Omega Engineering, Inc., Stamford, CT). During the incubations, the temperature varied less than 0.4°C. Tissue sections were treated at 20°C, 40°C, 45°C, and at 2° intervals between 50° and 60°C. Slides with the attached sections were immersed for 30 min in complete medium at the desired temperature. Then they were immediately transferred for another 30 min to antibody-containing medium at the same temperature. They were washed at the same elevated temperature in three changes of PBS and once more at room temperature. The sections were then treated with rhodamine-conjugated goat anti-mouse IgG for 45 min at room temperature, washed in four changes of PBS, and mounted in glycerol-PBS (95:5). The sections were viewed with a Nikon Fluorophot fluorescence microscope equipped with an epi-illuminator and a 590-nm filter. Photographs were taken with Kodak Tri-X film and developed with Diafine (Acufine, Inc., Chicago, IL).

The relative fluorescence of selected ocular tissues at different temperatures was measured by photometry. Readings were performed using the "spot" setting (1% of field) of the Microflex UFX-II photomicrographic attachment on the fluorescence microscope and a 20× neofluor objective. A photometer setting of ASA 160 was chosen since this gave measurable readings throughout the entire range of fluorescence found in the tissues. Readings were recorded as "seconds of exposure required" (i.e., an inverse function of the fluorescence intensity). Duplicate tissue sections were examined at each temperature. Nine separate readings were taken on the corneas—three each along the anterior, middle, and posterior parts; three readings were taken on the scleral tissue at either side of the cornea; and three readings were taken in the dermal portion of the eyelid.

Results

Anti-type I Collagen Antibodies Used

In these studies we used two different anti-type I collagen monoclonal antibodies. Both antibodies are specific for conformation-dependent epitopes in the triple-helical domain of type I collagen, but not in types II, III, IV, or V. Predigestion of type I collagen samples and corneal tissue sections with highly purified bacterial collagenase (20), which specifically degrades the triple-helical domain, destroys their binding (data not shown). In competition ELISAs with collagen samples that had been raised to progressively higher temperatures (Fig. 1), both gave similar results, producing a thermal denaturation curve for the collagen with a T_m of slightly less than 44°C and essentially a complete loss of binding by 47°C. To verify that the antibodies themselves had sufficient thermal stability for the proposed studies, samples of the antibody-containing hybridoma culture supernatants were raised to elevated temperatures for 0.5 h, cooled to room temperature, and tested for reactivity with sections of corneal tissues. Antibody I-I_1B6 is stable up to 60°C and becomes denatured at 65°C. Antibody I-BA1 is stable up to 65°C and becomes denatured at 70°C.

In Situ Thermal Denaturation Studies

Sections of unfixed corneas from late stage chicken embryos (18–19 d) were reacted with one of the two different anti-type I collagen monoclonal antibodies at 25°, 40°, 45°, and at each 2° interval from 50° to 60°C. Cornea, sclera, and the dermis of the eyelid were examined. The observations made by visual inspection of the tissues (discussed later), were

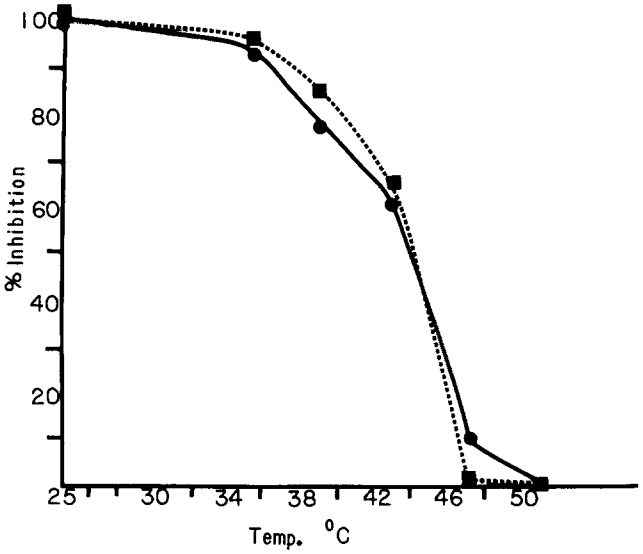

Figure 1. Thermal denaturation of the antigenic determinants against which the anti-type I collagen antibodies were directed was determined by an inhibition ELISA using collagen samples which had been heated to progressively higher temperatures as inhibitors. The loss of inhibition is indicative of denaturation of the epitopes. *Solid line*, ELISA values obtained with antibody I-BA1. *Dashed line*, ELISA values obtained with antibody I-I_1 B6.

