The extracellular matrix of the developing cornea: diversity, deposition and function*

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*We dedicate this paper to the memory of Steven Meier, a sometime colleague of J.B.L.B.
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Summary

This paper examines the role of the extracellular matrix (ECM) in the development of the cornea. After a brief summary of the corneal structure and ECM, we describe evidence suggesting that the differentiation of neural crest (NC) cells into endothelium and fibroblasts is under the control of ocular ECM. We then examine the role of collagen I in stromal morphogenesis by comparing normal corneas with those of homozygous Mov13 mice which do not make collagen I. We report that, in spite of this absence, the cellular morphology of the Mov13 eye is indistinguishable from that of the wild type. In the 16-day mutant stroma, however, the remaining collagens form small amounts of disorganized, thin fibrils rather than orthogonally organized 20 nm-diameter fibrils; a result implying that collagen I plays only a structural role and that its absence is not compensated for. It also suggests that, because these remaining collagens will not form the normal fibrils that they will in vitro, fibrillogenesis in the corneal stroma differs from that elsewhere.

The latter part of the paper describes our current work on chick stromal deposition using corneal epithelia isolated with an intact basal lamina that lay down in vitro ~3 μm-thick stromas of organized fibrils similar to that seen in vivo. This experimental system has yielded two unexpected results. First, the amount of collagen and proteoglycans produced by such epithelia is not dependent on whether its substratum is collagenous and we therefore conclude that stromal production by the intact epithelium is more autonomous than hitherto thought. Second, chondroitin sulphate (CS), the predominant proteoglycan, appears to play no role in stromal morphogenesis: epithelia cultured in testicular hyaluronidase, which degrades CS, lay down stromas whose organization and fibril-diameter distribution are indistinguishable from controls. One possible role for CS, however, is as a lubricant which facilitates corneal growth: it could allow fibrils to move over one another without deforming their orthogonal organization. Finally, we have examined the processes of fibrillogenesis in the corneal stroma and conclude that they are different from those elsewhere in the embryo and in vitro, perhaps because there is in the primary stroma an unidentified, highly hydrated ECM macromolecule that embeds the fibrils and that may mediate their morphogenesis.

Key words: collagen, cornea, eye, growth, morphogenesis, neural crest, Mov13 mutant, proteoglycan.

Introduction

The development of the eye is important for reasons that go beyond its intrinsic interest. The tissues of the eye, and particularly of the cornea, are unusually large and accessible, notably in the chick embryo; they are therefore relatively easy to study and thus provide model systems for investigating many aspects of vertebrate organogenesis. Indeed, so many developmental events occur as the eye forms (e.g. Romanoff, 1960) that its organogenesis may be considered as embryogenesis writ small. Among the most important of these events is the laying down of extracellular matrix (ECM): not only are collagen and proteoglycans (PGs) major structural components of the eye and, in particular, of the cornea, but they may well
play an important functional role in their development.

This paper thus focuses on the diversity and functions of ECM macromolecules present in the cornea. It examines evidence suggesting that these macromolecules control some aspects of neural crest (NC) cell differentiation and presents observations on the corneal abnormalities that we have observed in the Movl3 mouse which fails to make collagen I. Finally, it describes our work on a new system which allows stromal deposition to take place in vitro; a system that we have used to investigate some of the processes that occur as organized stroma is laid down in vivo. To set the scene, however, the paper starts with a summary of recent work on corneal development in the chick embryo.

Normal development of the eye

The structure of the chicken eye is well known, but its origins less so. It is convenient to view the eye as being composed of central and peripheral tissue, the former being directly concerned with vision and the latter being, by and large, the supporting part of the organ. The pigmented and neural retinae, and in due course, the iris, arise as an outgrowth from the brain that induces the superficial epithelium to become lens and corneal epithelium (see Hay, 1980). Most of the other tissue, the choroid, sclera, musculature, ossicles, nerves and, most notably, the endothelium and the fibroblasts of the cornea arise from the NC cells that surround the eye from the third day and differentiate from the fourth day onwards (Johnston et al. 1979).

