Chick corneal development in vitro: diverse effects of pH on collagen assembly*

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*This paper is dedicated by J.B.L.B. and D.J.S.H. to John Chapman on the occasion of his retirement
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SUMMARY

In vivo, the embryonic chick corneal epithelium lays down a stroma of collagen and proteoglycans whose fibrils are unusual as their diameter distribution peaks sharply about a mean of 20 nm. Such epithelia cultured on Nuclepore filters will also lay down a stroma containing 20 nm diameter fibrils, although there is only limited orthogonal organisation. We report here that collagen fibril morphology is critically dependent on the pH of the medium in which the corneal epithelium is cultured and that normal 20 nm diameter fibrils only assemble in a narrow band around neutral pH (approx. 6.9-7.4). At higher pH (7.6-8.1), fibrils in the distal region of the stroma more closely resemble those seen in non-corneal stroma as their diameters can be up to 200 nm even though fibrils near the basal lamina are only about 10 nm in diameter. At low pH (approx. 6.5), there are again wide fibrils, but with the hieroglyphic cross-sections typical of those seen in heritable disorders of N-terminal procollagen processing. Biochemical analysis by SDS-PAGE and fluorography confirms that N-terminal procollagen processing is deficient at this pH. At very low pH (approx. 5.8-6.2), there is little processing of procollagen and the stroma comprises filamentous material with the occasional banded structures typical of those formed by unprocessed procollagen at high concentration. Gel electrophoresis and peptide mapping showed that the collagens produced by the corneal epithelium of the primary stroma included types I, II and V and that total collagen production, as assessed by incorporation of [³H]proline, increased with pH, although the relative amounts of the different collagens produced remained essentially unchanged. While the biochemical data can account for the altered morphologies in the pH range 5.8 to 7.0, the sensitivity of fibril diameter to small changes around neutral pH remains unexplained, but points to the subtle, charge-based interactions necessary for the formation of 20 nm diameter fibrils in the developing cornea.

Key words: collagen, cornea, extracellular matrix, fibrillogenesis

INTRODUCTION

In most tissues, collagen fibril diameters are widely distributed around a mean of about 100 nm; in the developing chick cornea, however, the fibrils that mainly contain collagens I, II and V have diameters that peak sharply about a mean of 20 nm (Birk et al., 1988; Bard and Bansal, 1987). The mechanisms responsible for this unusual distribution in the cornea remain unknown, but are particularly important as the transparency of the cornea depends on both fibril uniformity and the low diameter (Maurice, 1957). Unfortunately, soluble corneal collagen reassembles in vitro to give wide rather than 20 nm fibrils (Bard et al., 1988; Birk et al., 1990) and the normal techniques for investigating collagen fibrillogenesis cannot therefore be used to study the process. We have, however, shown that the chick corneal epithelium will, when isolated from its stroma with an intact basal lamina and cultured under appropriate conditions, lay down a new stroma with 20 nm fibrils (Bansal et al., 1989a) that mimics the primary stroma laid down in the early chick eye (for review, see Hay, 1980). This system thus permits the process of corneal fibrillogenesis to be studied experimentally.

In this paper, we investigate how the corneal stroma laid down in vitro depends on the pH of the medium and report that relatively small changes in pH have surprisingly dramatic effects on fibrillogenesis, even though proportions of the various collagen types remain unaffected by pH. In the discussion, we explore the implications of these observations for collagen self-assembly and corneal fibrillogenesis in vivo.

MATERIALS AND METHODS

Embryos

Fertile eggs of S-line White Leghorn chicks were obtained from the AFRC Institute of Animal Physiology and Genetics, Edinburgh
Research Station, Roslin, Midlothian, UK, and incubated in a 38°C humidified incubator. Embryos were staged as required by age or according to the criteria of Hamburger and Hamilton (1957).

**Dissection and culture techniques**

Squares of corneas from 5.5-day chick embryos were dissected out in phosphate-buffered saline, incubated for 18-20 min at room temperature in dispase (0.25 mg/ml; grade II, Boehringer Mannheim) supplemented with DNase (5 µg/ml; grade I, Sigma, UK; this is added to minimise stickiness in the tissue due to DNA from disrupted cells) made up in phosphate-buffered saline, washed in three changes of fresh saline and left for 10 min to recover. Epithelia were then peeled off their stromas and placed lamina-downwards on ~3 mm² pieces of Nucleapore filter (pore size, 0.1 µm). These squares were put on the grids of a standard Falcon organ-culture dish containing about 0.75 ml medium supplemented with 10% fetal calf serum and 50 µg/ml ascorbic acid. Dishes were kept in a plastic box, lined with wet Kleenex to maintain humidity, and cultured in a gassed and humidified incubator for 20-24 h (for further details, see Bansal et al., 1989a).