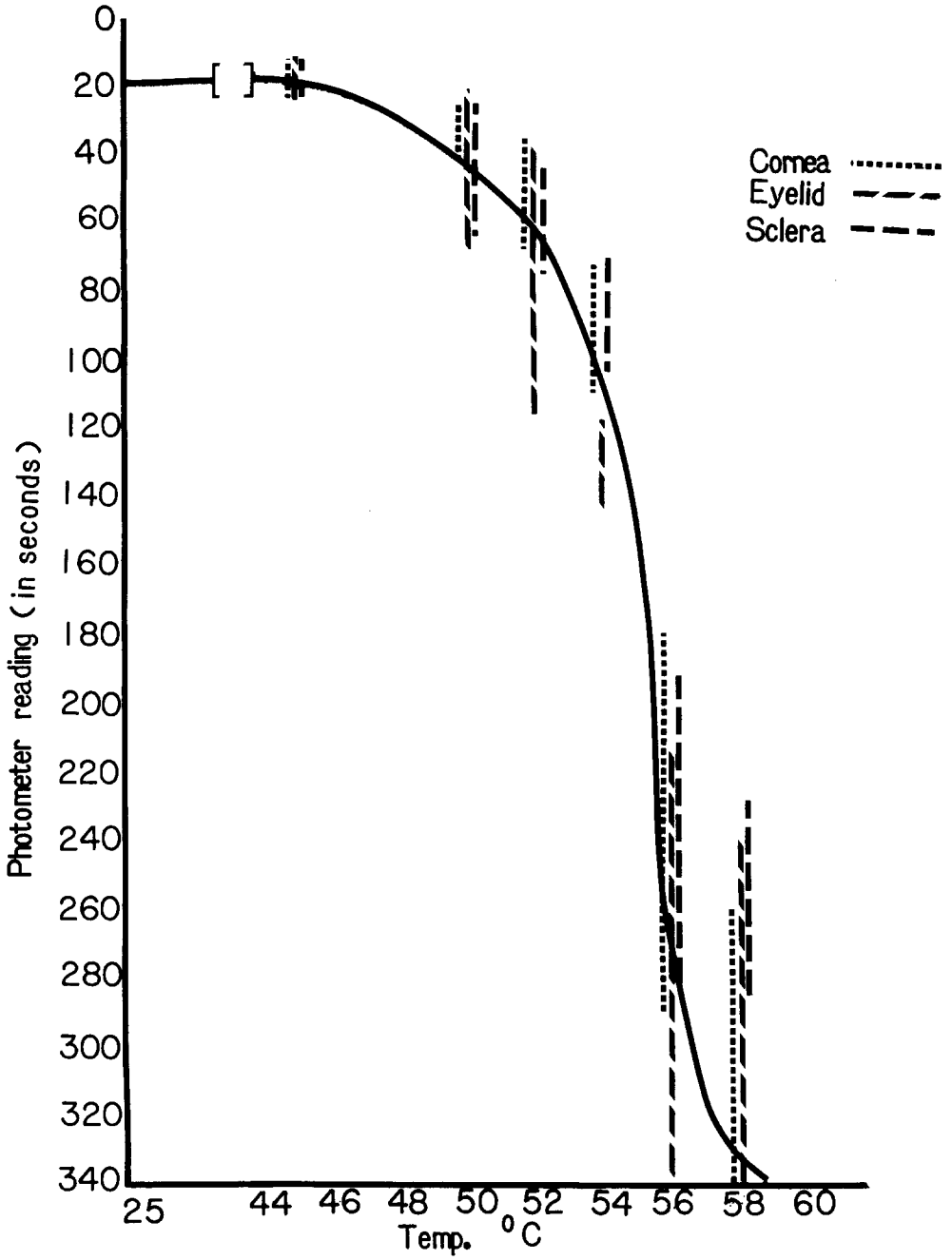

Figure 2. Photometer readings of the fluorescence signal obtained from cornea, eyelid, and sclera reacted with antibody I-BA1 at progressively higher temperatures. The vertical lines at each temperature represent the standard deviations of the measurements recorded for cornea, eyelid, and sclera. For details see Materials and Methods. Note that for the data obtained at 58°C for the eyelid and sclera, only the top halves of the standard deviation bars are shown.