The cornea itself is first apparent during the third day of development when, very soon after lens formation, the superficial epithelium starts to lay down, between itself and the lens, a stroma of orthogonally organized collagen and PGs (see Hay, 1980, for review), a structure which renders the stroma transparent (Maurice, 1957). This synthesis continues throughout embryogenesis with recently deposited fibrils being closer to the basal lamina than older ones. On the fourth day, peripheral NC cells migrate between this stroma and the lens to form the monolayer endothelium (Fig. 1), the posterior layer of the cornea (Bard, Hay & Meller, 1975). Initially, this endothelium seems to secrete posteriorly into the anterior chamber a PG matrix whose swelling causes the cornea to become convex (Bard & Abbott, 1978) and so able to act as a lens. Two days later, the endothelium secretes hyaluronan anteriorly into the stroma (Toole & Trelstad, 1971) causing it to swell and be colonized by a further cohort of NC cells (Bard & Hay, 1975). These cells differentiate into...
fibroblasts and lay down more collagen, the secondary stroma, on the scaffold of the primary stroma (Bard & Higginson, 1977; Birk & Trelstad, 1984). Later, nerves colonize the stroma (Bee, 1982) and the endothelium synthesizes a hyaluronidase which breaks down the hyaluronan and causes the stroma to compact and become transparent. Collagen continues to be laid down and some of it extends into the corneal periphery, perhaps to stabilize corneal shape (Linsenmayer, Gibney & Fitch, 1986).

Although it is generally thought that the corneal fibrils of the primary stroma are uniform in diameter and laid down in orthogonal layers of densely packed fibrils, the situation turns out to be different (Bard & Bansal, 1987). First, fibrils are not uniform in diameter: we have now measured fibril diameters close to, and distant from, the basal lamina and the latter are not only wider than the former but wider than they were when first laid down. This result suggests that newly synthesized collagen both forms fibrils de novo and adds to existing ones. Second, the collagen is not laid down in lamellae of parallel fibrils: SEM micrographs show no sign of such layers; instead, the collagen fibrils lie randomly along axes parallel and perpendicular to the choroid fissure and show only short-range order (Fig. 2). Third, the fibrils are not as closely packed as they appear in TEM sections or SEM specimens that have been critical-point dried. Such corneas have been dehydrated with alcohols which cause hydrated structures such as PGs to collapse. If, however, SEM specimens are freeze dried, a process which sublimates off bound water without causing the structure to collapse (Bard, McBride & Ross, 1983), the fibrils are both further apart than in critical-point dried stromas and seen to be embedded in an amorphous matrix (Fig. 3).

It is interesting to note that, although both the origins of the mouse eye and the structure of its mature cornea are similar to those of the chick (Swoboda & O’Shea, 1987), mouse corneal morphogenesis differs from that of the chick in two distinct ways (Haustein, 1983). The epithelium of the mouse does not lay down substantial amounts of collagen – there is no primary stroma – and there is no separate migration of cells that will form the endothelium. Instead, cells migrate into an apparently empty space (but see Pratt, Larsen & Johnston, 1975) between the lens and the overlying cornea and start to lay down collagen (~day 12) which, by about 16 days, is seen to be orthogonally organized. Only later (~day 17) do the posterior fibroblasts differentiate into the endothelium. The origin of the migrating cells is not known, but they are likely to be NC cells. The stromal expansion and contraction seen in the chick do not occur in the mouse, although the rabbit cornea condenses in the last week of development (Cintron, Covington & Kublin, 1983). The morphogenesis of the mammalian cornea seems simpler than that of the chick, but the reasons for the differences between them and also the amphibian double cornea (Bard & Abbott, 1978) remain unclear. A comparison of corneal development across the vertebrates could have intriguing evolutionary implications.

