The structure and membrane properties of the frog nictitans

Mauricio A. Lande and José A. Zadunaisky
With the technical assistance of
Susan Branson

The nictitating membrane of the frog is a semitransparent tissue which appears as an adaptation to terrestrial life and serves a protective function. The optical and electron microscopic studies indicate that it does not contain smooth muscle and that it is formed by an external stratified epithelium similar to that of frog skin, a thick stromal region containing vessels, nerves, and collagen organized in lamellae with fibers of uniform size with an arrangement resembling the corneal stroma, and an inner mucoid epithelium similar to the one of the conjunctiva. This membrane maintains a potential difference, positive inside (40 ± S.E. 5.6 mV. in vivo and in vitro. By means of $^{22}$Na fluxes a transport of sodium from outside to inside (0.568 μEq h⁻¹ cm⁻²) was found which is inhibited by ouabain and stimulated by vasopressin. This transport is located in the outer epithelium. The membrane does not swell remarkably in vitro, nor does it show a temperature reversal phenomenon. An extremely well-organized stroma and loss of pigmentation are the causes for its semitransparency. The amount of incidental light scattered by single fibers predicts complete opacity for the frog nictitans as much as in the case of the transparent cornea. The difference in fiber size and interfiber distance between the stromas of the nictitating membrane and that of the cornea could be related to their different degrees of transparency.

Key words: nictitating membrane, ultrastructure, cornea, light scattering, light transmission, collagen fibrils, cell membrane potential, active biological transport, cell membrane permeability, sodium transport, ouabain, vasopressin, temperature changes, frog, fine structure, transparency, resting potentials, swelling.

The nictitating membrane is found in the family Ranidae as a highly differentiated upper portion of the lower lid, which becomes thin and functionally semitrans-
fore permits partial vision. It is folded again partly by the protrusion of the eyeball, but chiefly by the contraction of a muscle which arises from the levator bulbi, the \textit{depressor membranae nictitantis}. The nictitating membrane arises during ontogeny from a small mass of undifferentiated tissue embedded within the tegument at the anterior border of the tadpole eye.\textsuperscript{1} Lindeman\textsuperscript{2} has shown that extirpation of this tissue prevents the nictitating membrane from forming during metamorphosis, whereas removal of the ventral border of the eye permits partial regeneration and formation of a perfect nictitating membrane. Hence, the anterior mass of tissue appears to be responsible for the development of this membrane.

In the present study, the ultrastructure of this membrane was examined, as well as some of its functional properties, mainly the presence of a potential difference and a sodium transport across it. Both sides of the membrane are lined by epithelium, each with different characteristics, which can be related to the electrical potentials and active transport of sodium from the external to the internal side present in the membrane. The electron microscopic structural study shows that the connective tissue is as highly organized as the corneal stroma. The collagen fibers are arranged in a similar manner. The main difference between this connective tissue and the one of the cornea is the diameter of the fibers and the size of the interfibrillar space. It is suggested that this dissimilarity could cause the difference in transparency when comparing both membranes.

**Methods**

Regular specimens of the American bull frog (\textit{Rana catesbiana}) were used. The frogs were kept in a tank with running tap water for a period of no longer than 4 weeks. The animals were immobilized by pithing the spinal cord as well as the brain, the latter in order to avoid eye and nictitating membrane reflexes during dissection. The upper lid was removed, and the nictitating membrane was dissected and immediately immersed in a Petri dish containing Conway-Ringer solution at room temperature, or mounted on a lucite chamber as described previously.\textsuperscript{3} Measurement of the potential differences was made with a 610C Keithley electrometer or with a Texas strip-chart recorder. An automatic short circuit current voltage clamp apparatus was also used.

Radioisotopic fluxes were measured under short-circuited conditions as follows: After equilibration (45 to 60 minutes) of short-circuit current at a stable value for a period of not less than 15 minutes, 3 \textmu l of \textsuperscript{22}Na 0.1N solution at a specific activity of 1 mc. per milliliter (New England Nuclear) was added to one of the sides of the chamber. One hour after the introduction of the labeled compound, samples of 250 \textmu l from the cold side and 10 \textmu l from the radioisotopic side were taken at hourly intervals during a period of 5 consecutive hours and counted with a Mark I Nuclear-Chicago liquid-scintillation system. Cal-

![Fig. 1. Measurement of the potential difference of the frog nictitating membrane in vivo.](image-url)
calculations were based on the known specific activity of the solution bathing the radioisotopic side and appropriate corrections for volume and exposed surface in the chamber. Unidirectional fluxes were measured in two groups of membranes, in one group from outside to inside and in the other group from inside to outside.