confirmed in a semi-quantitative manner by using the "spot" photometer on the microscope to measure the relative fluorescence intensity at several sites in each of the tissues (see Materials and Methods). The data obtained with antibody I-BA1 produced a curve for the progressive loss in fluorescent antibody signal, indicating a T_m for the epitope of 55°C (Fig. 2). This is slightly more than 10° higher than that which we determined for the epitope in solution. The maximum loss of the fluorescence signal occurred at 58°C. As determined by these data, which represent the average fluorescent signal throughout the tissue, no clear-cut differences were detected among cornea, sclera, or dermis of the eyelid (Fig. 2). The only experimental variation observed was that in some experiments, the curve appeared to be shifted ~1–2°C lower. The data obtained with the other antibody (I-I_1B6, not shown) were very similar, except that the absolute fluorescence signal at each temperature was slightly less.

Although no obvious tissue-specific differences in thermal stability were apparent from the photometer readings, visual examination of the sections showed that, in fact, regional differences in the antibody reactivity did exist within each tissue. These were only detectable in the temperature range during which the fluorescent signal was decreasing (52–56°C). Within the cornea, the most dramatic difference was the presence of two brightly fluorescent layers (Fig. 3, arrowheads).[2] Depending on the individual experiment, the two

2. At these elevated temperatures two additional regions of fluorescence were sometimes observed within the stroma. One was adjacent to the anterior (epithelial) surface and the other was adjacent to the posterior (endothelial) surface. These were not further investigated (but see Discussion).

