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THE ARRANGEMENT OF COLLAGEN FIBRILS IN THE IRIDESCENT CORNEA OF THE SCORPION FISH, *TAURULUS* (*COTTUS*) *BUBALIS*, AND THE TRANSPARENCY OF VERTEBRATE CORNEAL STROMA

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SUMMARY

1. The iridescent layer in the corneal stroma of the scorpion fish, *Taurulus* (*Cottus*) *bubalis* (Scorpaeniformes), is composed of alternating thin lamellae of normal stroma containing collagen fibrils and lamellae of an amorphous dense-staining material.

2. Iridescence is lost after conventional resin embedding procedures, but is retained after embedding in urea-glutaraldehyde polymer.

3. The retention of iridescence and the absence of gross thickness changes during embedding in urea-glutaraldehyde polymer are an indication that the fine structure of the cornea is less altered than by conventional resin embedding.

4. It is believed that in life the collagen fibrils in the cornea of the scorpion fish and the cornea of the frog and rabbit are slightly larger and much more closely packed than is revealed by conventional resin-embedded sections.

INTRODUCTION

The corneal stroma is made up of collagen fibrils running through a matrix of different composition and there has been discussion over why light passing through it remains virtually free of scatter.

If the collagen fibrils have essentially the same refractive index as the mucopolysaccharide matrix through which they run, no scatter would be expected (Smith, 1969). If, on the other hand, the fibrils and matrix differ in refractive index, another explanation for corneal transparency must be sought.

In 1957, Maurice suggested that the fibrils were arranged in a regular crystalline array when seen in cross-section. A feature of such a lattice is that a wave form passing through it can suffer destructive interference of the energy scattered from neighbouring dipoles. The scattered light would

thus be suppressed. Subsequent studies have not shown such a regular crystalline array but rather a quasi-regular, quasi-random arrangement. Nevertheless, a series of mathematical investigations using data from electron micrographs has indicated that the observed arrangement of fibrils is sufficiently regular to explain the transparency of the cornea (Hart & Farrel, 1969; Cox, Farrel, Hart & Langham, 1970; Feuk, 1970, 1971; Benedek, 1971; Farrel, McCally & Tatham, 1973).

All the work on corneal transparency in relation to structure shares a dependence on measurements made from electron micrographs, both to calculate the dimensions of the structures and to estimate the degree of hydration (hence the refractive index) of the fibrils and matrix. In the past it has been difficult to be sure that the dimensions seen under the electron microscope correspond to those that exist in nature.

A key to this problem may be found in the iridescence that is frequently observed in the corneas of shallow-water marine benthic and demersal teleost fishes. This iridescence is an interference phenomenon and a variety of anatomically distinct structures are responsible in different species (Lythgoe, 1975). Of particular interest in this context is the type of iridescent layer found in the scorpion fishes and their allies (Scorpaeniformes or Scleroparei). In the species under study here, the scorpion fish, *Taurulus* (*Cottus*) *bubalis* (Euphrasen), the iridescent layer is composed of alternating lamellae of apparently normal collagen stroma separated by lamellae of apparently amorphous dense-staining material. When the cornea is illuminated from above and viewed laterally or from below, a strong green-blue iridescence is visible. When a small overhead light source is used, the iridescence is broken up into one or two files of spots as shown in Pl. 1.

The colour of the iridescence depends in large part upon the spacing of the reflecting layers. If the colour of the iridescence is altered or lost during preparations for electron microscopy, it is possible that the spacing that is seen in the electron micrograph is not that occurring in life.

However, with due regard to the osmolarity of the glutaraldehyde fixative, the iridescence survives the fixation, almost without change, though it is lost during the subsequent dehydration through the alcohols before infiltration with resin.

Recently, Pease & Peterson (1972) have developed a technique for embedding which does not involve the dehydration procedures. In this technique, glutaraldehyde is itself polymerized at low pH with urea and the resulting plastic contains considerable amounts of water.

Scorpion fish cornea embedded in glutaraldehyde-urea polymer preserves its iridescence, but the dimensions of the structures involved in the iridescence differ from those in the conventional resin polymer.

METHODS

The frogs, *Rana temporaria*, were purchased from a commercial supplier and were held for not more than 4 weeks in the laboratory.

The scorpion fish, *Taurulus* (*Cottus*) *bubalis* (Euphrasen), were caught in inter-tidal rock pools at Rottingdean, Sussex, England. These were used within a week of capture.