Fig. 1 shows the system utilized for the in vivo measurement of electrical potential differences. The eye was removed, leaving the nictitans in place, and a nitrocellulose half cup with a lucite holder frame was placed in position so as to form a reservoir with the internal face of the nictitans. This cup was then filled with Ringer's solution. Connections to the electrodes and the meter were made with agar-filled polyethylene tubes in contact with the solution bathing the internal side and with a drop of the solution held by capillarity between the external epithelium and the agar of the outside bridge.

For optical histologic studies, the nictitating membrane was fixed in 5 per cent formal solution during 24 hours, dehydrated in alcohols, and embedded in paraffin. Sections were mounted on clean slides and stained with hematoxylin-eosin, Alcian blue, Masson, and periodic acid–Schiff.

Specimens for the electron microscopic study were fixed for 1 to 2 hours in 3 per cent glutaraldehyde in 0.05M sodium cacodylate buffer (pH 7.45, 630 mOsm.) and postfixed in osmium tetroxide. They were then dehydrated in ethanol solutions and embedded in Epon. Sections were cut with a Sorvall-Porter-Bloom ultramicrotome, poststained with uranyl acetate and lead citrate, and examined with a Siemens Elmiskop I-A electron microscope.

The immersion fluid utilized throughout was Conway-Ringer solution containing 104 mM Na and 2.5 mM K. In some of the experiments, sodium-free solution was utilized with the following composition: choline chloride 80 mM, potassium bicarbonate 2 mM, potassium chloride 3 mM, magnesium sulfate 1.2 mM, glucose 26 mM, calcium gluconate 1 mM, and sucrose 40 mM. The osmolarity of the sodium-free solutions was similar to that of the basic fluid, as tested with an Advanced Instruments osmometer.

Swelling experiments were made with central squared pieces of the membrane immersed in Ringer's solution at different temperatures. At the end of the incubation period the nictitating membranes were taken out of the solution, blotted gently on paraffin paper, and weighed. Dry weight was determined after keeping the membrane in a desiccator oven at 45° C. under vacuum until constant weight was reached in two successive determinations made 8 hours apart.

Results

Anatomy of the frog nictitating membrane. The observation of sections under the optical microscope showed that this membrane is composed of three main layers: The first is an external outer or superficial epithelium (Fig. 2) which is stratified and shows a well-developed cornified layer. This external epithelium is formed by 3 to 5 layers of cells and is continuous with the epithelium of the skin of the lower lid of the frog's eye. The second layer, a stromal layer, is composed of connective tissue proper, collagen fibers disposed in a ground substance. This external epithelium is formed by 3 to 5 layers of cells and is continuous with the epithelium of the skin of the lower lid of the frog's eye. The second layer, a stromal layer, is composed of connective tissue proper, collagen fibers disposed in layers in a ground substance. This region contains vessels, nerves, and typical fibrocytes which have all the appearances of the typical connective tissue.

Fig. 2. Section of the frog nictitating membrane prepared for optical histology. The outer portion shows the epithelium with several layers of nuclei, the thick stroma showing the lamellar structure and the inner epithelium. In the stroma the nuclei of connective tissue cells (nictocytes) and cross-sectioned vessels are seen.
Fig. 3. Cross-section through the stroma or connective tissue layer of the nictitating membrane. Note the regular distribution of the collagen fibers within each lamella. The presence of a fibrocyte (nictocyte) is observed. There is a great resemblance with the distribution of the collagen fibers in the corneal stroma. (×17,600.)
cells and are clearly oriented in the same plane of the epithelium. The third layer is an internal epithelial layer in contact with the surface of the cornea and sclera. This inner layer is 1 to 2 cells thick and constitutes a typical “serrated” epithelium very much resembling the epithelium of the conjunctiva. This epithelial layer stained positively with the PAS reaction and showed the presence of mucine and goblet cells which stained frankly with Alcian blue dye. Fig. 2 shows a microphotograph

