Developmental histochemistry of the rat eye

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A study of the development of the rat eye was undertaken with the histochemical demonstration of five oxidative enzymes to correlate patterns of oxidative enzyme activity with morphologic development. Succinic dehydrogenase, TPN and DPN diaphorase and two DPN-dependent enzymes, malic and lactic dehydrogenase, were demonstrated, and the histochemical patterns were examined in relation to the standard hematoxylin and eosin pattern obtained at seven periods during the development of the eye.

The immature rat eye, especially the developing retina, was found to have a significant level of oxidative enzyme activity. The pattern of enzyme localization in the outer layers of the retina appeared much before the morphologic differentiation of the retina. Mueller’s fibers could be demonstrated histochemically only at a very late stage of development. In some of the enzyme preparations, such as succinic dehydrogenase and TPN diaphorase, the development of the immature patterns differed, in some respects, from the development of the other enzyme patterns. As the retina and the rest of the eye matured, however, the histochemical patterns gradually approached those of the adult.

Except for the report of Pearse on the developing rat and chick retina, most of the studies on ocular histochemistry have been on fully developed adult eyes. A detailed embryologic study of the sequence of enzyme development to the adult pattern has not been recorded. The purpose of the present investigation is to elucidate by histochemical methods the development of the adult enzyme pattern. Five oxidative enzymes were localized in the eyes of rats in progressive stages of maturity.

Many vertebrates commonly used for laboratory investigation, such as the dog, cat, rat, and mouse, have eyes that are immature at birth. The rat, however, was chosen for this study for several reasons. It is readily obtained and easily bred. By taking daily vaginal smears, the date of conception may be determined so that the age of the embryos is known accurate to one day. The rat eye is quite immature at birth, and the development of the rat eye including its pattern or retinal vascularization is similar to that of many vertebrates including the human.

Materials and methods

Adult white albino rats were mated and the date of conception determined by taking daily vaginal smears. The first presence of epithelial cells and leukocytes in the vaginal smear was the criterion used to mark the date of conception.

At 13 and 17 days of gestation, the mothers were anesthetized with ether, and the embryo rats delivered by cesarean section. The embryo was decapitated and the entire head was quick frozen in an isopentane bath at -80°C. (dry ice-acetone mixture). The heads of newborn, 7-, 10-, and 14-
day-old rats were quick frozen in a similar fashion. The eyes of 21-day-old rats were enucleated and quick frozen. The heads and eyes were then immediately transferred to the cryostat. Frozen sections were cut at 6 μ on a Minot rotary microtome. The sections were mounted on coverslips, incubated in appropriate media, and then mounted in Kaiser's glycerogel.

Malic, lactic, and succinic dehydrogenases were determined according to the method of Nachlas and co-workers. Phenyazine methosulfate was added to the succinate incubation mixture. Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) diaphorase activity was determined by the method of Nachlas, Walker, and Seligman.

Results

Thirteen-day embryo. At this stage of development the rat retina, in hematoxylin and eosin section, is composed of undifferentiated epithelium with an indication of formation of the optic nerve layer. The lens has separated from the covering ectoderm and is still developing. The ciliary body has not yet formed.

The histochemical patterns obtained when the sections are incubated for lactic, malic, and succinic dehydrogenase are similar.

The developing retina shows a light, even distribution of formazan precipitate along most of the inner layer of the optic cup, with a much denser layer of precipitate along the outer periphery of the inner layer. There is also a dense precipitate of formazan along the outer layer of the optic cup. A zone of slightly denser precipitate than is present in the midportion of the inner layer is seen along the innermost border of the developing retina, the region that will differentiate into the nerve fiber and ganglion cell layers, as shown in Fig. 1.

There are fine particles of formazan scattered uniformly in the developing lens stroma with a much increased density of precipitate at the anterior surface, and a thin band of dense precipitate along the posterior periphery of the lens. A zone of increased density of precipitate is present along the anterior edge of the elongated posterior lens cells (Fig. 2).