The rabbit was a wild animal shot on a nearby estate and immediately taken into the laboratory. It was dead for about 30 min before the cornea was removed.

All sections were fixed in glutaraldehyde whose osmolarity was adjusted by the addition of sucrose to retain iridescence in teleost cornea after fixation (Lythgoe, 1975). The tissue blocks were post-fixed in osmium and embedded in Araldite (TAAB). The frog nictitans and rabbit cornea were embedded in Spurr resin which, like Araldite (CIBA), is an epoxy resin. The sections were stained in uranyl acetate and lead citrate in the manner described by Lythgoe (1975). Dehydration was in 5 min stages each of 30, 50, 70 and 90% ethanol and two changes of 30 min each of dry ethanol.

The urea-glutaraldehyde sections were fixed and stained in the same way, but were embedded in a polymerized mixture of urea and glutaraldehyde at pH 4·1–4·3 according to the first protocol of Pease & Peterson (1972). Collagen fibrils, but not other tissues, were often reluctant to take up the stain and the staining procedures were, in this case, repeated. The urea-glutaraldehyde polymer had a refractive index of 1·429. The TAAB resin had a refractive index of 1·559 and the Spurr resin 1·478.

The osmium post-fixative was omitted from some of the iridescent cornea preparations to ensure that iridescence was preserved into the polymer block. In fact, osmium post-fixative had little, if any, staining effect and did not alter the dimensions of the fibrils.

Fibril diameters were measured directly from enlarged photomicrographs. Care was taken to measure only those fields where the fibrils appeared circular in cross-section and, therefore, presumably were cut transversely. Neverthless, the shortest diameter was measured to avoid over-estimates of diameter that would result from measuring obliquely cut fibrils. The percentage of the stroma occupied by fibrils (the volume fraction) was calculated from the mean diameter of fibrils occupying a field of known area. In the histograms the fibril diameters are displayed in 3 nm groups. These groups are large enough to embrace the likely measuring error and all variation shown in the histograms is thus real.

The thickness of the rabbit cornea stroma was measured whilst it was being prepared for sectioning. Both the epithelium and endothelium of the cornea was stripped off with a scalpel blade leaving a sheet of stroma intact. This was taken through the embedding procedures described above except that at each stage the thickness was measured directly with an optical microscope by successively focusing on the lower and upper surface of the stroma, and reading the thickness from the fine focus scale. The propylene oxide step was omitted from the measurements since it evaporated too rapidly from the slide.

Comparative estimates of corneal swelling in epoxy-resin and urea-glutaraldehyde fixed material were also made by counting the number of fibrils within a known area on the photographic print. Fields were selected that showed only fibrils cut in cross section and the areas they occupied on the print estimated by weighing. Since it can be assumed that the number of fibrils in the stroma are unaltered by histological procedures, differences in the number of fibrils per unit area give a good estimate of the relative differences in volume between stroma prepared in different ways.

RESULTS

1. *Taurulus* (*Cottus*) *bubalis*

The colour of the iridescent layer

Iridescence survived fixation but was permanently lost at the dehydration stage of the resin impregnated corneas. In the urea-glutaraldehyde embedment, which does not involve dehydration, iridescence was maintained into the block to be sectioned. There was a slight yellowing of the iridescence. This could be caused by the yellowing effect of the glutaraldehyde on some proteins or by an increase in the refractive index of the two lamella types due to the impregnation of the urea-glutaraldehyde mixture.

The anatomy of the iridescent layer in *Taurulus* (*Cottus*) *bubalis* has been described by Lythgoe (1975). The layer is situated in the stroma. Iridescent reflexions are produced by the constructive interference of light reflected from successive boundaries between thin lamellae composed of apparently normal stroma and layers composed of amorphous dense-staining material. In resin embedded sections the boundaries between layers are quite sharp (Pl. 2); in urea-glutaraldehyde embedded tissues the boundaries are poorly defined (Pl. 3).

In the living eye, iridescence is most visible when the eye is viewed laterally and illuminated from above (Lythgoe, 1971: Locket, 1972; Lythgoe, 1975). This directionality is maintained after glutaraldehyde fixation but in the cornea embedded in the urea-glutaraldehyde polymer iridescence appears equally strong irrespective of the angle of illumination. This indicates that some distortion in the arrangement of the lamellae has taken place during embedding.