The medium used was mainly DMEM as its pH can be varied by adjusting its sodium bicarbonate content and the percentage of CO₂ in the incubator atmosphere. For pH 7.9 medium, 50 µl of bicarbonate stock (7.5%, w/v) was added per ml of DMEM medium and 5% CO₂ was used. For pH 7.2 media, we used either DMEM to which had been added 15 µl of bicarbonate per ml of medium and 7% CO₂ or MOPS-buffered F10 medium in an ungassed incubator. For low pH values of about 6, DMEM medium was used with the bicarbonate being omitted and with the corneas being cultured in 7% CO₂. Intermediate pH values were achieved by changing the the amount of bicarbonate added to DMEM medium. To confirm that the medium acidity was stable, its pH was checked after 3 and 24 h.

**Microscopy**

Cultured epithelia were first gently detached from their filters (it is hard to embed Nucleopore in resin) and then fixed in 2.5% cacodylate-buffered glutaraldehyde and postfixed in osmium tetroxide. Specimens for TEM were dehydrated and routinely flat-embedded in Araldite (Bansal et al., 1989a). Blocks were cut and split along an axis parallel to one side of the square so that the section would be parallel or perpendicular to the original orientation of the chorioid fissure. Thick and thin sections were cut on an LKB Ultratome V, the former were stained with toluidine blue from TEM micrographs of at least three stromas. For this, micrographs were printed at ×50,000 and the diameters of fibrils measured with a Brinel lens.

**Biochemical analyses**

To measure procollagen processing and the production of different genetic types of collagen, the culture medium was supplemented throughout the incubation with [5-³H]proline (Amersham, U.K.), to a final concentration of about 100 µCi/ml. Usually, about 10 epithelia were cultured together, with 3-4 epithelia per 3 mm² piece of Nucleopore filter. At the end of the incubation period, Nucleopore filters (with adherent epithelia) and culture medium were processed as follows.

For studies on the extent of procollagen processing, proteinase inhibitors (250 mM EDTA, 100 mM NEM, 10 mM PMSF in 1 M Tris-HCl, pH 7.4) were added to both the filters (20 µl, undiluted) and the medium (approximately 75 µl, final tenfold dilution). Further procedures were at 0-4°C. Following the addition of type I collagen carrier to the medium (final concentration 10 µg/ml, from a 400 µg/ml stock solution in 5 mM acetic acid), high Mr proteins were precipitated by addition of ammonium sulphate to a final concentration of 30% saturation (from a 90% saturation stock solution in 0.1 M Tris-HCl, 25 mM EDTA, pH 7.4) followed, after ~20 h, by centrifugation for 30 min at 15,600 g.

After discarding the supernatants, ammonium sulphate pellets and epithelia (attached to Nucleopore filters) were then extracted for 3 min at 100°C in 200 µl of 2% SDS, 0.125 M Tris-HCl, pH 6.8. Following reduction with 2-mercaptoethanol, the extracts were analyzed by discontinuous SDS-PAGE (Laemmli, 1970) with 5% acrylamide in the separating gels. Gels were processed for fluorography (Mellor et al., 1991) and exposed to pre-flashed Kodak X-Omat XAR5 film.

To measure the relative amounts of different collagen types, epithelia and culture media were collected as above, except that proteinase inhibitors were not added. All subsequent procedures were at 0-4°C. Proteins in the medium were precipitated with ammonium sulphate (as above) and the ammonium sulphate pellets and epithelia (attached to Nucleopore filters) were extracted, each in 250 µl of 0.5 M acetic acid containing 100 µg/ml pepsin (Boehringer Mannheim). Extractions were for 18-24 h on an orbital shaker. After centrifugation for 5 min at 15,600 g, extracts were separated from insoluble material and both were freeze-dried.

Finally, all samples, including pepsin extracts of both medium and epithelia as well as pepsin-insoluble material, were dissolved in 2% SDS, 0.125 M Tris-HCl, pH 6.8, and immediately heated to 100°C for 3 min, followed by SDS-PAGE and fluorography, as above.