Fig. 4. Outermost portion of the exterior epithelium of the frog nictitating membrane. The general aspect is that of frog skin epithelium with a cornified layer on top stained darker in this figure. Parts of two cells are seen, presence of mitochondria is observed in one of them while the granules of the granulosa and intracellular fibrils are seen in the other. Desmosomes are clearly observed at the points of contact between cells. (×15,000.)
Fig. 5A. Section through the stroma of the nictitating membrane. The size of the fibers was found to be 700 Å, while the spaces between the fibers were 140 Å. (x40,000.)

Fig. 5B. Section through the stroma of the frog cornea shown at a slightly larger magnification. The size of the fibers is smaller here, 283 Å, while the spaces in between them are larger, 223 Å, than in the nictitans. (x48,000.)
of a section through the center of the nictitating membrane where it has a thickness of about 0.20 to 0.24 mm.

The sections observed under the electron microscope revealed some very interesting findings, mainly at the level of the stroma which very much resembled the stroma of the cornea, as shown in Fig. 3. For the sake of an organized description, the layers will be described in the same order utilized above for the optical observations.

The outer epithelium shows well-developed cell junctional complexes, with tight junctions and a number of desmosomes. The general appearance of the cells is similar to that of the epithelium of frog skin, as described by Farquhar and Palade. The cells contain numerous
granules, such as those observed in the granulosa of skin epithelium. Intracellular fibrillar elements are also seen, as well as mitochondria and a well-developed endoplasmic reticulum. Fig. 4 shows a section through the outer epithelium.

The region of the stroma showed the presence of a lamellar structure composed of layers of collagen fibers organized in a fashion extremely similar to the cornea of the frog or other vertebrates. Fig. 3 shows a section through the stroma where the highly organized collagen structure can be appreciated, as well as the presence of a connective tissue cell, fibrocyte or, in this case, it could be called nictocyte, in a similar mode of nomenclature as the keratocyte of the cornea. At higher magnification the similarity with the organization of the corneal lamellae is even more striking. However, in Fig. 5, A and B, where sections from the frog corneal stroma and the stroma of the nictitating membrane of the same animal are shown together, at similar magnifications, it is possible to see some differences. The size of the fibers is greater in the stroma of the nictitating membrane, as compared to the ones of the cornea, and the spaces in between them appear to be smaller.

Actual measurements of the diameter of the fibers in the electron micrographs of both nictitating membrane and cornea of the frog showed mean values of 700 ± 36 Å for 400 fibers of the nictitating stroma and 265 ± 26 Å for 400 fibers of the corneal stroma. The spaces between the fibers from border to border of adjacent fibers was 140 ± 23 Å for the nictitating membrane and 223 ± 14.6 Å in the cornea. Some implications of these differences with respect to transparency of the cornea and semitransparency of the nictitating membrane are presented in the discussion.

The stroma of the nictitating membrane, furthermore, has irrigation and innervation as mentioned above. In some sections of the stromal area, vessels such as the one shown in Fig. 6 were seen slightly displacing the harmonious distribution of the lamellae. Myelinated and nonmyelinated axons are seen in the section shown in Fig. 7. The inner epithelium consisted of a secretory or mucoid type layer of cells with numerous projections as shown in Fig. 8 which produced the “serrated” shape in the sections observed with the optical microscope. The cells’ membranes show a tortuous path and numerous granules are seen in the apical portion of the cells. The brush border and the very well-developed endoplasmic reticulum give these cells some of the characteristics of the secretory cells found in mucosal epithelium. A fine basement membrane separates the inner epithelium from the stroma.