The mean thickness of each pair of stroma and amorphous lamellae in resin sections is almost double those in urea-glutaraldehyde sections. The thickness of the lamellae was measured from enlarged electron micoscopic photographs, but only those lamellae that were cut at right angles, as judged by the appearance of the fibrils, were measured. In all, 194 lamella pairs were measured in the urea-glutaraldehyde preparations and 156 lamella pairs in the resin sections (Text-fig. 1). The mean thickness of the urea-glutaraldehyde lamella pairs was 116 nm and 217 nm for the resin pairs. In both types of preparation the thickness of the amorphous and the stromal lamellae was about equal.

In percentage terms the lamellae pairs in resin were 87 % greater than in urea-glutaraldehyde. Measurements of the area occupied by each individual fibril when cut in cross-section showed that in resin each fibril occupied 80% more area than it would in urea-glutaraldehyde.

The diameter and packing of the fibrils in the stromal lamellae are shown for both resin and urea-glutaraldehyde sections in Pls. 2 and 3 and diagrammatically in Text-fig. 2. In the resin sections 441 fibrils had a mean diameter of 19·5 nm and occupied a volume fraction, i.e. proportion by volume of the stroma occupied by fibrils, of 0·26. In the urea-glutaraldehyde sections 533 fibrils had a mean diameter of 21·3 nm and occupied a volume faction of 0·45.

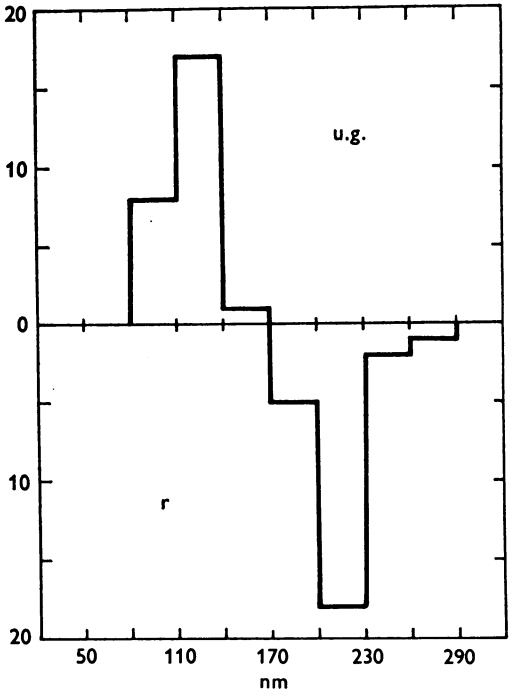


Fig. 1

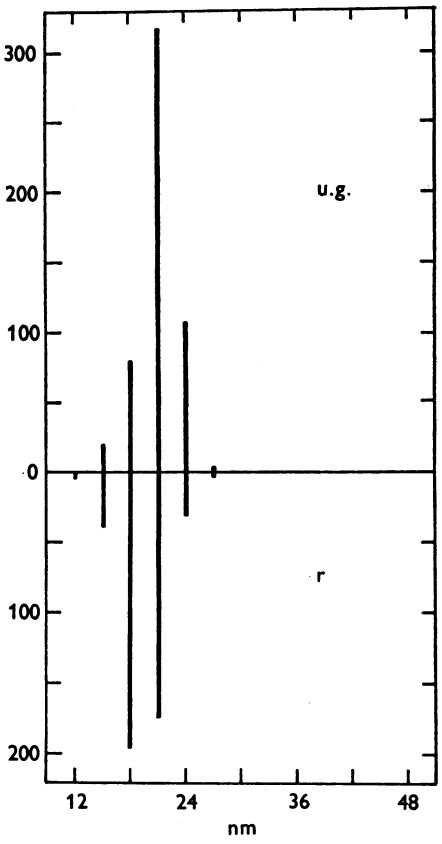


Fig. 2

Text-fig. 1. The thickness of lamella pairs in the iridescent layer of the scorpion fish cornea. Data are shown for urea-glutaraldehyde (u.g.) embedded sections and resin (r) sections. Measurements from twenty-seven fields in each embedment are shown and represent measurements of 195 pairs in urea-glutaraldehyde and 154 in resin. The mean pair thickness in urea-glutaraldehyde is 115·9 nm and 216·9 in resin.

Text-fig. 2. The diameter of the fibrils in the stroma type of lamella in the iridescent layer of the scorpion fish cornea. In the urea-glutaraldehyde (u.g.) sections 533 fibrils had a mean diameter of 21·27 nm and in the resin (r) sections 441 fibrils had a mean diameter of 19·52 nm.