The role of extracellular matrix

With this structural background, we can now turn to the chemical composition of the ECM in the cornea. The ECM constituents identified in chick, mouse and bovine corneas include collagens I–VI (Lee & Davidson, 1984; Linsenmayer, Fitch & Mayne, 1984; Linsenmayer et al. 1986; Pratt & Madri, 1985; von der Mark et al. 1977), heparan, chondroitin, dermanatan and keratan sulphates (Hart, 1978; Funderburgh, Caterson & Conrad, 1986; Hyldahl, Aspinall & Watt, 1986; Meier & Hay, 1973), hyaluronan (Toole & Trelstad, 1971), fibronectin and laminin (see Yamada, 1981), but this list, although long, is unlikely to be complete (Zak & Linsenmayer, 1985; Bard & Ross, 1986). As to their localization, Scott & Haigh (1985) have demonstrated that keratan sulphate binds to the ‘a’ and ‘c’ bands of the collagen fibril and chondroitin and dermatan sulphate to the ‘d’ and ‘e’ bands, while Linsenmayer et al. (1985) have provided evidence that collagens I and V may be in the same fibril. The exact functions of the ECM components are still unclear but it now seems that, among other roles, they may influence fibril orientation (Coulombre & Coulombre, 1975) and NC cell differentiation.

ECM and NC cell differentiation

The NC cells in the eye differentiate into a wide variety of cells (Johnston et al. 1979) and there is now evidence that ECM components may mediate the differentiation of some at least of these types. Thorogood, Bee & von der Mark (1986), in particular, have pointed to the strong correlation between the pattern of collagen II expression in the head and the future pattern of chondrogenesis: at the periphery of the retina, for example, collagen II is transiently produced when and where NC cells become determined as scceral cartilage.

In the chick cornea, NC cells form endothelial and fibroblastic cells. The former originate as cells which migrate between the basal lamina of the lens, which is composed inter alia of collagen IV and laminin, and the primary stroma, which at this stage contains collagens I and II together with various PGs. After these cells have formed a monolayer, they differentiate into the endothelium without, it may be noted, having contacted any cells directly. Furthermore, as
those NC cells which do not colonize this region remain mesenchymal, it seems likely that interactions between the NC cells and the ECM are responsible for endothelial differentiation (see Bard et al. 1975).

The differentiation of NC cells into fibroblasts when they colonize the acellular primary stroma also seems to be under ECM control. Here, however, there is some experimental data to supplement the circumstantial evidence (Fig. 4). If these cells are removed from the cornea within 4 or 5 h of their entry and cultured, some will pigment (Campbell & Bard, 1985). This observation confirms that the fate of the NC cells is controlled by unknown ECM components in the acellular environment of the stroma. This conclusion is supported by the observations of Weston, Ciment & Girdlestone (1984) who showed that NC cells formed fewer pigment than neural cells when cultured on collagen, a situation reversed on fibronectin. More detailed analysis of how the NC cells differentiate into such a wide variety of cell types in the eye will require markers for the earliest stages of the different cell types.

The Movl3 mutant

The NC cell observations implicate ECM macromolecules in differentiation, but say nothing about the role of collagen I, the predominant member of the family. One way to explore its function would be to remove it and see how the eye copes with the loss. This cannot be done biochemically, but a chance experiment has allowed it to be done genetically. Some years ago, Jaenisch and his co-workers introduced the Moloney virus into a mouse embryo and one provirus inserted into the first intron of the collagen I gene which then ceased to produce messenger in the homozygote (Hartung, Jaenisch & Breindl, 1986). Such mice appear to develop identically to controls until they die, usually at about 13–14 days, because their heart vessels collapse (Löhler, Timpl & Jaenisch, 1984). Homozygotes form normal tissues and any function for which collagen I is thought to be required, e.g. ducted gland morphogenesis, seems to be mediated through other interstitial collagens (Krauthwil et al. 1986).