**Potentials.**

*In vitro.* When the central portion of the isolated nictitating membrane is mounted in a lucite chamber and bubbled with air, it maintains a potential difference that stabilizes in values of 40 ± 5.6 mv. after 40 to 60 minutes with a resistance of 1,400 ohms (750 to 1,750). The outside of the membrane, which in the animal is exposed to the environment, is positive with reference to the inside, which is in contact with the surface of the cornea and sclera. Air was chosen as bubbling gas since no

Fig. 8. The inner epithelium of the frog nictitating membrane is shown in cross-section. The apical border of the cells, in contact with the corneal surface, has multiple projections, microvilli, with numerous granules (probably of mucine). The cells have an elaborate endoplasmic reticulum and in general a lobulated nucleus. The tortuous contacts between the cells are strengthened by desmosomes which are not so frequent as in the outer epithelium. However, really tight junctions between the cells at the border were not observed. A fine and well-stained basement membrane is observed separating the mucoid epithelium from the first stromal lamella, (×8,000.)
Fig. 8. For legend, see opposite page.
significant difference in values of potentials or resistance is found when a mixture of 5 per cent CO₂ and 95 per cent O₂ is used, and values of pH are above 7.4.

In vivo. Potentials were registered in 6 animals, as described in the Methods section and as shown in Fig. 1. They were of the same orientation as those in vitro, and the average stable value obtained was 18.3 ± 2.6 mv. This potential difference was not found in nictitating membranes of other vertebrates in which measurements were attempted in vitro, such as in ducks and rabbits.

Short-circuit current. Fig. 9 shows the normal evolution of the short-circuit current in vitro. Once equilibrium is reached, after approximately 45 minutes the values remain relatively stable for several hours with a slow constant slope. In sodium-free bathing solution, the short-circuit current drops more than 95 per cent, as shown in Fig. 10. The effect of vasopressin (0.25 U. per cubic centimeter) is also shown in the same figure; it produces an increase in the short-circuit current of about 70 per cent of the control values.

Fig. 11 shows the effect of anoxia produced by bubbling pure nitrogen instead of air into the chambers. Anoxia produces an average decrease of the short-circuit current of about 78 per cent. The effect of ouabain (10⁻⁴M) added to the internal or corneal side of the preparation consists of a reduction of 74 per cent after 180 to 240 minutes from the addition of the drug.

Radioisotopic fluxes. Table I shows the results obtained when transmembrane fluxes were measured using ²²Na. As can be observed from the comparative values of net transport and short-circuit current registered simultaneously, there is a good match between them; therefore sodium carries most of the current in this system.

Swelling. Table II shows the water content of the nictitating membrane, determined by 3 groups of 5 membranes each, kept in regular Conway-Ringer solution for periods of 6, 12, and 24 hours, respectively, at room temperature. Comparison of these values with an identical group immersed briefly in the same solution (time zero) did not show a significant difference. A fourth group immersed for a period of 48 hours showed an increase in water content of 6.26 per cent.
Fig. 10. In the lower trace the effect of sodium removal and replacement by choline on the short-circuit current of the nictitating membrane is shown. Recovery is obtained if the membrane is bathed again in regular, sodium containing Ringer’s solution. In the upper trace the stimulating effect of vasopressin on the current is clearly observed. The vertical bars indicate ±S.E.M. and N refers to the number of experiments.

Fig. 11. The inhibitory effect of ouabain on the sodium transport of the nictitating membrane is shown in the earlier of the two tracings. The great reduction in transport rate produced by removal of oxygen and bubbling of nitrogen into the solutions bathing the nictitans is observed, as well as the recovery in the presence of oxygen. The bars indicate the ±S.E.M. and N refers to the number of experiments.
Table I. Sodium fluxes across isolated nictitating membrane of Rana catesbiana

<table>
<thead>
<tr>
<th></th>
<th>No. of experiments</th>
<th>Mean Na flux</th>
<th>Mean short circuit current</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{22}$Na influx</td>
<td>5</td>
<td>0.617 ± 0.060</td>
<td>16.54 ± 1.61</td>
</tr>
<tr>
<td>$^{22}$Na efflux</td>
<td>5</td>
<td>0.049 ± 0.006</td>
<td>1.33 ± 0.18</td>
</tr>
<tr>
<td>$^{22}$Na net flux</td>
<td></td>
<td>0.568</td>
<td>15.31</td>
</tr>
</tbody>
</table>