Detailed measurements of the fibrils running through the normal collagen stroma in the scorpion fish cornea were not attempted since the stroma is somewhat un-ordered in structure. However, in general appearance the normal stroma embedded by the two methods resembled that in the iridescent layer.

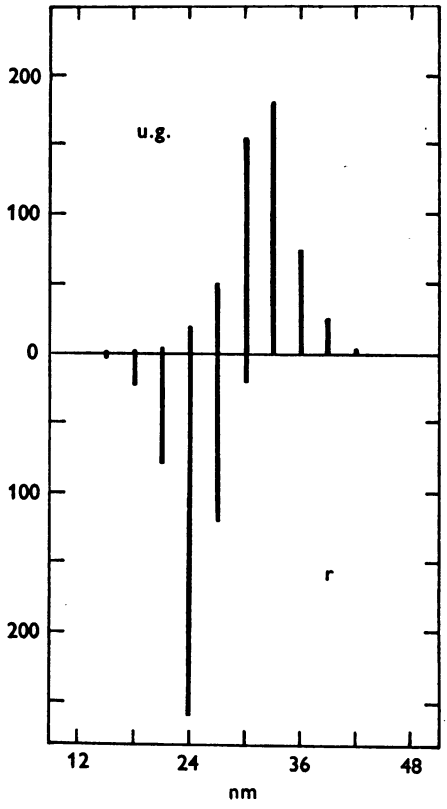


Fig. 3

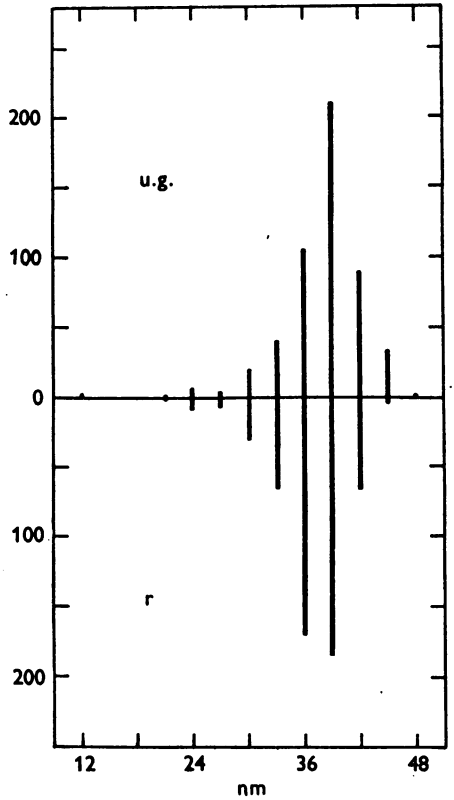


Fig. 4

Text-fig. 3. The diameter of the fibrils in the corneal stroma of the frog. In the urea-glutaraldehyde (u.g.) sections 508 fibrils had a mean diameter of 32·00 nm, and in the resin (r) sections 507 fibrils had a mean diameter of 25·62 nm.

Text-fig. 4. The diameter of the fibrils in the nictitans stroma of the frog. In the urea-glutaraldehyde (u.g.) sections 516 fibrils had a mean diameter of 38·7 nm. In the resin (r) sections 532 fibrils had a mean diameter of 37·00 nm.

2. *The frog Rana temporaria*

The corneal stroma

In the resin-embedded sections (Pl. 4) 507 fibrils had a mean diameter of 25·6 nm and a volume fraction of 0·34. In the urea-glutaraldehyde sections

(Pl. 5) 508 fibrils had a mean diameter of 32·0 nm and a volume fraction of 0·61 (Text-fig. 3). Estimates of relative stroma volume derived from measuring the cross-sectional area occupied by individual fibrils showed that in the resin sections the stromal volume was 29 % greater than in the urea-glutaraldehyde sections.

The nictitans stroma

I confirm the finding of Lande & Zadunaisky (1970) that in the nictitans, resin sections show that the fibrils are much more closely packed than in the corneal stroma (Pl. 6). A difference is that in *Rana catesbeiana* studied by these authors, the nictitans fibril had a mean diameter of 70 nm, which is more than three times those in the cornea. In *R. temporaria* the mean fibril diameter of 532 fibrils in resin is 37·0 nm compared to 25·6 nm for the corneal fibrils (Text-fig. 4). In the urea-glutaraldehyde section (Pl. 7) a count of 516 fibrils in the nictitans stroma had a mean diameter of 38·7 nm and a volume fraction of 0·64 (Text-fig. 4).

