THE INNER LIMITING MEMBRANE OF THE RETINA*

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The micro-anatomy of the region between the retina and the vitreous has been a source of discussion and interest for more than a century. Work has, in general, been concerned with the inner surface of the retina and has involved extensive investigation of what is normally termed the inner limiting membrane. The basis for this name was very probably provided by Pacini, who referred to a homogeneous structure on the vitreous surface of the retina as the membrana limitante. The same author, however, mentions at least twelve other workers who apparently preceded him with morphological studies on the retina (Pacini, 1845). Indeed, earlier references to the inner retinal surface can readily be collected; for example, Gottsche (1836) recognized a lamellar tissue in close relationship to the inner nervous elements of the retina and Michaelis (1837, 1842) wrote of what he called the "serous inner layer" of the retina. It is difficult to be certain when ordered structure was first observed within the region of the membrana limitante, but the first description is almost certainly due to Hannover (1840), who wrote of a glass-bodied membrane composed of large, transparent, six-sided "cells". Pacini also described a similar appearance, but the formation and minute relations of the membrane were not considered in greater detail until the description of the radial fibres by Heinrich Müller (1851) prompted both Schultze and Schelske to suggest that the mosaic of cells described by Hannover was in fact related to the flat vitrad ends of the radial fibres. Schultze went so far as to state, in a classical work on the structure and function of the retina, that the membrane is formed by these fibres (Schultze, 1859); Schelske was content merely to write that the morphology of the two structures did, to some extent, correspond, and that the suggestion made by Schultze was a possibility. The same author went on to show that the average diameter of the clear spaces in the mosaic formation varied approximately between 3 x 20 microns, and further that the spaces were elongated in the long axis of the great vessels to a maximum size of 42 microns (Schelske, 1863).

In 1871, the great anatomist Gustav Retzius published the first of two articles on the inner limiting membrane; he first extensively reviewed the existing work on the subject and then went on to describe experiments on

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the retinae of man, the cat, and the rabbit, using a silver impregnation technique which he attributed both to Schelske and von Recklinghausen. He also made detailed drawings showing the characteristic mosaic formation as revealed by the silver, and stated quite categorically that the membrane was formed by the ends of the radial fibres (Retzius, 1871). Over 30 years later he published a second work describing the appearances of the mosaic formation in a large variety of vertebrate species, again using a silver nitrate impregnation method (Retzius, 1904). This, together with his first paper, probably represents the most extensive original work ever carried out on the subject. In the same year, however, Tornatola (1904) also published a preliminary note in which he stated that, although the mosaic present in the plane of the inner limiting membrane conformed to the contour of the radial fibres in some specimens, he was not of the opinion that the inner surface of the retina was covered either by a glass membrane or by a membrane composed of the conjoined ends of the radial fibres. He wrote, instead, that connexions between the vitreous and the retina existed and that these could be demonstrated by special glial stains. Some doubt had therefore been cast on the original concept that the internal limiting membrane was formed by the radial fibres, and this was apparent even some years later, for Salzmann, in a discussion on the nature of the membrane, wrote that it was as much related to the vitreous as it was to the retina, and further that surface views of the structure showed only the impressions made by the radial fibres, so that it could not be inferred that the radial fibres gave rise to the membrane by the fusion of their vitrad ends (Salzmann, 1912). The contrary view was again expressed when van der Stricht (1922) published an immensely detailed work on the subject. The author used a silver nitrate impregnation technique similar to that used by the earlier investigators and came firmly to the conclusion from observations made on a variety of species, that the membrane was formed as a direct extension of the fibres of Müller. Furthermore, he stated that the extension was formed by the intercellular cement surrounding the radial fibres. Eight years later, Menner (1930) came to an essentially similar conclusion, but Police (1932), in a treatise on the morphology of the radial fibres, stated that they played no part in the formation of either the inner or the outer limiting membrane, although he conceded that connexions probably existed. In assessing this work it may be of interest to note that the author was abusively criticised by Polyak (1941) for his views on the relationship between the visual cells and the radial fibres. The view that the two structures are separate was again endorsed by Wolff (1937), who wrote that the inner limiting membrane was distinguishable from the Müller fibres since Mallory’s triple stain shows the inner limiting membrane to stain blue and the radial fibres red. Finally, Polyak (1941), in his great work on the retina, stated without equivocation that the inner limiting membrane was formed by the expanded ends of the radial fibres.
A review of the literature, therefore, reveals a considerable difference of opinion on the nature of the inner limiting membrane. On the one hand there are those who consider that it is formed by the radial fibres, and on the other those who see it as an entirely separate structure, composed either of a single sheet of cells or of featureless hyaline material. By far the most prevalent opinion and that which has found its way into the majority of textbooks is the view that the membrane is formed by the radial fibres.