These Movl3 mice thus allow corneal development to be examined in the absence of collagen I. Detailed analysis of their corneas and the rest of their eyes shows that, at day 13, their cellular organization is indistinguishable from that of normals. Both stromas have small amounts of collagen but they can sometimes be distinguished because the collagen is marginally less well organized in some homozygotes than in controls (Bard & Kratochwil, 1987).

Although older mutant mice usually die, one pair of homozygous mice embryos has survived to 16 days together with three normal siblings. At this age, there is substantial collagen production in wild types and heterozygotes so the homozygotes should demonstrate whether the absence of collagen I affects corneal development. We have found no differences among thick sections of the five eyes, but an obvious one in TEM micrographs: normal corneas had far more stromal collagen than homozygotes (Figs 5, 6). Moreover, such collagen as was present in Movl3 corneas was thinner and more disorganized than that in controls (Figs 7, 8). A histogram of normal fibril diameters (Fig. 9) peaks sharply at about 20 nm whereas that of homozygote fibrils peaks at about 6 nm (Fig. 10) and has a wide spread (Bard & Kratochwil, 1987).

These observations imply, first, that collagen I has only a structural role in the development of the mouse cornea; second, that production of the other collagens is not increased to compensate for the loss and, third, that, as very few 20 nm fibrils form in its absence, collagen I is an important component of the normal corneal fibril. This last conclusion is surprising.

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Fig. 4. A Nomarski micrograph of stage-27 NC cells removed from a cornea and cultured for 20 days. Some cells have pigmented (arrow). ×215 (from Campbell & Bard, 1985).
as the other collagens found in the cornea (II, III and V) will all form normal, if relatively thick, fibrils in vitro (Birk & Silver, 1984; Adachi & Hayashi, 1985). We shall consider this point again later.

**Laying down organized primary stroma**

Although the Movl3 observations illustrate some roles of collagen I, they do not explain the processes of normal stromal morphogenesis.

Unanswered questions include why the fibrils are orthogonally organized and why they are so narrow and uniform in diameter as compared to most tissues where collagen fibrils have a diameter spread of 40–150 nm. Other questions include: how the precise structure of the stroma can grow while maintaining its organization, what are the morphogenetic roles of the many collagenous and other macromolecules, how do such large molecules move through the basal lamina and how does the epithelial substratum affect stromal synthesis? The answers to these questions are not known and, indeed, there has been surprisingly little speculation about them. The major insight into stromal morphogenesis has probably been that of Trelstad & Coulombre (1971) who suggested that stromal organization derives from a complex self-assembly process, and our data (Bard & Bansal, 1987) certainly support this view.

Were the corneal epithelium of the chick able to lay down in vitro the stroma that it synthesizes in vivo, some of these questions could be investigated directly because one could correlate directly biochemical and morphological studies on stroma laid down de novo. The remainder of this paper describes such a system, the stroma that it generates and how stromal morphology may be altered under experimental conditions. But first, we compare our system with that of Meier & Hay (1973, 1974) who first showed that such an enterprise was realistic.

Meier and Hay removed chick corneal epithelia from their underlying stromas with trypsin/collagenase, an enzyme mix which degrades the basal lamina and causes the epithelium to bleb, and investigated biochemically the stroma laid down by cultured epithelia (1973). They also found that the amount of ECM that such epithelia synthesized depended on their substratum: collagen and PG production was, over a 24 h period, two to three times greater on collagenous than on noncollagenous substrata such as Millipore filters (1974). The authors were not, however, able to tell whether this result reflected some sort of inductive or promotional interaction or whether the collagen, by binding to and stabilizing the epithelium, was substituting for the lost basal lamina (Sugrue & Hay, 1986).

Our system differs from that of Meier and Hay in several ways: first, epithelia are separated from their underlying stroma with dispase rather than collagenase/trypsin; this enzyme leaves an intact basal lamina. Second, epithelia are very carefully flattened onto substrata to mimic their in vivo geometry and so encourage the deposition of organized stroma. Third, we have used Nucleopore rather than Millipore filters as an inert surface because they are translucent and thus easy to use, and because Nucleopore filters, unlike Millipore filters, do not cause corneal epithelia to reorganize by losing their basal lamina, bilayering and secreting collagen internally (unpublished data).