Estimates of the relative stromal volume occupied by each fibril indicated that in the resin sections the stromal volume was 23 % greater than the urea-glutaraldehyde sections.

The very close packing of the *R. catesbeiana* nictitans stroma fibrils shown in resin sections by Lande & Zadunaisky (1970) and in the present urea-glutaraldehyde sections of *R. temporaria* show packing so close that the fibrils must often be touching (Pl. 7). The crystal-like lattice arrangement that might be expected with a closest-packing arrangement does not, in fact, occur, except in restricted areas, because of irregularities caused by the variation in diameter of the fibrils.

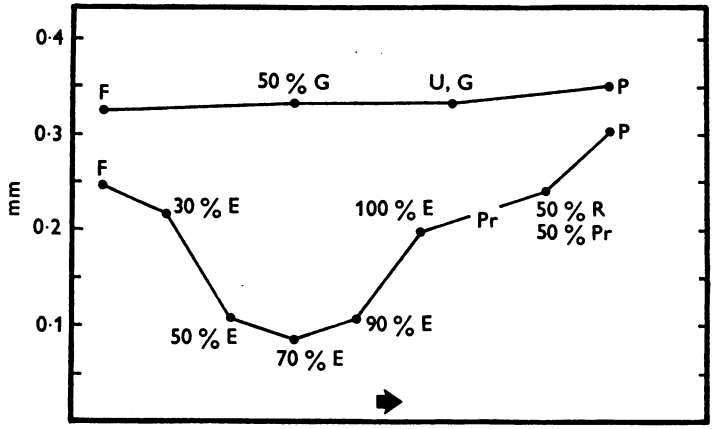
3. *Rabbit corneal stroma*

The changes in stroma thickness during embedment in epoxy resin and urea-glutaraldehyde were measured directly (Text-fig. 5). During urea-glutaraldehyde embedment there was a slight but steady increase in thickness so that the final thickness in block was about 7 % greater than in the recently fixed materials. During epoxy resin embedment there was considerable shrinkage during ethanol dehydration that was most pronounced in the 50–90 % ethanol stages. The relationship between stroma thickness, ethanol concentration and time was not investigated. During the infiltration with epoxy resin and polymerization there was a continued increase in thickness until in the block ready for sectioning the corneal stroma was 23 % greater in thickness than in the freshly fixed material.

An alternative value of corneal swelling derived from fibril counts in a known area indicates that the stroma has a 20 % greater volume in the resin block than in the urea-glutaraldehyde block. Allowing for a 7 %

increase in thickness observed directly during the urea-glutaraldehyde embedding (Text-fig. 5), it is estimated that a 28·5 % increase in stromal volume results from epoxy resin embedding.

These results are similar to those of Cox *et al.* (1970) who reported considerable shrinkage during ethanol dehydration. Their data show that the final thickness of the cornea in the epoxy resin block was 18–34 % greater than in the living animal. These authors showed that the thickness of the living cornea was unchanged by fixation.



Text-fig. 5. The change in thickness in rabbit corneal stroma from fixation into the urea-glutaraldehyde or epoxy resin block fully polymerized and ready for cutting. F, fixed; E, ethanol; Pr, propylene oxide; R, epoxy resin; G, glutaraldehyde; U, urea; P, polymerized. The duration of the various steps is given under Methods section.

DISCUSSION

All the quantitative explanations for the transparency of the vertebrate cornea rely upon measurements made from dehydrated material embedded in resin. If, as seems possible, this type of preparation yields misleading results, some re-assessment of these explanations is necessary.

Both the collagen fibrils and the matrix in which they are embedded are hydrated structures. Therefore, it is to be expected that any procedure involving dehydration would cause more alterations in dimensions than one that does not. Indeed, an examination of Text-fig. 5 shows that there is considerable shrinkage during dehydration and a subsequent swelling during infiltration with resin and its subsequent polymerization. No such drastic dimensional changes were recorded in material embedded in urea-glutaraldehyde polymer.