To identify collagen types and collagen precursors, samples were analysed by SDS-PAGE as above, and then further analysed in a second dimension after treatment with cyanogen bromide. The procedure was essentially that of Sokolov et al. (1989), except that individual gel tracks were cut out of the first slab gel, and were processed with CNBr prior to loading, with the aid of a 1% agarose plug, on a second discontinuous slab gel system with 12% and 5% acrylamide in the separating and stacking gels, respectively. After electrophoresis in the second dimension, gels were processed for fluorography as above.

Standards of ³H-labelled chick type I procollagen were prepared from embryonic tendon cells as previously described (Mould et al., 1990) and its varied processed forms, pN-collagen, pC-collagen and collagen, were prepared by digestion with procollagen C-proteinase (Hojima et al., 1985) and/or procollagen N-proteinase (Hojima et al., 1989). Pepsinised collagen I was prepared from 17-day chick embryo tendons as follows (all procedures being at 4°C). After a preliminary extraction with 4 M NaCl, 50 mM Tris-HCl, pH 7.4 (measured at room temperature), tendons were extracted overnight in 0.5 M acetic acid containing 1 mg/ml pepsin (Boehringer, gastric mucosal enzyme). After centrifugation, the supernatant was collected, neutralised and dialysed against 0.7 M NaCl, 0.5 M acetic acid. Precipitated collagen was pelleted by centrifugation, dissolved in 0.5 M acetic acid and dialysed against 3.5 M NaCl, 50 mM Tris-HCl, pH 7.4. The neutral-salt precipitate was pelleted by centrifugation, then dissolved in 0.5 M acetic acid and dialysed against 0.1 M acetic acid before freeze-drying. Standards of pepsinised chick collagens II and V were prepared from sterna and gizzard, respectively, as described (Mayne and Zettergen, 1980; Reese and Mayne, 1981).

To measure the relative amounts of newly synthesised collagenous material present in the various extracts, [³H]hydroxyproline was determined by HPLC on a Bio-Rad HPX-87C cation exchange column (Stimler, 1984). After addition of unlabelled collagen as carrier, ³H-labelled proteins were precipitated from both pepsin and SDS extracts by addition of ethanol to a final concentration of 75%, followed, after standing overnight at 4°C, by centrifugation for 15 min at 15,600 g. All pellets, including insoluble material remaining after pepsin or SDS extraction of epithel-
lia, were then hydrolysed in 6 M HCl for 20 h at 110°C. Hydrolysates were then dried down in a Speedivac rotary evaporator, dissolved in water and filtered through Acrodisc filters (pore size 0.2 µm, Gelman Science) before application to the HPLC column.

RESULTS

The observations are divided into two groups. The first describes the morphologies of the stromal fibrils laid down when chick corneal epithelia are cultured in medium whose pH ranges from about 8 down to 6. The second includes the biochemical investigations that we have undertaken to investigate why pH should have such a dramatic effect on fibrillogenesis in the corneal stroma.

Fibrillogenesis in pH 7.2 medium

Epithelia cultured basal-lamina-downwards on a Nucleopore filter in pH 7.2 DMEM or F10 medium for 24 h lay down a stroma some 4 µm thick that extends across most of the cell sheet (Fig. 1a). As has previously been shown (Bansal et al., 1989a), this stroma contains a mass of distinct, uniform fibrils and there is some evidence of local orthogonal organisation (Fig. 1b). The diameters of these fibrils are very similar to those observed in vivo, peaking sharply at about 20 nm (Fig. 2).

Fibrillogenesis in high pH medium

When epithelia were cultured in pH 7.8-8.0 DMEM, the resulting stromas were very different from those that formed at pH 7.2. Instead of containing normal, uniform 20 nm diameter fibrils, these stromas had a mass of fine filaments near the basal lamina and wider fibrils distal to it (Fig. 1c,d). The histogram of fibril diameters confirms that collagen assembly has gone awry (Fig. 2). At pH 7.8, the peak in the diameter distribution at 10 nm is from fibrils near the basal lamina, while the flat tail from fibrils in the distal part of the stroma extends to 200 nm. As distance from the lamina is likely to be a measure of the time since fibrillogenesis began (Bard and Bansal, 1987), these thin, abnormal fibrils are newly formed while the broad ones reflect those that were first laid down. This situation is apparent in both 12 and 24 h cultures (not shown), so demonstrating that fibrils are thin when they first assemble and then thicken, although it is not clear whether this happens by accretion or by fusion of fibrils. Alternatively, the thin fibrils may have a different collagen-type composition from that of the thicker fibrils.