5-day corneal epithelia cultured for 22 h on Nucleopore filters will lay down a 2–4 μm thick stroma in which the collagen is layered parallel to the basal lamina and, to some extent, orthogonally organized (Fig. 11). Moreover, not only has this stroma a PG profile similar to that seen in vivo (Figs 12, 14), but the fibril diameter distribution is very similar to, if a little broader than, that in situ, peaking at 20 nm (Figs 13, 15). The culture system thus provides a good model system with which to study the processes that occur as stromal deposition takes place de novo and we have used it to investigate whether collagen substrata stimulate stromal production by intact epithelium, what role CS plays in stromal morphogenesis and how collagen fibrillogenesis takes place in the stromal environment.

Is there a collagen-substratum effect on stromal production?

We have compared collagen and PG deposition by 5-day epithelia on Nucleopore filters with that on a range of collagenous substrata that includes 5-day stroma, killed by freezing and thawing three times.

![Fig. 11. A TEM micrograph of a 5-day corneal epithelium (e) cultured for 22 h on a Nucleopore (N) filter (0.1 μm pore size). The epithelium has a clear basal lamina (arrow) to which the stromal fibrils are parallel. ×13,900.](image-url)
Figs 12, 14, 16. Dissociative Sepharose CL-4B chromatography of PGs laid down in the stroma by 5-day corneal epithelia *in vivo* (Fig. 12) and *in vitro* (Fig. 14) cultured on Nucleopore filters in DMEM medium with only 10% of the normal sulphate content, with and without 30 i.u. ml⁻¹ testicular hyaluronidase. Fig. 16 shows the PGs released into the culture medium.

Figs 13, 15, 17. Diameter distributions of collagen fibrils (>400) in 5-day stromas (Fig. 13), in stromas laid down in routine medium over 22 h (Fig. 15) and in such medium with 30 i.u. ml⁻¹ testicular hyaluronidase (Fig. 17).

For these experiments, we have measured [³H]proline incorporation into hydroxyproline (Sugrue & Hay, 1986) and [³⁵S]sulphate incorporation into proteoglycans over a 6 h period in order to study any substratum effect because, once a collagenous stroma has been produced, it acts as a collagenous substratum for the epithelium. We have also measured 22 h incorporations to compare our results directly with those of Meier & Hay (1974) and the results are given in Table 1. They show that total ³⁵S-incorporations into PG by cultured epithelia with intact basal laminae over both 6 h and 22 h periods are independent of whether or not the underlying substratum is collagenous.

We have also found that proline incorporation into hydroxyproline by 5-day epithelia cultured on Nucleopore filters is the same as that cultured on dried collagen, but that incorporation on hydrated collagen is ~50% higher than on the solid substrata after both 6 h and 22 h. The reasons for this difference are not known but they clearly have nothing to do with the collagenous nature of the substratum.

These results demonstrate that the amount of stroma synthesized by epithelia cultured with an
Table 1. 5-day corneal epithelia

<table>
<thead>
<tr>
<th>Substrata</th>
<th>6 h culture</th>
<th>22 h culture</th>
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</thead>
<tbody>
<tr>
<td><strong>(A)</strong>[^h]proline incorporation[^a] into hydroxyproline (disintegrations/min×10^-4)</td>
<td></td>
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<tr>
<td>Solid substrata</td>
<td></td>
<td></td>
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<tr>
<td>Nucleopore filter</td>
<td>8.4</td>
<td>37.5</td>
</tr>
<tr>
<td>Dried collagen</td>
<td>8.0</td>
<td>40.5</td>
</tr>
<tr>
<td>Hydraled substrata</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitated collagen</td>
<td>12.65</td>
<td>69.0</td>
</tr>
<tr>
<td>Killed stroma</td>
<td>13.8</td>
<td>51.5</td>
</tr>
</tbody>
</table>

| **(B)**[^35]S/sulphate incorporation[^b] into PG (disintegrations/min×10^-3) |            |            |
| Nucleopore filter          | 16.7        | 32.4        |
| Dried collagen             | 17.3        | 41.0        |
| Precipitated collagen      | 16.9        | 39.3        |
| Killed stroma              | 16.1        | 37.6        |

These experiments used 18 epithelia per class; the figures include counts from the cell sheet, stroma and medium.