The expectation that hydrated tissues retain their *in vivo* dimensions when embedded in water-containing urea-glutaraldehyde polymer (Pease

& Peterson, 1972) is strongly supported by the retention of iridescence in the *Cottus* cornea during all the stages of preparation for electron microscopy right up to the sectioning stage. Iridescence is lost during dehydration through the alcohols in the conventional resin embedding methods. It does not reappear during the swelling that occurs during polymerization.

Iridescence occurs when there is constructive interference between the light rays reflected from the interfaces of layers of different refractive index which are of the order of a light wave-length in thickness. In animals the reflector is generally a regular multilayer stack (Land, 1972). The colour of the iridescent reflexions depends upon the thickness of the component layers, the refractive index of each layer and the angle of incidence of the light.

The retention of iridescence into the urea-glutaraldehyde block is strong evidence that the dimensions of the tissue involved have not been changed by the embedding procedures. The loss of iridescence during dehydration before resin embedment suggests that the dimensions have been changed during that process. This view is supported by the direct measurements of corneal thickness changes during dehydration (Text-fig. 5). No such large changes occur during urea-glutaraldehyde embedment.

If it is indeed true that the urea-glutaraldehyde sections reveal an acceptably accurate picture of the separation between the fibrils, it follows that at some stage during the process that leads to the conventional resin embedment there is a marked increase in the volume of the matrix. This swelling appears to occur during infiltration with epoxy resin and its subsequent polymerization.

There are two kinds of explanation for the transparency of the corneal stroma. The most simple is that the refractive index of the collagen fibrils and the mucopolysaccharide matrix through which they run have virtually the same refractive index and thus the system does not scatter light because it is optically homogeneous. The second kind of explanation accepts that the fibrils and matrix differ in refractive index but uses scattering theory and diffraction optics to show that the arrangement and spacing of the fibrils is such that scattered light is suppressed.

The original theory invoking diffraction theory was proposed by Maurice (1957) who considered that the early electron micrographs from which he had to work indicated that the fibrils were arranged in a crystal-like lattice. Light scattered from a fibril was then suppressed by light scattered by neighbouring fibrils by destructive interference. A similar approach was made by Feuk (1970) who believed that the fibrils were arranged in an open disturbed hexagonal lattice and also used diffraction theory to explain the cornea's transparency.

However, it became clear that the fibrils, as seen in resin embedded

material, are, in fact, arranged in a quasi-regular–quasi-random array where the distance of each fibril from its neighbours varies in an apparently random manner between fixed limits. Detailed analysis of the optics of such an arrangement by Hart & Farrel (1969) and Cox *et al.* (1970) showed that there was still sufficient regularity in fibril arrangement for the scattered light from individual fibrils to be suppressed by destructive interference and thus for the stroma to be transparent.

In the urea-glutaraldehyde sections which I believe more closely represents the situation in life, the arrangement of the fibrils can be described as the closest possible packing of fibrils of unequal diameter. Feuk (1970) considered the possibility that the fibrils were arranged in a closest-packing array and mentions the theoretical possibility, which he did not describe in detail, that such a structure could also be transparent.

Benedek (1971) concluded that irrespective of the refractive index of the collagen and ground substance, the cornea would be transparent provided that the non-homogeneities in refractive index are less than half the wavelength of light (λ). Farrel, McCally & Tatham (1973) essentially agree with this view when they conclude that scattering will not take place in a quasi-ordered array where the spacing between fibrils is small compared with λ/π.

If it is true that the fibrils in the cornea are too small and too densely packed to scatter light whatever their refractive index, it becomes less important to try to estimate the refractive index of the fibrils and their matrix by a combination of chemical and electron microscope studies. There is little agreement in the literature as to what the correct refractive index figures are (Maurice, 1957; Smith & Frame, 1968; Smith, 1969; Maurice, 1970; Smith, 1970). Part at least of the disagreement comes from the differing values these authors accept for the volume fraction of the fibrils and hence their hydration. It is clear from Table 1 that the data obtained by different workers varies considerably, perhaps as a result of difference in dehydration schedules during embedment.

To estimate the refractive index of the collagen and ground substance it is also necessary to introduce chemical data on the composition and hydration of the material surrounding the fibrils. Here again, there is little agreement on the correct values. For the uniform refractive index theory to be proved it is necessary to show that the refractive index of both fibrils and matrix are the same. With so little agreement on the basic data it is difficult to reach a firm conclusion one way or the other.

The very large changes in corneal thickness that Cox *et al.* (1970) reported during epoxy, resin embedment are confirmed here. However, in the past it has been tacitly assumed that the relative volume occupied by fibrils and matrix is the same in both the fresh and embedded cornea.