Fibrillogenesis in low pH medium

As the pH of the medium is dropped below about 6.9, further unusual features are apparent in the stroma. At pH 6.85, the very great majority of the fibrils have, as expected, 20 nm diameter fibrils, but are now less well organised; there are also the occasional, small convoluted hieroglyphic-like aggregate. At pH 6.7, this effect is more pronounced in that there are many more of these aggregates, while the relatively few fibrils that are present maintain 20 nm diameters, but seem far less well organised than those at higher pH in that they no longer seem to be preferentially oriented parallel to the basal lamina (Fig. 3a).

At about pH 6.5, the organisation of the stroma has changed yet again as it no longer contains any 20 nm fibrils (Fig. 3b,c). Instead, there are broad, banded loosely organised fibrillar structures and, in addition, many more of the hieroglyphic-like, nonstriated aggregates that are similar to but larger than those seen at pH 6.7 (Fig. 3b) and may be cross-sectional views of the loose fibrillar structures. These aggregates are similar to those seen in dermatosparaxis, which are derived from the assembly of inadequately processed procollagen (Holbrook and Byers, 1982; Hulmes et al., 1989; Smith et al., 1992). The matrix within which these fibrils are embedded also contains small dense aggregates within a low-staining background.

As the pH is dropped to 6.2 and below, the morphology changes once more. The stroma now contains some ill-formed fibrils embedded in a mainly amorphous matrix. The fibrils are up to about 80 nm wide and show a 60 nm spacing marked by a strong line of positive staining (Fig. 3d,e); these fibrils are characteristic of those formed by unprocessed procollagen at high concentration (Mould et al., 1990). The matrix in which these fibrils are embedded is mainly amorphous, although it contains the occasional structure that seems to be a hollow sphere about 30 nm in diameter from which radiates thin filaments about 50 nm in length (open arrow; Fig. 3d). At pH 5.8, the small amount of matrix laid down no longer contains any fibrils; instead, it seems to contain lateral aggregates of fine filaments. These have the length expected of procollagen molecules and the lateral association is roughly analogous to the SLS form of collagen (Fig. 3f). This is probably the lowest pH at which the epithelium can survive as basal lamina tend to detach from its basal surface, while its constituent cells tend to have pyknotic nuclei and to separate from one another.

Biochemical observations

It was not clear whether the changes that pH effects on stromal morphology reflect an alteration to the milieu in which fibrillogenesis takes place or whether it results from some effect on collagen synthesis. To distinguish between these options, we have investigated whether changing the pH of the medium might affect the total amounts of collagen produced, the extent of processing of procollagens to collagens or the relative proportions of the different collagen types. In all cases, epithelia were cultured in the presence of [3H]proline in appropriate media for 24 h and both the epithelia and their culture medium then analysed.

Different collagen types were identified in pepsin extracts, subsequent SDS extracts and pepsinised culture media. As shown in Fig. 4, pepsin extracts revealed two prominent bands when analysed by SDS-PAGE and fluorography. Comparison with chick collagen standards showed that the strongest bands co-migrated with the α1(I) and α2(II) chains of pepsinised collagens I and II, respectively, while the lower band co-migrated with α2(I). A third, weaker and slowly migrating band also appeared in a position corresponding to the α1(V) chain of pepsinised collagen V (McLaughlin et al., 1989). Compared with the pepsin extracts, the proportion of total band intensity at the α1(V) position was greatest in the SDS extracts of pepsin-insoluble material, while, in the pepsinised media, this pro-
portion was essentially zero (Fig. 4). According to the total amount of radioactivity in pepsin-resistant bands, total collagen production increased systematically in the pH range 6.2 to 7.9. This increase was observed in both the pepsin and the subsequent SDS extracts, as well as in the medium.