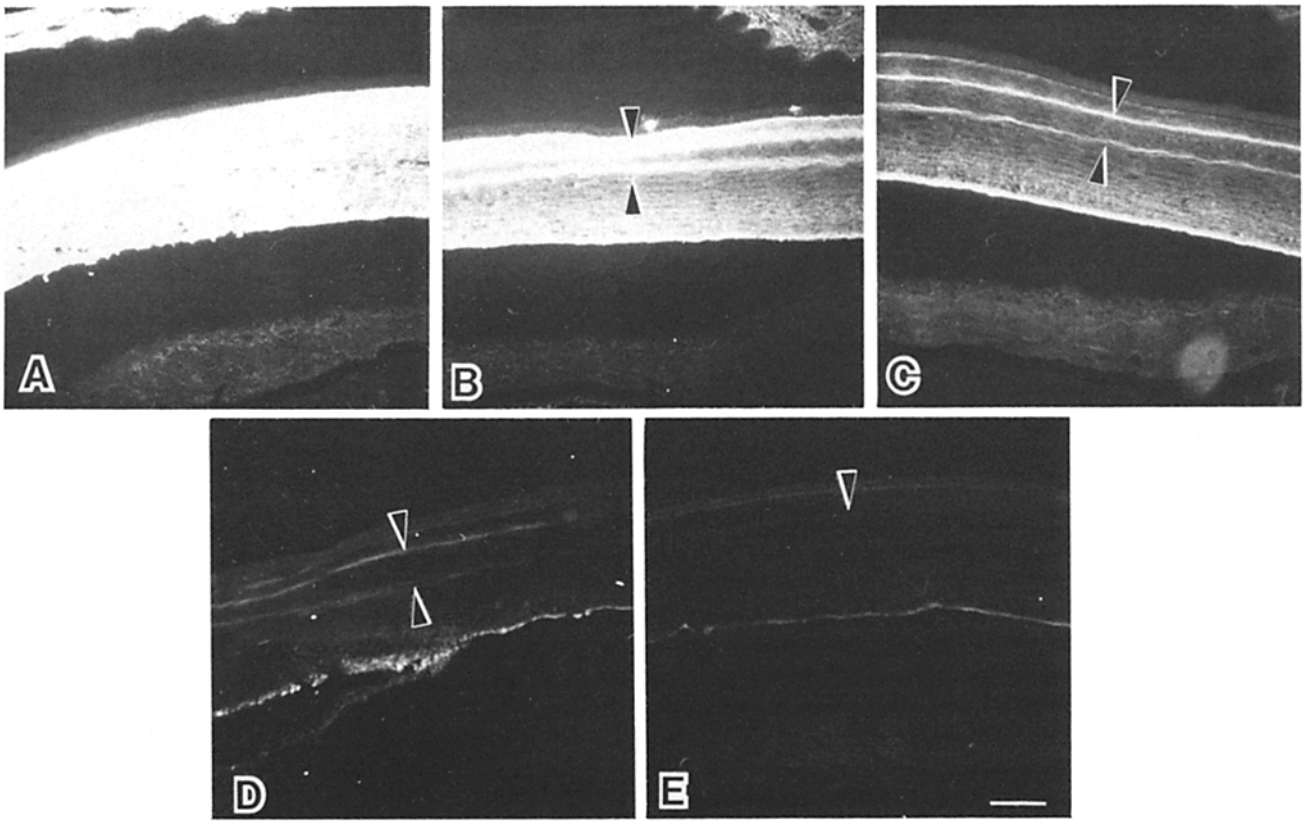

Figure 3. Fluorescence micrographs of sections of 18–19-d embryonic chick cornea reacted with monoclonal antibody I-BA1 at progressively higher temperatures. (*A*) 50°C; (*B*) 52°C; (*C*) 54°C; (*D*) 56°C; (*E*) 58°C. In *B*, *C*, and *D*, the arrowheads designate the two fluorescent layers of interest. In *E*, the arrowhead designates the anteriormost layer. In *A* and *B*, a portion of the eyelid is also seen in the uppermost part of the figure. All are oriented with the epithelial surface at the top. Bar, 100 μm.

major layers of thermally stable fluorescence were most clearly visible at either 52° or 54°C. At times the layers were still slightly visible at 58°C. In the numerous tissue sections and embryos we have examined, the two layers are always present and appear at the same general positions within the stroma. At higher magnification (not shown), each layer consists of several of the alternating lamellae of fibrils or fibril bundles which make up the cornea.[3]

As can be seen in Fig. 4 (middle montage, 54°C), the layers stretch uninterrupted from one edge of the cornea to the other. Within the sclera (Fig. 4) and the eyelid (not shown), at these elevated temperatures, the presence of intensely fluorescent structures, which appear to be large fibers or fibril bundles, also became apparent (most clearly visualized in the enlarged frames of the cornea–scleral junction at the bottom of Fig. 4). At one end, the scleral bundles appear to be in close association with one of the layers of stable corneal collagen. At the other end, they terminate at a scleral ossicle, appearing to insert into its upper and lower surfaces.[4] In Fig. 4, the two ossicles (*o*) are cut in cross section and can be seen as brightly fluorescent structures. (Their intense fluorescence results both from their content of type I [bone] collagen, and their tendency to exhibit autofluorescence.) In control sections reacted with antibody at 50°C or lower, neither the thick scleral collagen bundles nor the stable corneal layers are visible (see Fig. 4, top montage, 40°C).

A trivial explanation that might account for the appearance of the corneal layers and the stable scleral bundles could be that these regions of the cornea simply have a greater collagen concentration, and thus a higher epitope density per unit area. The observed differences, then, might be produced if increasing the temperature at which the antibody reactivity was performed simply lowered the affinity of the antibodies, effectively resulting in a lowered concentration of antibody bound. To examine this possibility, we reacted sections at room temperature with several different dilutions of antibody. The resulting sections simply showed a general decrease in fluorescence throughout the cornea and sclera. There was no sign of either the corneal bands or the scleral bundles.