The work reported in this article has been carried out in order to determine the appearances of the inner retinal surface, using different methods, and to relate these appearances to the morphology of the immediately adjacent structures.

**Methods**

(1) **Photo-sensitized Silver Impregnation of the Wet, Whole Retina.**—Several of the workers mentioned above used a method involving exposure to light with impregnation by silver nitrate. The exact mechanism of this reaction has never been satisfactorily explained; but it must consist, at least in part, of selective reduction of the applied silver solution, so that certain structures in the treated tissue become differentially visible because they are coated to a different degree by a deposit which includes metallic silver. The method to be described has proved reliable at least in one respect, that it will show similar structures in consecutive specimens provided that some care is taken to repeat the process exactly:

Pieces of human retina were taken from eyes received for routine pathological examination, provided that there was no evident retinal damage and the eye had not been in 10 per cent. formal saline for more than 3 days. The eyes from animals, with the exception of those of the cat, were taken from an abattoir, the retinae being removed unixed and placed as rapidly as possible flat on a slide into 10 per cent. formal saline at room temperature. In the case of the cat, the eyes were removed under intraperitoneal Nembutal anaesthesia.

**Silvering Procedure.**—The eye is bisected coronally as near as possible to the equator, and the posterior half is held in position under a dissecting microscope and the retina removed whole. It is then placed with the inner surface uppermost on a slide, the vitreous body is then removed and the retina immersed, mounted on the slide, in 10 per cent. formal saline. A coverslip is gently lowered on to the inner surface of the specimen to assist flattening. If the retina is fixed before removal from the eye it will not flatten sufficiently without first making several radial incisions throughout its full thickness from the region of the disc to the periphery. Fixation is continued for approximately 72 hours. It is not necessary to be precise about the time, but if fixation is continued for a much longer period (more than one week) it is found that some clarity of surface detail is lost. On removal from the formal saline, the slide containing the specimen is again placed under a low-power microscope, superfluous fluid is removed, and the specimen covered gently with a solution of 1 per cent. silver nitrate. A flocculent white precipitate of silver chloride forms immediately, which is washed off by repeated application of silver nitrate until both the surface of the retina and the covering fluid remain quite clear. A monofilament lamp (6 v. 6 a.) is then trained on the surface of the specimen from a distance of 9" and is focused with an appropriate converging lens until an image of the filament is seen to cover the specimen as evenly as possible. No heat-absorbing filter is used.
On exposure to the light, the silvering process begins. At first a faint metallic sheen develops on the inner retinal surface, and then the remaining tissue gradually begins to change its appearance; first becoming a light fawn colour and then, over a period of about 10 minutes, deepening to a characteristic sepia tone. The depth of this colour, together with its translucent quality, is the sole macroscopic indication of successful impregnation and if the process is continued for a longer period, an opaque dull brown colour develops, which on subsequent microscopic examination is shown to consist of a granular and structureless black precipitate. Finally, the silver nitrate solution is washed off with 10 per cent. formol saline until once again the resultant white precipitate is removed. The specimen is then mounted on a sealed slide in either formol saline or gelatin and is examined by transmitted light and oil immersion. All solutions are used at room temperature.