To identify the collagen types present in the extracts, peptide maps were generated by electrophoresis in the second dimension following treatment with CNBr. Fig. 5 shows the results for the pepsin extracts compared to peptide maps of pepsinised chick collagen standards. The presence of collagens I and II in approximately equal amounts was confirmed by the appearance of characteristic CNBr peptides, in agreement with previous observations on chick corneal primary stroma (Linsenmayer et al., 1977). Fig. 5 also indi-
cates that the slowly migrating band in Fig. 4 is the $\alpha_1(V)$ chain of pepsinised collagen V. There is also evidence for the presence of the $\alpha_2(V)$ of pepsinised collagen V co-migrating with $\alpha_1(I)$ and $\alpha_1(II)$. Similar experiments identified collagens I, II and V in the SDS extracts of pepsin-insoluble material.

As might be expected from the morphological data, pH had clear effects on the extent of procollagen processing (Fig. 6). At pH 6.0, after overnight incubation, comparisons with procollagen standards showed that procollagens I and II remained either unprocessed or only partially processed to their respective pN-collagens. The identity of the pN-collagen bands was confirmed by comparison with standards (Fig. 6a) and by peptide mapping with CNBr, followed by SDS-PAGE in the second dimension (Fig. 7). The band identified as pN$\alpha_1(I)/pN\alpha_1(II)$ was found to have the same peptide map as that of the $\alpha_1(I)/\alpha_1(II)$ band. At pH 6.3, processing to pN-collagen was almost complete, although only limited conversion to fully processed collagen was observed. At pH 7.0, extensive processing of procollagens I and II to the corresponding pN-collagens was observed, although densitometry of the fluorograms showed that approximately 36% remained in the form of pN-collagen. The pattern of processing at pH 7.7 was similar, although the relative amount of pN-collagen was reduced to about 18%.

At higher pH (7-8), where only small differences in processing were observed, we measured collagen-type ratios to see if changes here might correlate with changes in fibrillogenesis. The relative amounts of the different collagen types in the pepsin, the subsequent SDS extracts and pepsinised media were measured by densitometry of fluorograms (e.g. Fig. 4). These amounts were calculated on the basis that the intensity of the 2(V) band is half that of the $\alpha_1(V)$ band and that the intensity of the $\alpha_1(I)$ band is twice that of the $\alpha_2(I)$ band. As shown in Table 1, we found no significant effect of pH on the relative amounts of collagens I, II and V, which were in the approximate proportions 10:9:1 and 2:2:1 in the pepsin and the subsequent SDS extracts, respectively, with overall proportions of about 6:6:1. In the pepsinised media, the corresponding proportions were approximately 2:1:0. Analysis of the $[^{3}H]$hydroxyproline content showed that 99% of newly synthesized collagens produced by the cultured epithelia were extracted by pepsin or, subsequently, by SDS.

A further effect of pH is also evident in Fig. 6. The presence of high molecular mass material in the stacking gel is indicative of extensive cross-linking. As samples were reduced with $\beta$-mercaptoethanol (which breaks disulphide bonds) prior to electrophoresis, it is likely that this material is cross-linked by lysine-derived bonds. Cross-linked forms of collagens I and II, or their precursors, as well as collagen V, were also apparent in Figs 6 and 7. The extent of this cross-linking was negligible at pH 6.0 and 6.3, but increased markedly at pH 7.0 and 7.7. Analysis by HPLC showed that 97% of total $[^{3}H]$hydroxyproline was extracted from the cultured epithelia by SDS in the presence of proteinase inhibitors.

Table 1. Effect of high pH on the relative amounts of different collagen types produced by isolated epithelia in vitro

<table>
<thead>
<tr>
<th>Extraction procedure</th>
<th>Number of experiments</th>
<th>pH</th>
<th>Collagen I</th>
<th>Collagen II</th>
<th>Collagen V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsin</td>
<td>12</td>
<td>7.2</td>
<td>49.5 (9.8)</td>
<td>46.2 (8.9)</td>
<td>4.3 (2.3)</td>
</tr>
<tr>
<td>Pepsin</td>
<td>14</td>
<td>7.9</td>
<td>49.5 (3.4)</td>
<td>45.5 (4.2)</td>
<td>5.0 (2.6)</td>
</tr>
<tr>
<td>SDS*</td>
<td>14</td>
<td>7.2</td>
<td>39.3 (6.4)</td>
<td>42.8 (6.7)</td>
<td>17.9 (6.3)</td>
</tr>
<tr>
<td>SDS*</td>
<td>15</td>
<td>7.9</td>
<td>43.1 (6.9)</td>
<td>38.2 (4.7)</td>
<td>18.7 (4.3)</td>
</tr>
<tr>
<td>Medium†</td>
<td>8</td>
<td>7.2</td>
<td>68.0 (6.2)</td>
<td>32.0 (6.2)</td>
<td>–</td>
</tr>
<tr>
<td>Medium†</td>
<td>7</td>
<td>7.9</td>
<td>65.6 (4.3)</td>
<td>34.2 (4.4)</td>
<td>0.2 (0.5)</td>
</tr>
</tbody>
</table>