[^a]: The collagen-production experiments are scaled to 10μCi ml^-1 of[^h]proline (activity ~32Ci mmol^-1)

[^b]: The PG-production experiments used 10Ci ml^-1 of[^35]S/sulphate (activity ~30Ci mg^-1)

intact basal lamina is only marginally affected by the nature of the substratum. They thus confirm the view of Sugrue & Hay (1986) that the collagen–substratum effect shown by epithelia separated from their stromas with collagenase is not one of promotion or stimulation, but of partial compensation for loss of the basal lamina.

A measure of this compensation is a comparison between total collagen production in our system and in that of Meier & Hay (1974). Such a calculation, of course, can only be very approximate because experimental protocols were very different. Allowing for isotope activity, numbers of epithelia, time of culture and assay methods, incorporation by epithelia with an intact basal lamina is approximately four to five times greater than by such epithelia lacking a basal lamina on noncollagenous substrata but only about 50% more than that by such epithelia cultured on collagenous substrata. Our analysis therefore suggests that exogenous collagen compensates quite well for a lost basal lamina.

The role of chondroitin sulphate in stromal deposition

One advantage of our system is that it allows epithelia to be cultured in the presence of enzymes that degrade specific stromal components. We have therefore been able to examine how stromal morphogenesis is affected when epithelia are cultured with testicular hyaluronidase (Sigma, 30 i.u. ml^-1) which degrades CS, the major sulphated PG present in the 5-day corneal stroma.

After 22 h of culture in the presence of 30 i.u. ml^-1 testicular hyaluronidase (Sigma, UK), the PG profile is very different from that of controls: the peak containing CS is much diminished and moved to the low Mr part of the spectrum and its[^35]S/sulphate incorporation is reduced by more than 70% (Fig. 14). In the medium, a high Mr peak is replaced by a wider low Mr peak of degraded glycosaminoglycan (Fig. 16). These observations make it clear that the enzyme has drastically altered the PG composition of the stroma.

Although such a loss might be expected to have a significant effect on stromal morphology, examination of sectioned material in the TEM shows that stromas laid down de novo by treated epithelia are indistinguishable from controls, having the same diameter distribution that peaks at 20nm (Fig. 17). Moreover, and of particular surprise considering that the major sulphated PG has been removed, the fibrils are still well separated (Fig. 18). These results thus suggest that CS plays no major role in normal stromal deposition or fibril separation. This conclusion is so counterintuitive, given the large amount of CS in the primary stroma, that it has seemed sensible to seek a role for CS and one possibility is in mediating stromal growth.

The growth of the primary stroma

We do not know how the stroma grows, but the solution cannot be trivial because of the geometry of the system: consider an early cohort of collagen secreted below the epithelium and forming fibrils that extend across the corneal diameter. Two days later, this collagen layer will be some 20μm below the...
Fig. 19. The effect of epithelial growth on stromal morphology: if the fibrils cannot reorganize, the stroma becomes conical. This does not happen! (From Bard & Bansal, 1987).

epithelium and, although it should not have increased its width, that of the corneal epithelium will have doubled. Were this scenario correct, the cornea should be conical (Fig. 19), but it is not and we may therefore conclude that the fibrils in the stroma reorganize as the cornea grows.