We examined whether the stability depended upon a normal fibrillar structure and a normal complement of collagen cross-links. Fibrillar organization was altered by pretreating the tissue sections with dilute HAc, a process known to be disruptive to fibril organization (2, 7); covalent cross-link

Also, there was not always a general, uniform decrease in fluorescence throughout the remainder of the anterior and posterior portions of the stroma. Sometimes the anterior portion was slightly more fluorescent and sometimes the posterior.

3. For a more complete description of the arrangement of the alternating lamellae of collagen fibrils or fibril bundles within the avian cornea, see references 1, 3, 5, and 24.

4. With respect to this assay, however, the stability of the corneal layers does not require the presence of these scleral structures. Corneas removed and processed in the absence of sclera still exhibited the layers of stable collagen.

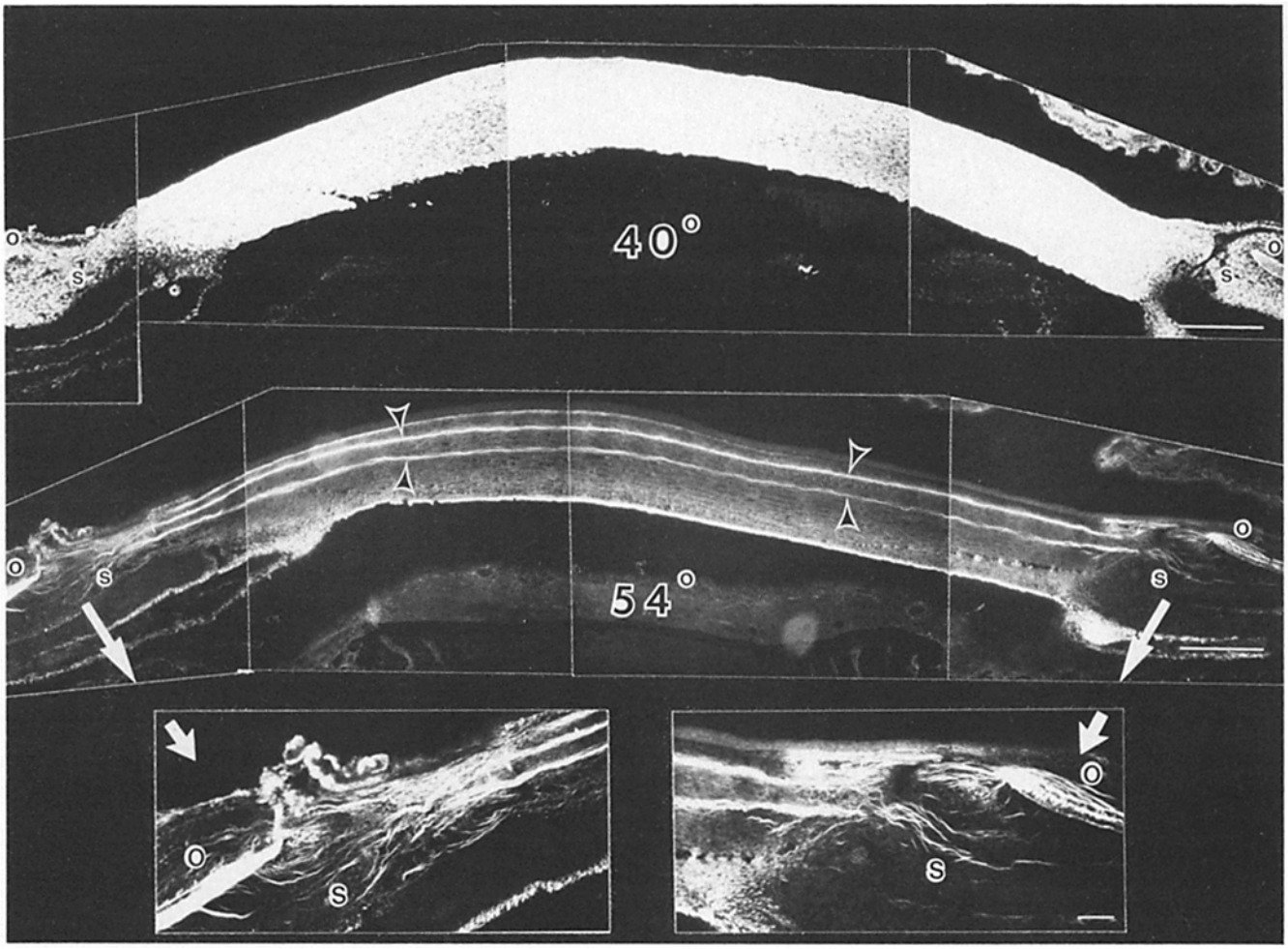

Figure 4. Fluorescence micrographs of 19-d embryonic chick corneas reacted with monoclonal antibody I-BA1 at either 40°C (*top montage*) or 54°C (*middle montage* and *bottom frames*). The two bottom frames (pointed out by the white arrows) are enlargements of the cornea–scleral junction region of the middle montage. (The scleral fibrils and ossicle at the right of the cornea have been displaced slightly anteriorly during the tissue preparation.) All are oriented with the epithelial surface at the top. (*Arrowheads*) Corneal layers. *S*, scleral region. *O*, scleral ossicle. Bars, (*top and middle*) 250 μm; (*bottom*) 50 μm.

formation was reduced by administering the lathyritic agent B-APN to developing embryos in situ. After either treatment, tissue sections reacted at the elevated temperatures still showed the presence of the corneal bands. The only notable difference was that in the lathyritic sections, the bands were most visible at 50°C, suggesting that the absence of a normal complement of cross-links slightly lowered the stability of all the corneal collagen. Enzymatic predigestions of sections to remove glycosaminoglycans (testicular hyaluronidase followed by keratanase) or noncollagenous proteins (trypsin) were also ineffective at destroying the layers.