(2) Methylene Blue Staining of the Isolated Radial Fibres.—The specimen shown in Fig. 6 (below, p. 430) was prepared in the following way:

A fragment of fresh unfixed cat retina was placed, inner surface downwards, on a well-albuminized coverslip, a non-albuminized slide was placed over the specimen, and the whole immersed in 10 per cent. formol saline. After approximately 72 hours, the upper-most non-albuminized slide was removed and the albuminized coverslip holding the specimen was mounted under a dissecting microscope. One edge of the retina was then raised and pulled off the coverslip. This process appears to pull the conical tips of the radial fibres out of the retina so that they remain adherent to the layer of albumen on the coverslip, which is then covered with 1 per cent. aqueous methylene blue solution for 10 seconds and mounted on a slide so that the wide inner ends of the radial fibres are pointing towards the objective of the microscope. 10 per cent. formol saline is then run between the coverslip and slide, and the whole is sealed.

These preparations are not permanent.

Results and Their Interpretation

(1) Inner Retinal Surface as seen in the Wet Preparation.—The photosensitized silver method described above will impregnate only certain tissues in any given specimen; thus a general picture of retinal architecture is revealed by differences in colour and depth of tone. The appearances produced by this technique are seen in Fig. 1 (opposite), which is a low-power photomicrograph (× 90) of part of an unsectioned cat retina. It will be noted that the nerve fibre bundles are not coloured by the silver and are shown up by contrast with the radial fibres, which have taken up the silver readily and appear, at this resolution, as dark lanceolate clumps of tissue protruding towards the inner retinal surface between the nerve fibre bundles. Similarly, the branched main vessel passing diagonally across the field also appears light in tone, since there are no radial fibres on its inner or vitrad surface to take up the silver. The mosaic pattern referred to in the introduction is not resolved at this magnification, and it is necessary to use an objective numerical aperture of at least 0.65 before the details of its structure can be satisfactorily examined. The photomicrograph shown in Fig. 2 (opposite) was taken with the aid of an oil immersion system giving a total magnification in the plane of the photographic negative of 384. The mosaic is resolved and presents a characteristic appearance, being formed by irregular granular
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Fig. 1.—Whole mount of cat retina treated by the photosensitized silver method, inner surface uppermost. (× 90).
A main vessel traverses the field diagonally from right to left and the nerve fibres run parallel to it.
The dark zones between the nerve fibres are the bundles of radial fibres stained by silver.

Fig. 2.—Whole mount of human retina treated by the photosensitized silver method, inner surface uppermost (× 384).
Transmitted light.

lines of silver-impregnated tissue continuously interconnected to enclose clear, polygonal spaces. These (the champs polygonaux of van der Stricht) are either uncoloured and translucent, or of a light sepia tone and semipaque; both varieties are shown in this specimen. This pattern is seen only on the innermost part of the retina and as the microscope is racked downward towards the inner retinal surface, it is the first regularly ordered
structure to be observed, apart from a layer of translucent granular material which covers its vitreous aspect. The granules which form this layer are similar morphologically to those which constitute the dark lines of the mosaic pattern with the exception that they are neither so deeply coloured by the silver, nor so regular in size. Figs 3 and 4 show similar appearances in the sheep and ox retina with a higher magnification (×712).

Fig. 3.—Whole mount of sheep retina treated by the photosensitized silver method, inner surface uppermost (×712). Transmitted light.

Fig. 4.—Whole mount of ox retina treated by the photosensitized silver method, inner surface uppermost (×712). Transmitted light.
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If a specimen is mounted so that the coverglass is not touching the inner retinal surface, the clear spaces within the mosaic are seen to be out of focus with respect to one another. The reason for this appears to be that the plane of each mosaic is very slightly tilted with respect to the general plane of the retina, thus the inner retinal surface exposed after removal of the vitreous is seen as a contiguous but irregular series of minute facets. Similarly, if reflected light is used, a series of tilted mirror-like surfaces is seen. The difference in angle of each individual facet is best demonstrated if the numerical aperture of the objective used is sufficiently large to imply a depth of field in the object space smaller than 1 micron. Similarly, if the numerical aperture of the objective is known, then the depth of focus in the object space can be calculated. Thus, by the use of a calibrated fine adjustment, the vertical distance between two very small objects can be measured to an accuracy of plus or minus the calculated depth of field, provided that the mechanism of the fine adjustment gives rise to no lost motion and that the observer suffers from no refractive error. This principle has been made use of in the preparation of the photographs shown in Figs 5A and 5B, which are taken from exactly the same region of a cat retina, using an objective with a numerical aperture of 1·3, giving a depth of field of approximately 0·5 micron.