Values are percentages (and standard deviations) based on densitometric scanning of fluorograms, and calculated assuming that the intensity of the $\alpha_2(V)$ band is half that of the $\alpha_1(V)$ band, and that the intensity of the $\alpha_1(I)$ band is twice that of the $\alpha_2(I)$ band.

*Insoluble material remaining after pepsin extraction (about 25% of total extractable $[^{3}H]$collagen) was subsequently extracted with SDS (see Materials and Methods).

†Culture medium was precipitated with ammonium sulphate and treated with pepsin. About 40% of total radio-labelled collagens were found in the medium, irrespective of pH, as determined by densitometry of fluorograms.
Fig. 3
DISCUSSION

The observations show that the morphology of the fibrils laid down in vitro by the chick corneal epithelium is highly pH-dependent and that the normal 20 nm diameter fibrils are only laid down in a very narrow pH range. In particular there are large changes in fibril diameter in the pH range 7.2 to 8.0 and, in this context, a clear explanation is provided for an earlier observation (Bansal et al., 1989b), that corneal epithelia cultured in DMEM gave broad fibrils while 20 nm fibrils were deposited in F10. It is now obvious that these earlier results derived from the relatively high pH of the standard DMEM medium (which starts off alkaline as it was intended for rapidly growing cells that produce their own CO$_2$) and the results presented here show that, if the pH of the DMEM is maintained at 7.2, stromal morphology and biochemistry are the same in both DMEM and F10 media.

The very great range of morphologies that relatively small changes in pH can elicit is surprising and there are several possible and overlapping mechanisms that may contribute to these diverse forms of assembly, with the environmental pH affecting the process of fibrillogenesis either directly or indirectly. Direct effects are pH-dependent changes in the charge distributions on the collagen molecules or on the other macromolecular constituents of the stroma. Indirect effects include the pH dependence of the enzyme activities involved in procollagen processing and collagen crosslinking, and possible biosynthetic changes in the production of different matrix components, including both the amounts and proportions of the different collagen types as well as the non-collagenous material.

Procollagen processing and cross-linking

There is a clear correlation between morphology in the pH
range 5.8 to 7.2 and the extent of procollagen processing. At the lower limit of this pH range, extracellular, non-fibrillar bundles were observed that are reminiscent of the procollagen bundles previously seen in secretory vesicles (Marchi and Leblond, 1983), and our biochemical observations confirm that there is very little processing of procollagen at pH 6.0. The pH within these procollagen secretory vesicles is unknown, but, by analogy with other secretory systems (Anderson and Orchi, 1988), it is likely to be low. If so, our observations suggest that the presence of procollagen bundles in secretory vesicles derives from their low internal pH inhibiting procollagen processing and encouraging bundle formation.

As the pH is raised above 6.0, D-periodically banded fibrillar structures appear in the matrix. The prominently staining single band in each D repeat is a characteristic feature of the fibrillar structures formed by highly purified type I procollagen in vitro when assembled at high concentrations in physiological buffer conditions (Mould et al., 1990), and here the strongly staining band is probably due to the procollagen C-propeptide. In the pH range 6.5 to 6.7, D-periodic structures are again seen, but without the single strongly staining band, and these appear as hieroglyphics in cross-section. Similar structures have been observed in a number of heritable connective tissue disorders where N-terminal procollagen processing is known to be defective (Holbrook and Byers, 1982; Smith et al., 1992). Limited persistence of the N-propeptide is sufficient to account for the altered morphology, as has recently been shown in a study where mixtures of type I collagen and pN-collagen generated by C-terminal processing of purified biosynthetic precursors were allowed to self-assemble in vitro (Hulmes et al., 1989). Again our biochemical data on corneal epithelia are consistent with these studies, since procollagen C-terminal processing takes place above pH 6.3 and gives pN-collagen/collagen mixtures in which the proportion of collagen increases with pH. The increased extents of procollagen processing and cross-linking are consistent with
the pH optima (all about pH 8.0) of the extracellular enzymes of collagen biosynthesis, i.e. procollagen N-proteinase (Hojima et al., 1989), procollagen C-proteinase (Hojima et al., 1985) and lysyl oxidase (Kagan, 1986).