There is some evidence that fibrils within a stroma can reorganize themselves: fixed stromas are highly convoluted (Fig. 1; Bard et al. 1975) and it has not been clear whether these convolutions exist in vivo or whether they are a fixation artifact due to shrinkage. We have done a simple experiment to distinguish between these possibilities by preparing stromas for SEM in three ways that give different amounts of shrinkage and have looked to see if these differences correlate with the extent of folding (Bard & Bansal, 1987). The diameters of specimens that are critical-point dried shrink by ~30% and fold extensively while specimens that are freeze dried shrink by ~15% and only wrinkle. In contrast, epithelia that are stabilized by being supported on filter paper while being freeze dried neither shrink nor fold, although they tear a little. These observations therefore imply that, in vivo, the stroma has no folds and that, as shrinkage occurs, fibrils form folds by sliding over one another. It is also of note that, even in the folds, the fibrils remain orthogonally organized (Bard & Bansal, 1987).

We suggest that similar movement occurs as the stroma grows and that it is lubricated by CS. This PG is a plausible candidate for this role because it has the type of hydrated structure that would allow it to act as a lubricant (Walker et al. 1970).

Collagen fibrillogenesis in the primary stroma

The cornea stroma is remarkable because its collagen is orthogonally organized and because the fibrils are so narrow and uniform in diameter. We end this paper by briefly considering whether the processes of collagen fibrillogenesis in the cornea are similar to those elsewhere. Two facts in particular argue that it is not. First, in the absence of collagen I in the Mov13 mutant cornea, the remaining collagens will not form 20 nm fibrils or, indeed, normal fibrils even though collagens II, III and V will form such fibrils in vitro and in the absence of collagen I (Adachi & Hayashi, 1985; Birk & Silver, 1984). Second, soluble collagen

Fig. 20. Fibrils precipitated in vitro from soluble bovine corneal collagen. ×50 000 (from Bard, 1969).

Fig. 21. TEM micrograph of a 5-day epithelium cultured in DMEM-sulphate-free medium for 22 h. The stromal collagen is disorganized and the fibrils have a wide range of diameters. ×33 200.

Fig. 22. SEM micrograph of a freeze-dried 5-day stroma after it had been incubated in testicular hyaluronidase (300 i.u. ml⁻¹) for 6 h. Note that the matrix is still present between the fibrils. ×12 000.
from bovine corneas, extracted in acetic acid and reprecipitated in phosphate buffer, forms fibrils with diameters of up to 100 nm (Fig. 20), that is, they form the type of fibril usually observed elsewhere in the embryo (Bard & Freeman, cited in Bard, 1969).

These results together suggest that conditions for fibrillogenesis in the cornea are different from those elsewhere and we have evidence suggesting that this difference may be due to the presence of an unidentified macromolecule in the corneal stroma. Although the collagen fibrils laid down by the corneal epithelia in vitro usually have a 20 nm diameter and are layered parallel to the basal lamina, we have found that this morphology is drastically altered if the epithelia are cultured in sulphate-free medium. Not only are fibrils very much wider than under usual circumstances, but they are also disorganized and packed closely together (Fig. 21). Such culture conditions will clearly explain stromal deposition and growth. The final observation demonstrates that the matrix material in which stromal fibrils are seen to be embedded (Fig. 3) is not CS. If 5-day stromas with their epithelia removed after dispase treatment are incubated in a high concentration of testicular hyaluronidase (300 i.u. ml⁻¹) at 37°C for 4 h, any CS present would be degraded. If such stromas are then freeze dried and examined in the SEM, they are found to be indistinguishable from controls (compare Figs 21, 22) and their collagen fibrils remain embedded in the pervasive matrix that is absent in critical-point dried stromas.

The identity of this material is unknown: it cannot be CS and the other likely candidate, keratan sulphate, seems to be absent in the chick stroma until day 7 (Funderburgh et al. 1986). It may be that the question of why collagen forms 20 nm fibrils that are orthogonally organized in the corneal stroma will remain unanswered until this material is identified and its role in collagen fibrillogenesis understood.

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