Fig. 5 (A and B).—Whole mount of cat retina treated by the photo-sensitized silver method, inner surface uppermost (×1060). The field is the same in both photographs, but the plane of optimum focus in Fig. 5B is 3 microns closer to the vitreous than in Fig. 5A. (See text.)
In the first (Fig. 5A), optimum focus was located in the uniform granular layer referred to above and it can be seen that the mosaic formation is blurred. In the second (Fig. 5B), the focus was located in the mosaic region by an indicated downward movement of the microscope of 3 microns; it will be seen that the granular layer is no longer clear but that the mosaic is in focus. If the plane of focus is moved still further into the retina, the mosaic pattern disappears from view after a movement of 2 microns; no further ordered structure being seen until the expanded ends of the radial fibres come into view between 7 and 10 microns deep to the retinal surface. These, however, are not seen clearly, because the light rays forming their image are diffused slightly by passage through the intervening tissue; they are shown as dark blurred patches in Figs 5A and 5B.

To obtain a more exact picture of the arrangement of the radial fibres, it is best to remove them according to the technique described above. Fig. 6 is a photomicrograph of a specimen prepared in this way and shows the radial fibres end-on as dark polygonal patches separated by clear zones and arranged in ranks between the spaces normally occupied by the nerve fibre bundles.

Fig. 6.—Inner ends of the radial fibres removed from a cat retina and stained methylene with blue (wet preparation, ×360).
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(2) Relationship between the Radial Fibre and the Inner Retinal Surface.— The differences of opinion concerning the relationship between the radial fibres of Müller, the inner surface of the retina, and the vitreous face have arisen for three main reasons:

(a) Because the contour of the polygonal areas in the mosaic sometimes conforms to the end-outline of a radial fibre;

(b) Because the exact location of the mosaic pattern has not been defined;

(c) Because many of the existing observations have been made on dehydrated tissue only.

(a) In Figs 1 and 6 the radial fibres are seen to be arranged in closely packed rows or clumps protruding towards the inner retinal surface between the nerve-fibre bundles and the blood vessels. They do not curve around the inner vitreal aspect of the nerve fibre bundles or the vessels completely, but pursue a moderately straight course towards the inner retinal surface. This is seen in Fig. 7, which shows a vertical section of cat retina cut at right angles to the long axis of the nerve fibres. The radial fibres pass directly to the inner retinal surface, where they terminate in trumpet-shaped expansions.

Fig. 7.—Vertical section of a cat retina cut in a plane at right angles to the longitudinal axis of the nerve fibres, showing radial fibres passing around a main vessel. The space between the expanded ends of the radial fibres at the inner retinal surface is seen. (Lieb’s haematoxylin, × 360.)
(b) Similarly, in Fig. 1, it is clear that the radial fibres are not present on the vitreous aspect of either the nerve fibres or the vessels. It is possible, therefore, to define zones in the retina where the vitreal ends of the radial fibres are not present at the inner retinal surface. However, examination of a whole retina impregnated by the photo-sensitized silver method shows the mosaic pattern to be evenly continuous over the entire internal surface, including the inner aspect of the nerve fibre bundles, the vascular tree, and the optic disc, with the exception of the central pit in the human; so that the mosaic and the radial fibres must at least be morphologically separate both for the above reason and also because the two structures are seen to be at least 7 microns apart in the wet specimen with no ordered continuous structure between them. In contrast, however, the appearances seen in Fig. 7 make it difficult to accept that they are separate, for in this dehydrated specimen, at the resolution used, the termination of the radial fibres apparently coincides exactly with the inner retinal surface, implying either a difference between wet and dry retinal tissue or that the mosaic structure does not appear in sections.