The striking changes in fibril morphology that occur between pH 7.2 and 8.0 are more difficult to account for in terms of procollagen processing alone, even though there is a drop in the relative amount of pN-collagen from about 36% to 18% as the pH is raised between these limits. Fleischmajer et al. (1987) have proposed a role for the N-propeptide in fibril-diameter limitation, and the lower pN-collagen content would be consistent with the increased diameters that are observed at pH 8. Limited support for such a role for the N-propeptide comes from in vitro experiments (Hulmes et al., 1989) in which mixtures of pN-collagen and collagen were allowed to assemble in vitro after C-terminal processing of their respective biosynthetic precursors. When the proportion of pN-collagen was low, near-cylindrical fibrils were formed and the fibrils were narrower than those consisting entirely of fully processed collagen. Fibrils in this in vitro system were, however, much wider than the 20 nm fibrils observed either in the primary corneal stroma in vivo or in the model system described here at pH 7.2. It is difficult to see how a simple N-propeptide-mediated, steric-exclusion mechanism (Chapman, 1989) alone could account for these observations: it would require that 20 nm fibrils consist entirely of pN-collagen in order for their surfaces to be completely coated in N-propeptides, yet the observed proportion of pN-collagen at pH 7.2 is no more than 36%. Furthermore, purified pN-collagen forms sheets, not cylindrical fibrils, and there must therefore be additional mechanisms operating within the stroma that preserve the normal cylindrical shape.

There is, however, a real difficulty in trying to extrapolate from studies on the assembly of purified collagen in vitro to the process of fibrillogenesis in vivo and for two reasons. First, it is not possible to recreate in vitro the complex spatial and temporal aspects of fibril formation found in vivo that allow collagen to interact with other stromal components and, second, the kinetics of assembly are very different in the two systems. Protein concentrations within the developing stroma are likely to be several orders of magnitude greater than those attainable when purified components are assembled in vitro and nucleation rates for fibrils are more highly concentration dependent than are growth rates (Oosawa and Akasera, 1975). Mean fibril diameters are thus likely to decrease with increasing protein concentration and so be less in vivo than in vitro for kinetic reasons. Observations on the concentration dependence of type I collagen assembly after enzymatic removal of the C-propeptide from pC-collagen support this hypothesis (D. J. S. Hulmes, K. E. Kadler, Y. Hojima and D. J. Prockup, unpublished). Together, these limitations imply that in vitro observations can do no more than point to the underlying mechanisms that regulate fibrillogenesis in vivo.

**Collagen types**

Our observations show that the total amount of [3H]proline incorporated into newly synthesised collagens increases systematically with increasing pH. If there are no changes in the specific activity of proline precursor pools, we can conclude that total collagen production also increases with pH. It is thus unlikely that total collagen production is significant in the control of fibril morphology, since broad fibrils were observed, albeit with different morphologies, on either side of pH 7.2, the optimal pH for normal fibrillogenesis, even though the amounts of collagen (including processing intermediates) were different.

The observation that collagen V had been laid down in vitro was unexpected because immunohistochemical observations on primary stromae have failed to show the presence of this collagen type (Linsenmayer et al., 1984). One possible explanation for our result is that the epithelia had aged in culture and had switched to producing the collagen V seen in secondary stroma (first deposited at 6-6.5 d). While this possibility cannot be excluded, it should be pointed out that the maximum age of the eggs when used was 5.5 d and that many were younger. Moreover, TEM micrographs showed no difference in morphology between fibrils laid down after 12 h and after 24 h in vitro (i.e. there is little time for a switch). An alternative explanation for the difference is that the amount of collagen V could be too low to be detected by immunohistochemistry here, particularly in view of the various procedures required to unmask antigenic sites (Linsenmayer et al., 1984, 1990; Birk et al., 1988) and as our quantitative studies show that collagen V represents only 8% of total collagen. It is noteworthy that collagen V is poorly extracted by pepsin compared to the subsequent SDS extraction, a result suggesting that collagen V is relatively inaccessible and/or tightly bound to other matrix components. The possibility that small amounts of collagen XI may also be present here cannot be excluded from the data shown in Fig. 5. The peptide maps of collagens V and XI are, however, sufficiently distinct to demonstrate unequivocally the presence of collagen V (see Reese and Mayne, 1981).