Seventeen-day embryo. In the eye of the 17 day embryo, in hematoxylin and eosin section, the lens is almost fully formed. The developing cornea and sclera can be identified. In the retina, the nerve fiber layer and the beginning differentiation of the ganglion cell layer can be recognized.

Fig. 1. Demonstration of succinic dehydrogenase in the retina and lens of the 13 day rat embryo. (×32.)
The ciliary body is in the very early stages of development (Fig. 3). Similar histochemical patterns are obtained when the sections are incubated for each of the five enzyme systems.

The retina shows four major zones of formazan precipitate, as shown in Fig. 4. The outer layer of the optic cup, the developing pigment epithelium, has a dense precipitate. The inner layer of the optic

Fig. 2. Higher magnification of lens in section incubated with lactate and DPN. Note presence of formazan along the anterior and posterior surface and along the anterior edge of the elongated posterior lens cells; 13 day rat embryo. (×125.)

Fig. 3. Section of anterior pole of a 17 day rat embryo eye incubated in malate and DPN. Activity is present along the periphery of the lens, ciliary body, iris, corneal epithelium and endothelium, and in several zones in the developing retina. (×32.)
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The optic cup shows three main zones of precipitate. The outermost periphery of the inner layer of the optic cup contains formazan precipitate equally as dense as the outer layer. Next to this outer zone of dense precipitate is a large zone of much lighter precipitate. This intermediate zone, which extends to the developing ganglion cell and nerve fiber layers, has a light deposit of formazan precipitate, except for a slight increase in the density of the deposit in the developing ganglion cell layer.

Fig. 4. A section of the retina of the 17-day-old rat embryo demonstrating TPN diaphorase activity in discrete zones. PE, developing pigment epithelium from the outer layer of the optic cup. R, adjacent area of the inner layer of the optic cup. (×125.)

Fig. 5. Demonstration of succinic dehydrogenase activity in the developing ciliary body (CB), lens epithelium (L), iris, and corneal epithelium (Ep) of a newborn rat. Precipitate is also present in the epithelium of the eyelid. (×125.)
density of the precipitate toward the outermost part of the zone. The innermost zone of precipitate, the area which corresponds in location to the developing ganglion cell and nerve fiber layer of the retina, has numerous local areas of dense precipitation.

The posterior epithelium of the developing ciliary body and iris, the part that is continuous with the retina and is of ectodermal origin, shows a dense precipitate in contrast to the stroma of the iris and ciliary body which is of mesodermal origin and has a much lighter deposition of formazan.

The lens shows a dense zone of precipitate along its entire surface. A light uniform precipitate is present in the remainder of the lens.

The corneal epithelium shows a dense deposit of formazan. The rest of the cornea shows a light uniform granular precipitate. The corneal epithelium does not show as dense a precipitate in the demonstration of TPN diaphorase as with the other enzyme stains. There is, however, a sufficient increase in the intensity of the precipitate in the epithelium to distinguish it easily from the remainder of the cornea.

Newborn rat. At birth, the retina of the rat is still poorly differentiated in hematoxylin and eosin section. The internal and external nuclear layers are not yet well defined and appear as a single broad zone of nuclei. The ganglion cell layer is separated from the broad nuclear zone by a narrow molecular layer. There is no evidence of any differentiation of the visual cells. The cornea, ciliary body, and lens can be easily identified.

The histochemical patterns present in the retina, cornea, and lens are identical with those of the 17 day embryo. Although this rat is 5 days older than the 17 day embryo, no difference can be detected histochemically (Fig. 5).

Seven-day-old rat. The eye of the post-partum 7-day-old rat, with the exception of the retina, appears well differentiated in hematoxylin and eosin section. The retina is, except for the photoreceptors, almost completely developed morphologically. The external and internal nuclear layers are completely separated by a definite external molecular layer. Very few mitotic figures are found, and these are present at the ora. The rods are very short and, at this stage of development, are only 3 \( \mu \) long at the fundus and 1.5 \( \mu \) long at the ora.12

The histochemical distribution of formazan precipitate in the retina, ciliary body, iris, lens, and cornea is essentially the same for each of the five enzyme systems tested.