(c) Dehydration of tissue for normal histological preparation automatically implies a shrinkage, common to cellular and noncellular components alike. The existence of a "ground substance" in the central nervous system has for long been a source of exacting controversy; recent work has shown however that it probably is present and further that it consists of mucopolysaccharides, and possibly mucoproteins although the balance of the evidence is in favour of the former (Hess, 1953). Furthermore, it has been suggested that the supporting substance of the central nervous system is capable of particularly delicate adjustments in volume by alteration of its water content (Taft and Ludlum, 1929), a property probably due in part to the convoluted spiral molecular shape of hyaluronic acid, which is almost certainly a constituent. If, therefore, it could be demonstrated that the morphology of the region containing the mosaic pattern is altered by treatment with hyaluronidase, it would suggest that the appearances are wholly or partially attributable to extracellular components.

(3) Effect of Hyaluronidase.—Several experiments were performed to test the possibility that the mosaic pattern may be altered by treatment with hyaluronidase, all of which conformed to the following general pattern:

A rectangular fragment of kitten retina was taken from a fresh specimen and divided into two equal squares, one being retained in normal saline and the other immersed in a solution of hyaluronidase in normal saline (1,500 I.U./ml.) for a minimum period of 2½ hours at room temperature. Both specimens were then subjected to the photo-sensitized silver method in exactly similar fashion. On examination, the control fragment appeared normal, whereas the piece treated with hyaluronidase showed an almost complete absence of the granular layer overlying the mosaic. Even prolonged treatment with hyaluronidase (24 hours
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at room temperature) failed to alter the appearance of the lines forming the pattern, which in fact were seen with greater clarity in the absence of the granules (Fig. 8).

**FIG. 8.**—Whole mount of kitten retina treated first with hyaluronidase and subsequently by the photo-sensitized silver method (× 340 enlarged). No granular layer is apparent.

With the provisos that such relatively coarse procedures can only be used as the broadest indication and the possibility that the enzyme is capable of bringing about a change in its refractile properties alone, it seems probable that the granular layer overlying and to some extent infiltrating the mosaic is partially formed by mucopolysaccharides of the hyaluronic acid type.

(4) **Presence of Reticulin.**—Further evidence for the thesis that the inner limiting membrane is a separate extracellular structure would be the demonstration within it of reticulin or argyrophil fibres. There is at the present time some dispute among molecular biologists as to the exact nature of this material, but its morphological characteristics and the methods used to demonstrate them are not seriously in doubt. A number of human retinae embedded in celloidin and stained for reticulin by Wilder’s method (Wilder, 1935) was examined for the presence of reticulin fibres in the region of the inner retinal surface. A typical result is seen in Fig. 9 (overleaf), showing the characteristic branching argyrophil pattern of reticulin in an oblique section of the inner retinal surface. The fibres do not appear to be regularly ordered and at no time was any resemblance to the mosaic pattern seen in preparations stained for reticulin. Unfortunately, it is not possible to relate the reticulin fibres to the mosaic pattern with any precision, for the mosaic is best seen in the wet whole specimen and reticulin in the dehydrated and embedded tissue. Furthermore, the presence of reticulin in this region does not necessarily imply that the inner surface of the retina is constructed from the same material, for it may well represent a transition zone between retina
and vitreous. In any case it would appear pointless to distinguish the exact level at which one structure changes into the other; it is far more probable that, in the living eye, the retina merges gradually into the outer face of the vitreous body and that the reticulin which is demonstrable in this region may be as much a part of the outer vitreous as of the inner retina. This is not to deny that a plane of cleavage (Fig. 10) exists between the retina and the vitreous in pathological states; indeed, there is considerable evidence to support genuine vitreous detachment as a sequel to exudative inflammatory lesions giving rise to an intravitreal cellular infiltration and subsequent contracture (Duke-Elder, 1940). Furthermore, it is easier to remove the vitreous from an excised eye if some hours have elapsed since death, again suggesting a plane of potential separation which is accentuated by the processes of post mortem autolysis.