An additional feature of the system used here is the production of collagen II in amounts relative to collagen I that are similar to those found in vivo. These findings differ from those of Ruggiero et al. (1990) where a similar in vitro system was used, but no collagen II was observed in the matrix as assessed by immunohistochemistry. The two systems differ in the presence (here) or absence (Ruggiero et al., 1990) of a basement membrane and the use of different substrata. When chick corneal epithelia were cultured on a collagen IV substratum, abundant matrix was produced (Ruggiero et al., 1990), but the organisation of the matrix was less than observed here at pH 7.2. These studies thus underline the importance of the culture conditions and the possible role of the basement membrane in mediating matrix organisation. From our biochemical studies with collagen V, which also appear to be in conflict with the immunohistochemical studies, the apparent absence of collagen II reported by Ruggiero et al. (1990) should be verified biochemically.

It is noteworthy that the relative amounts of collagens I, II and V appeared to remain unchanged in the pH range 7.0 to 8.0, although there were large changes in the fibril diameter distributions. Studies on the reconstitution of collagen fibrils in vitro with mixtures of collagens I and V have supported the notion that fibril diameters are limited by increasing proportions of type V collagen (Birk et al.,
of about 100 nm. It thus seems unlikely that direct effects of collagen in vitro. In studies over the pH range 7 to 8 (Wood and Fleischmajer, 1973), the diameters of fibrils reconstituted from dermal collagen (types I and III) decreased with increasing pH, in contrast to the results reported here, but in all cases the diameters of the reconstituted fibrils were much larger than 20 nm and had a mean of about 100 nm. It thus seems unlikely that direct effects of pH on collagen-fibril interactions can account for our observations, although there may be complex effects of pH on the interactions between different collagen types and the non-collagenous stromal components. It will be of interest to determine by immunolocalisation whether the different diameter fibrils produced at abnormal pH show differences in collagen type composition.

Interactions with other stromal components

The corneal stroma also contains small amounts of collagens IV, VI and IX (Linsenmayer et al., 1990; Fitch et al., 1991), a cornea-specific collagen (Marchant et al., 1991) and a number of non-collagen components of which the best characterised are the proteoglycans (Scott, 1991), but the morphogenetic significance of each remains unclear. Although dermatan/chondroitin sulphate proteoglycans are the major proteoglycans in the 6-day chick embryo stroma, the role of their glycosaminoglycan (GAG) chains in collagen assembly is minimal (Bansal et al., 1989a) since fibril formation is normal when epithelia are cultured in the presence of testicular hyaluronidase (which degrades dermatan/chondroitin sulphate) or β-xyloside (which interferes with proteoglycan synthesis). Similarly, no effect on fibrillogenesis is observed when epithelia are cultured in the presence of keratanase, which degrades keratan sulphate proteoglycans (Bansal et al., 1989a). These experiments do not, however, exclude a possible role for the proteoglycan cores in the control of fibril formation. The small proteoglycans decorin (Vogel et al., 1984), fibromodulin (Hedbom and Heinegard, 1989) and lumican (Blochberger et al., 1992) are all known to inhibit collagen fibril formation in vitro and, in the case of decorin, this is a property of the protein core (Vogel et al., 1984). There may also be additional components, resistant to hyaluronidase and keratanase, that are important for fibril assembly (Bansal et al., 1989a).

If the mechanisms responsible for fibrillogenesis in the cornea are to be understood, further work will have to be done. This will involve identifying the relevant non-collagenous components in the stroma and elucidating their interactions with collagen. Further analysis of the kinetics of normal fibrillogenesis will also be needed and, here, the ability of our model system to lay down collagen of a wide range of morphologies may well be helpful. As the epithelium can lay down fibrils in vitro that are typical of both corneal (~20 nm) and non-corneal (~100 nm) tissues, the system may allow elucidation of the mechanisms responsible for laying down constant-diameter fibrils (Bard and Chapman, 1969, 1973) as well as the additional tier of control that further limits the diameter to 20 nm in the cornea.

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