The retina shows several distinct zones of precipitate. The pigment epithelium and adjacent retina, the area of the developing rods, appear as two intense adjoining bands of precipitate. A faint, diffuse granular distribution of formazan is present throughout both nuclear layers, with a narrow zone of increased density of formazan in the developing outer plexiform layer. This latter area is most readily demonstrated by the TPN diaphorase preparation. The innermost layers of the retina, the developing inner plexiform, ganglion cell, and nerve fiber layers, show dense precipitation. There is some variation in the amount of precipitate present in the region of the ganglion cells. Heavy deposits of formazan are present in the ganglion cell layer in a distribution similar to the site of the ganglion cell nuclei in the DPN diaphorase and malic and lactic dehydrogenase preparations (Fig. 6). In the demonstration of TPN diaphorase and succinic dehydrogenase, these same areas in the ganglion cell layer, the sites of the nuclei of the ganglion cells, are in marked contrast to the adjacent areas of the retina by their lack of precipitate. This is most marked in the demonstration of TPN diaphorase (Fig. 7).

The ciliary body and iris show a large amount of activity with each of the five enzymes tested. In each of the histochemical demonstrations, the pattern of formazan precipitation is similar. The epithelium of the ciliary body and iris shows a much more intense precipitate than the more anterior parts derived from mesoderm (Fig. 8).
Most of the enzyme activity that can be demonstrated in the lens is present in the epithelium just under the lens capsule. The lens capsule shows a thin but definite band of formazan precipitate. A small amount of diffuse granular precipitate is present throughout the lens stroma.

The cornea shows a similar pattern with each of the enzymes demonstrated. The epithelium and endothelium are most ac-

Fig. 6. DPN diaphorase activity in the seventh postpartum day rat retina. Dense precipitate is present in the area of the pigment epithelium (PE) and immature photoreceptors (Ph). Note large clumps of precipitate in the ganglion cell layer (arrow). (x125.)

Fig. 7. TPN diaphorase activity in the seventh postpartum day rat retina. Note the absence of large clumps of precipitate in the ganglion cell layer in contrast to Fig. 6. (x125.)
tive. A slight amount of precipitate is present in the corneal stroma in a lamellar pattern (Fig. 9).

**Ten-day-old rat.** The eye of the 10-day-old rat, as seen in hematoxylin and eosin section, has almost entirely finished its morphologic development. Except for the final differentiation of the rods, the retina is adult in histologic appearance. Rods, at this stage, are 5 to 6 µ long and are just be-

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**Fig. 8.** Part of the anterior pole of the eye of a seventh day postpartum rat eye incubated with lactate and DPN. Precipitate is present in the ciliary body (CB), iris (I), corneal epithelium (Ep) and endothelium (En), lens epithelium (LE), and lens capsule (LC). (×32.)

**Fig. 9.** Cornea of a seventh day postpartum rat eye incubated with lactate and DPN. Precipitate is present in the epithelium (Ep), endothelium (En), and scattered throughout the stroma in a linear lamellar pattern. (×125.)
gining the period of rapid elongation to complete their development. Inner and outer segments of rods can be discerned. At this stage of development, the histochemical patterns of the enzymes studied are very similar to the adult patterns. The patterns of formazan precipitation are the same for each of the enzymes and will be described together.

In the retina, the distribution of precipitate at the posterior pole more nearly resembles the adult than does the region near the ora. The precipitate is densest in the pigment epithelium, the area of the developing rods, the outer and inner plexiform layers, the ganglion cell, and nerve fiber layers. An evenly distributed granular deposit of precipitate is present in the inner and outer nuclear layers, the outer nuclear layer having a somewhat lighter over-all deposit of precipitate. There are areas of clumps of precipitate evenly distributed in the ganglion cell layer in the approximate location of the cell nuclei in that layer. This pattern is present with all the enzymes studied, including TPN diaphorase (Fig. 10).