TABLE 1. Comparison of current data

Species and tissue	Fibril diameter (nm)		Fibril volume fraction (%)		Authority
	Resin	Urea-glutaral-dehyde	Resin	Urea-glutaral-dehyde	
Shark, *Squalus acanthias* Cornea stroma	26·8–32·7	—	35	—	Goldman & Benedek (1967)
Cornea – Bowman's layer	26·0–32·5	—	30–50	—	Goldman & Benedek (1967)
Scorpion fish *Cottus* (*Myxocephalus*) *bubalis*					
Iridescent layer	19·5	21·3	26	45	Present work
Frog, *Rana catesbeiana*					
Cornea stroma	22·3	—	36·5	—	Lande & Zadunaisky (1970)
Nictitans stroma	70·0	—	66·5	—	Lande & Zadunaisky (1970)
Frog, *Rana temporaria*					
Cornea stroma	25·6	32·0	34	61	Present work
Nictitans stroma	37·0	38·7	44	64	Present work
Rabbit					
Cornea stroma	24–35	—	33	—	Smith & Frame (1968); Smith (1969)
Cornea stroma	—	—	25	—	Feuk (1970)
Cornea stroma	20·0	—	10	—	Cox *et al.* (1970)
Man					
Cornea stroma	20–23	—	10–20	—	Schwarz & Graf Keyserlingk (1969)

The data presented here do not support this assumption, but indicate that the diameter of the fibrils is slightly reduced whilst the corneal stroma as a whole suffers at least a 20% increase in volume during epoxy-resin embedment.

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EXPLANATIONS OF PLATES

PLATE 1

Corneal iridescence in the scorpion fish, *Taurulus* (*Cottus*) *bubalis*. The eye is illuminated from directly overhead. The iridescence is a blue-green colour. Magnification × 6.

PLATE 2

Part of the iridescent layer in the cornea of the scorpion fish, *Taurulus* (*Cottus*) *bubalis*. Epoxy resin was used as an embedding medium. Lamellae composed of apparently normal stroma alternate with lamellae composed of apparently amorphous material that does not contain fibrils. The bar represents 250 nm.

PLATE 3

As for Pl. 2, but urea-glutaraldehyde was used as the embedding medium. Note that the boundaries between the lamellae are less well defined than in the resin sections. Most fibrils appear to be hollow and in places (arrow) appear to be surrounded by an unstaining sheath. The bar represents 250 nm.

PLATE 4

Corneal stroma of the frog cut from material embedded in epoxy resin. The bar represents 250 nm.

PLATE 5

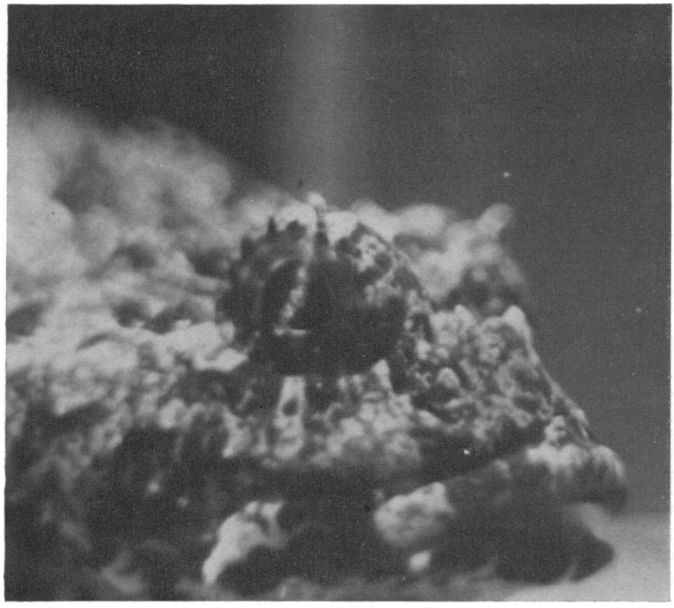
As for Pl. 4, but cut from material embedded in urea-glutaraldehyde. The bar represents 250 nm.

PLATE 6

Nictitans stroma of the frog cut from sections embedded in epoxy resin. The bar represents 250 nm.

PLATE 7

As for Pl. 6, but cut from material embedded in urea-glutaraldehyde. The black bar represents 250 nm.



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