In the cornea, low temperature produces an increase in the rate of swelling and opacification which is reversible. Attempts were made to reproduce a temperature reversal phenomenon in the nictitating membrane. The results are shown in Table III. Three groups of 5 nictitating membranes each were treated as follows: The group considered at time zero was simply immersed in Conway-Ringer solution and immediately dried for the determination of water content as previously described; a second group was maintained for 6 hours at 4° C. and then subjected to similar treatment; the third group was kept at 4° C. for 6 hours, then returned to room temperature for 6 more hours, after which they were also dried and weighed. None of the 3 groups showed any significant differences, as observed in Table III, despite the different times of immersion in fluid and different temperatures at which they were maintained. The water content of these 15 nictitating membranes did not differ significantly from the water content found in the 25 membranes previously described, undergoing similar determination while maintained constantly at room temperature for identical periods.

Discussion

The nictitating membrane in amphibians, such as Rana catesbiana, utilized in these studies has no muscle included in its thickness, and in this sense it differs from the third lid or nictitating membrane of higher species such as birds, rabbits, or cats, to mention the best known animals having nictitans. The nictitating membrane appears in amphibians most probably as an adaptation to terrestrial life. The obvious function of protection of the eye from the environment is emphasized by the fact that frogs always close up the nictitating membrane when they dive into water.

This membrane is semitransparent when compared to the cornea. One reason for this partial opacity could be the presence of vessels and nerves in the depth of the collagenous stroma. However, these anatomical elements are of such a big size and low frequency that they most probably would not affect the transparency of the whole membrane.

The highly organized nature of the connective tissue which gives the necessary thickness to the nictitans of the frog is striking and only found in some instances, such as in the stroma of the cornea7 and the basement lamella of amphibian larvae.8 The fibers are arranged in well-organized lamellae and the regular size of the fibers gives this tissue its corneal-like orthogonal arrangement.

The relationship between light scattered by a nonhomogeneous body of the type of the nictitating membrane and the size and organization of the fibers of the stroma has been studied by Maurice.9 If the fibers are considered to scatter light independently, then Maurice’s equations should apply:

\[
\Delta S = k \cdot \Delta t = \frac{d^2 \pi \rho^2}{\lambda^3} \left[ 1 + \frac{2}{(n'_1 + n'_2)^2} \right] \left( n'_1 - n'_2 \right)^2 \cdot \Delta t
\]

and

\[
S = 1 - e^{-\Delta S}
\]

In order to find $S$, the light scattered by all the tissue, the following values were used for the frog nictitating membrane:
Table II. Water content of frog nictitating membranes immersed in Ringer's solution at 20° C.

<table>
<thead>
<tr>
<th>Time (hr.)</th>
<th>Water content, % wet weight</th>
<th>No. of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>80.46 ± 1.66</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>82.23 ± 1.88</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>82.72 ± 1.84</td>
<td>10</td>
</tr>
<tr>
<td>24</td>
<td>78.55 ± 1.98</td>
<td>5</td>
</tr>
<tr>
<td>48</td>
<td>86.72 ± 2.38</td>
<td>5</td>
</tr>
</tbody>
</table>

Table III. Water content of frog nictitating membrane immersed in Ringer's solution at different temperatures

<table>
<thead>
<tr>
<th>Time (hr.)</th>
<th>Water content, % wet weight</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>80.22 ± 1.76 (5)*</td>
<td>20° C.</td>
</tr>
<tr>
<td>6</td>
<td>83.21 ± 1.97 (5)</td>
<td>2-5° C.</td>
</tr>
<tr>
<td>12†</td>
<td>82.02 ± 2.44 (5)</td>
<td>20° C.</td>
</tr>
</tbody>
</table>

*Number of experimental determinations.
†First 6 hr. at 2 to 5° C., plus 6 hr. at 20° C.

volume fraction of the fibers, \( d_0 \), 66.5 per cent of the total, mean radius of the fibers, \( \rho = 350 ± 17 \) Å, figure arrived at by measuring the diameter of 400 fibers in prints obtained from electron micrographs from different specimens; the wavelength of the light \( \lambda \) was taken here as 5,000 Å; the index of refraction of the interstitial ground substance \( n_i \) and the collagen fibers \( n_c \) were taken as 1.345 and 1.550 from Maurice's data, since analysis of the nictitating membrane was not made for this purpose.