The iris and ciliary body show a pattern similar to that of the adult, with a much denser precipitate present in the epithelium.

The lens has a dense precipitate along the anterior epithelium with a thin band of precipitate along the capsule. The posterior epithelium and capsule show much less precipitation of formazan, but more than the lens stroma. The lens stroma has a very light diffuse granular precipitate throughout.

The histochemical pattern of the cornea is identical to that of the adult. The epithelium and endothelium show a dense precipitate. The corneal stroma contains areas of light precipitate arranged in a linear and lamellar pattern.

**Fourteen-day-old rat.** As seen in the hematoxylin and eosin section, the eye of the 14-day-old rat is nearing the completion of its development. The rods are rapidly increasing in length and are almost adult in size. The remainder of the eye has reached maturation morphologically.

The histochemical patterns of the iris, ciliary body, lens, and cornea are identical to the patterns of the fully matured adult.

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![Fig. 10. Demonstration of TPN diaphorase in the 10-day-old postpartum rat retina. Note the decreased amount of precipitate in the developing rods (R) and the presence of clumps of precipitate in the ganglion cell layer (arrow). Precipitate is also present in the pigment epithelium (PE) and choroid (Ch). (x125).](https://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/933629/)
The retina shows a basic pattern similar to the adult eye, but not identical to it.

In the retina, the patterns of formazan precipitate are similar in the demonstrations for lactic and malic dehydrogenase and DPN diaphorase. In these preparations, a dense precipitate is present in the pigment epithelium, the ellipsoids of the rods, the outer and inner plexiform layers, the ganglion cell, and nerve fiber layers. A small amount of diffuse granular precipitate is present in the outer nuclear layer with a larger amount of diffusely distributed granular precipitate in the inner nuclear layer.

In the demonstration of succinic dehydrogenase, the region of the outer limbs of the rods show a moderate density of precipitate. The remainder of the retina appears similar to the malic and lactic dehydrogenase and DPN diaphorase preparations.

The patterns of precipitate for TPN diaphorase differ from those produced by the other enzymes in that in the TPN diaphorase preparation the greatest density of precipitate is in the pigment epithelium, the outer plexiform, ganglion cell, and nerve fiber layers. The ellipsoids of the rods show only a moderate amount of precipitate. A diffuse granular precipitate is present in the outer and inner nuclear layers. The densest amount of precipitation is present in the nerve fiber layer adjacent to the vitreous.

The ciliary body and iris are very active. The posterior portions have a denser precipitate than the anterior portions in each of the preparations.

The cornea of the 14-day-old rat resembles that of the 10-day-old rat. The epithelium and endothelium show more activity than the stroma. The same linear lamellar pattern of precipitate is present in the stroma.

The anterior lens epithelium and capsule show a heavier precipitate than the posterior, and the lens appears similar to that of the 10-day-old rat.

Twenty-one-day-old rat. The 21-day-old rat, as seen in hematoxylin and eosin section, has finished its morphologic maturation. The rods, which are the last segments of the retina to complete their maturation, have reached adult length.

The histochemical patterns for lactic, malic, and succinic dehydrogenase are entirely adult at this stage of development. In the retina there is dense formazan precipitate in the pigment epithelium, the ellipsoids of the rods (except for TPN diaphorase), the outer plexiform, ganglion cell, and nerve fiber layers. There is much less precipitate in the outer nuclear layer than in the 14-day-old retina. No precipitate can be detected in the outer segments of the rods in the preparations for lactic and malic dehydrogenase and DPN diaphorase. In the succinic dehydrogenase preparation, precipitate is present in the outer segments...
of the rods in this retina as in the 14-day-old retina.

The TPN diaphorase preparation shows precipitate most densely in the pigment epithelium, the outer plexiform, ganglion cell, and nerve fiber layers. The ellipsoids of the rods show moderate activity. Most striking, however, is the demonstration of dense precipitate in Mueller's fibers which, even in a 6 \( \mu \) section, can be traced from the inner limiting membrane almost to the level of the outer limiting membrane (Fig. 11).