To determine when during embryonic development the stability of the corneal layers was acquired, sections of corneas from embryos of 8 to 14 d of development were reacted with the antibodies from 50 to 54°C. The first embryonic stage at which a stable corneal layer could be distinguished was 12 d (not shown). Only the anteriormost layer could be detected at this time, and even it was quite diffuse. Within the sclera, a rudimentary ossicle could also be detected at this time, consistent with earlier observations of Coulombre (4). At this stage, however, no large scleral collagen bundles were detectable at elevated temperatures. By 13–14 d, the stage at which the individual scleral ossicles interdigitate with one another to form a continuous ring (4), the anteriormost corneal layer was more clearly evident, especially at the lateral border near the sclera (Fig. 5), and the other layer was becoming evident. That the layers appeared to be considerably wider at these stages is probably due, at least in part, to the fact that the cornea had not yet completely undergone compaction, a process during which the corneal thickness is considerably reduced. Within the scleral region itself, the ossicles were more well formed, as would be expected from the results of Coulombre (4), and the large scleral collagen bundles were now clearly evident. From these observations, it appears that the stable corneal collagen layers do not develop abruptly, but instead form slowly over a period of days. This also seems to be true for the associated scleral structures.

Discussion

When the two antibodies we used in this investigation were examined for their ability to bind in solution to collagen molecules that had been heated to progressively higher tem-

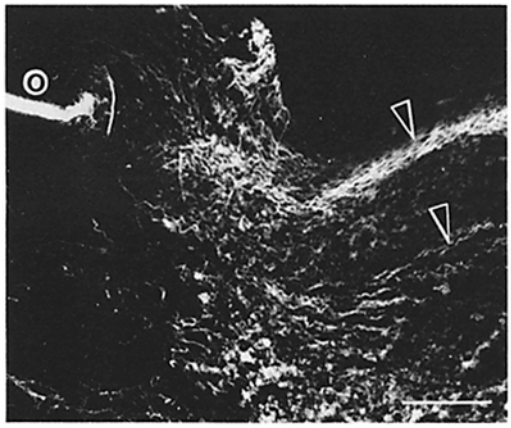

Figure 5. Fluorescence micrograph of a section of a 14-day embryonic corneal–scleral junction, reacted with monoclonal antibody I-BA1 at 52°C. Epithelial surface at the top. Arrowheads designate the stable corneal layers. *O*, scleral ossicle. Bar, 100 μm.

peratures, both behaved similarly, producing a thermal denaturation curve with a T_m of ~44°C and a complete loss of binding by 47°C. This is only ~1.5° higher than the T_m that has been reported for chick type I collagen determined by circular dichroism (CD) spectroscopy (10), and is within the range of the slight differences we have observed in other studies in which we have compared antibody binding to CD spectroscopy for determining thermal denaturation of a collagen in solution (14, 21).