The value of light scattered obtained from the above treatment was greater than 99 per cent, that is to say, the nictitating should be an extremely opaque body. However, it is only slightly more opaque than the very transparent cornea of the frog. If the same formula and integration are applied to the cornea of the frog, utilizing a value of 36.5 per cent for the volume fraction of the fibers \( d_0 \) and a mean radius of the fibers of 132.5 ± 13 Å (obtained as for the nictitating membrane) results for the light scattered greater than 90 per cent of the incident light are obtained. In both cases, then, some explanation is required to account for the transparency of these membranes. It is possible that destructive interference of the light scattered individually by the fibers of the stroma does occur as proposed for the rabbit cornea by Maurice,1 and this is the reason for the transparency of the frog cornea and the semitransparency of the nictitating membrane. The high regularity of the stroma of the nictitans, however, would provide a very good crystal-like structure which could behave as completely transparent if destructive interference is the explanation.

Recent observations of Goldman and Benedek10 and Goldman and associates11 indicate that in spite of the presence of randomly oriented fibers in the corneal stroma of the shark, the cornea as a whole behaves as a transparent object. This permits the supposition that the relationship \( \lambda/2n \) is an important factor or the only determinant of the transparency of these tissues, and indicates that a critical value of particle size is an important factor in transparency. When the size of the fibers of collagen in the nictitating membrane is compared with those of the frog or even the rabbit cornea, it is found clearly that the ones of the nictitating membrane are greater in diameter. The relationship \( \lambda/2n \) for the frog cornea gives a value of 1/18.1, while for the nictitating membrane it gives 1/7.1. It is likely then that the greater thickness of collagen fiber would give a certain degree of opacity to the nictitating membrane of the frog.

The epithelium of the outer or superficial portion of the frog nictitating membrane is a sodium-transporting epithelium as much as that of frog skin.12 The potential difference observed both in vivo and in vitro can be interpreted as the consequence of the transport of this positive cation from the outside toward the inside. The presence of the active transport of sodium is confirmed by the evidence presented: the drop of the potential differ-
ence and the short-circuit current when sodium-free solutions are placed on the outside of the membrane and their return to the original values in the presence of sodium, the presence of a net sodium flux measured with the use of radioisotopic sodium under short-circuit current conditions, the inhibitory effect of low doses of ouabain placed in the inside solution, and the stimulatory effect of vasopressin. The reversible effects of anoxia reveal the aerobic nature of the energy source of the transport of sodium in this membrane.

The simple action of scraping the epithelial surface of the nictitating membrane produces a reduction to near zero values of the potential differences or the short-circuit current, while the same operation made only on the internal conjunctival epithelium produced little or no effect. The pump then is most likely located in the epithelium.

The fact that another epithelium located in a series is present in this membrane made the study of the ultrastructure of further interest, especially a search for tight junctions in this membrane. The passage of the ions from the outside through the outer epithelium is the function of the active transport mechanism. Diffusion of ions and water through the stroma most probably occurs and the salts then diffuse into the plasma of the vessels circulating in the nictitating stroma. When the membrane is placed in vitro in a chamber, the diffusion into the vessels disappears, and the stroma has to be in equilibri-um with the fluid of the inside solution. This also implies that the inner epithelium has to be permeable to ions and water. If the inner epithelium were impermeable, it would be somewhat difficult to understand how the ions and water could get to the internal side of the membrane. This is the reason why it was of interest to see if tight junctional complexes were present in the inner epithelium, since it is at these points that the control of the passive permeability occurs. It was found, as described in the Results, that no tight junc-

The nictitating membrane of the frog appears as an adaptation to terrestrial life. Some anatomical and functional modifications of a skinlike structure take place, such as the loss of pigmentation and the reorganization of the connective tissue in order to make it transparent, the acquisition of a mucoid inner epithelium for lubricating and protective functions at the level of contact with the cornea, and the presence of a tendon for flexibility and strength. Meanwhile, the capacity of the epithelium to transport salts from the surrounding medium into the blood stream appears to be fully preserved.

REFERENCES