A further characteristic of reticulin is that it stains positively with certain specific variants of the periodic acid-Schiff method. Vertical sections show
that the inner retinal surface stains strongly with this method which is additional evidence for the presence of reticulin and may, according to some observers, serve to distinguish it from collagen which is alleged to stain lightly if at all (Robb-Smith, 1957). Similarly, vertical sections show the inner limiting membrane to take up the acid aniline blue as used in the Mallory trichrome method, whereas the vitrad extensions of the radial fibres are coloured a deep red, which is not only another method of demonstrating reticulin but again indicates that the membrane and the radial fibres are separate structures. The fact remains, however, that the radial fibres are firmly attached to the inner limiting membrane and may in fact be pulled out of the retina if the membrane is detached artificially—a phenomenon which is frequently seen as an artefact through handling during histological preparation and also as a result of post mortem degeneration (Fig. 11).

Finally, repeated searches for nuclear and cytoplasmic detail within the mosaic have proved consistently negative, with the exception of the vitreous cells of Hannover which are easily distinguishable. It may be concluded therefore, that the inner limiting membrane is a separate and specific extracellular structure containing both hyaluronic acid and reticulin and that it is separate from the radial fibres and not formed by lateral extension of their vitrad ends although these are firmly attached to it.

(5) Nature of the Mosaic Pattern.—If, therefore, this region of the retina is non-cellular, it is of interest to consider, first the nature of the mosaic pattern and secondly the relationship between the inner retinal surface and the equivalent parts of the central nervous system elsewhere.

As the retina differentiates from the neural ectoderm, fibrillary connexions are seen to develop between the inner surface of the primitive retina and the combined ectoderm and vascular mesoderm which together form the primary vitreous; this process is apparent at the 12-mm. stage of the embryonic development, at which time the fibrils, which run in an approximately radial direction, are continuous with the vitrad ends of the
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differentiating radial fibres although they are seen to disappear during subsequent maturation.

It is interesting to speculate, therefore, that the mosaic pattern may be the last vestigial imprint of these fibrils and that it is artificially brought into view first by the removal of the vitreous, which is in itself an artificial process, and secondly by the silver technique used which shows it up differentially, possibly because the tissue forming the pattern protrudes for a short distance beyond the level of the clear spaces it encloses. This latter possibility is unfortunately impossible to verify with the optical microscope since the necessary resolving power is not obtainable, and it is equally difficult to record with the electron microscope since wet specimens are electron opaque and are in fact rapidly damaged by the incident electron beam.

The relationship between the inner aspect of the retina and the embryologically equivalent zone of the spinal cord is clear if it is allowed that both arise from the same surface of the primitive central nervous system: the cord by an outgrowth and fusion of the neural crest, and the retina by invagination of the anterior surface of the outgrowing optic vesicle. Thus the outer surface of the cord is equivalent to the inner surface of the retina. It is scarcely surprising, therefore, that there is a striking morphological resemblance between what is usually termed the external limiting membrane of the cord (Strong and Elwyn, 1953) together with the feet of the attached bipolar spongioblasts, and the inner aspect of the retina together with the attached radial fibres (Fig. 12).

Fig. 12.—Transverse section of spinal cord of 44-mm. human embryo shown by the Golgi silver technique. Reproduced from Strong and Elwyn (1953) by courtesy of the publishers.
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The peripheral feet of the spongioblasts expand in a similar way to the inner ends of the radial fibres, both are arranged in closely packed pali- sades, and both show lateral or, in the case of the cord, circumferential fibrillar expansions.

Summary

(1) To indicate that controversy exists both in regard to the nature of the inner limiting membrane and to its relationship with the radial fibres, a brief review of the literature is presented.

(2) A photosensitized silver impregnation technique is described which is suitable for the delineation of certain structures in wet preparations of whole retinæ.

(3) The morphological appearances of the inner retinal surface are described and photomicrographs of the mosaic pattern on the inner limiting membrane shown.

(4) The relationship between the structures of the inner retinal surface and the radial fibres of Müller is discussed, and evidence is presented which suggests that the two are separate and that the inner limiting membrane is not formed by the radial fibres.

(5) The possibility that the inner limiting membrane is an extracellular reticulin-containing structure is considered.

(6) The suggestion is made that the mosaic pattern revealed by the photosensitized silver technique is concerned with the attachment of the vitreous body to the surface of the retina.

(7) The morphological resemblances between the inner surface of the retina and the outer surface of the spinal cord are noted.

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