For the cornea, sclera, and dermis in situ, the photometrically measured and statistically averaged decrease in antibody binding with increasing temperature gave a T_m of ~54°C. We have only performed these measurements on late stage chick embryos, so we do not know whether this value would be different for adult animals or animals of different species. The data, however, do appear to fit quite well with previous determinations made on the shrinkage temperature of intact rat tail tendons (8, 23) and fibrils reconstituted from tendon collagen (8).

Although there was no clear-cut difference in the averaged melting temperature of the type I collagen within these three tissues, visual inspection showed that differences do exist, and that such differences can occur even within the same tissue. At the elevated temperatures, the most striking differences observed were the appearance of the stable layers within the cornea, and the appearance of the thick collagen bundles within the sclera and the dermis. None of these differences were apparent until a temperature was reached at which a portion of the collagen population had become denatured.

We describe the scleral and dermal collagen bundles which become obvious at the elevated temperatures as being thick in appearance. We do not know for certain, however, that they have a larger diameter than the total population of bundles which are present within these tissues since, at the level of the fluorescence microscope, the requisite measurements cannot be made. In addition, we do not know whether individual scleral fibrils traverse the entire distance from ossicle to corneal band, and if so, whether they are directly continuous at one end with those of the periosteum surrounding the scleral ossicle, and at the other end with those of the stable corneal layers. The fibrils within the stable corneal layers are presumably of much smaller diameter than those within the sclera; how these could produce direct continuity with one another poses interesting problems in fibril morphology and fibrillogenesis.

Within the corneal stroma, in addition to the two major stable layers (discussed below), two other regions exist in which thermally stable collagen was observed. One of these, which was always observed, was located in the posterior cornea, adjacent to Descemet's membrane; the other, whose presence was variable, was in the anterior part, adjacent to Bowman's membrane. Possibly such interfacial regions where two matrices abut one another are inherently stable. Consistent with this, we previously noted that the type I collagen in the stromal area adjacent to Descemet's membrane appeared to be more resistant to digestion with vertebrate collagenase than was the type I collagen in the rest of the stroma (7).

Each of the two stable layers of interest appears to be comprised of several of the alternating lamellae of collagen fibrils or fibril bundles which make up the cornea.[3] The stability of each layer appears to be inherent to the cornea itself, since they are still present in corneas examined in the absence of any scleral structures. However, attempts to destroy the bands by altering the organization of the collagenous and noncollagenous components of the cornea have thus far yielded negative results. But, as is always the case for negative results, these experiments do not entirely eliminate a direct involvement of the parameters tested. The acetic acid pretreatment may not be a severe enough disrupting agent to produce the requisite alterations in fibrillar structure; the administration of B-APN does not prevent all aldehyde-derived cross-link formation—it only lowers the relative content; and the enzymatic pretreatments of sections to remove selected noncollagenous components are unlikely to have quantitatively removed their respective substrates. Other alternatives exist which have not yet been examined. One is differences in interactions (2, 7, 13) among the collagen types we know to be present in the mature embryonic cornea (i.e., type V [14] and type VI [18]). Another is a suprafibrillar organization such as the multifibrillar 0.5–2 μm bundles recently described in the embryonic avian cornea (1; see also reference 3).

Our observations indicate that there appears to be an association of the corneal bands, scleral collagen bundles, and scleral ossicles. It is of interest that Slonaker (22) has suggested that contraction of the ciliary muscle, which underlies the scleral ossicles, could alter the diameter of the bony ring and thus change the curvature of the cornea during accommodation. From their morphological appearance, the thick scleral collagen bundles seem to form a bridge from the scleral ossicles to the stable corneal bands. Thus, they potentially could transduce the force generated by changes in the scleral ring to the stable corneal bands. Tension on the stable corneal bands, then, could be responsible for adjusting the curvature of the cornea, but this remains to be tested. Alternatively, the bands may act to stabilize the shape of the cornea.

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