The histochemical patterns of the ciliary body, iris, cornea, and lens are identical to the patterns of these structures in the 14-day-old eye.

Discussion

The eye of the 13-day-old embryo, the youngest rat studied, shows significant histochemical activity. In the retina, the greatest density of precipitate is present in the regions that will become the pigment epithelium and the photoreceptors, although at this stage only undifferentiated epithelium is present. Even though sharp differentiation of the layers is obvious in the enzyme sections, it is significant that the delineation of the photoreceptors and pigment epithelium cannot be demonstrated with hematoxylin and eosin or other standard histologic stains. Therefore, enzyme preparations are a useful adjunct to methods used in study of ocular embryology.

As the development of the retina is observed in later stages, the histochemical pattern remains the same in the region of the photoreceptors and the pigment epithelium. In the 7-day-old rat retina, the rods can be identified, and, although the rods are very short, the formazan precipitate can be seen to be mainly in the rods. It is significant that this histochemical pattern is obvious several days before morphologic differentiation of the retina can be detected by routine histologic stains. The localization of enzyme activity in this part of the retina, therefore, is determined very early in development.

The differentiation of the retina begins at the inner surface of the optic cup at the posterior pole and spreads gradually outward and toward the ora. The development of the adult histochemical pattern correlates well with this scheme of differentiation. As the inner layers of the retina develop, they show increased density of precipitate and approach the adult pattern.

Mueller's fibers, although not demonstrated histochemically in their entirety until the twenty-first postpartum day, show, in the 14-day retina, precipitate in the area of the ganglion cell and nerve fiber layer.

In the TPN diaphorase demonstration in the retina, the ellipsoids of the rods show progressively less activity as the rods develop. In the seventh day postpartum retina, the precipitate in the ellipsoids in the TPN diaphorase preparation appears as dense as that of DPN diaphorase and the other enzymes studied. A gradual progressive disappearance of TPN diaphorase activity in this area is observed beginning with the 10-day postpartum retina. This was in marked contrast to the other four enzymes which continued to show much activity in this region.

TPN diaphorase activity in the inner layers of the retina appears different from the other enzymes. Areas in the ganglion cell layer in the seventh postpartum day retina were devoid of formazan precipitate. However, these identical areas show dense precipitate with the other 4 enzymes. In the 10-day-old retina, the next stage studied, a dense precipitate is present in this area in all the enzyme preparations. This suggests that the cells, as they mature, may acquire or develop enzyme activity at different rates or at different stages of development, and that the eye undergoes an enzymatic differentiation in addition to a morphologic one.

The similarity of localization of the enzymes demonstrated in this study is not unexpected. As was pointed out by Farber and co-workers and Nachlas and associates, the specific DPN- and TPN-dependent dehydrogenases cannot be selec-
tively demonstrated histochemically since the precipitate demonstrates the site of the dehydrogenase with the accompanying diaphorase. The present study demonstrates that malic and lactic dehydrogenase are both present in areas that contain DPN diaphorase. The localization of succinic dehydrogenase is not dependent upon a diaphorase if phenazine methosulfate is added to the system, and therefore the pattern produced is specific for succinic dehydrogenase. The similarity of the succinic dehydrogenase pattern to those of the DPN-dependent enzymes indicates that there are areas of the morphologically undifferentiated rat retina which are already differentiated enzymatically.

In the present study, considerable lactic dehydrogenase activity was found in the 10-day-old rat retina and earlier, whereas Pearse, using a basically similar technique, reported a lack of significant activity in a 10-day-old postpartum specimen. Pearse, however, did report a large amount of lactic dehydrogenase activity in his 13-day-old specimen, a finding that is in agreement with the results reported here.

The cornea shows the adult histochemical pattern early in the development of the rat eye. The epithelium, endothelium, and stroma have precipitate localization characteristic of the adult at 7 days of age post partum.

The lens, during its early development, shows enzyme activity along its periphery. The actively proliferating lens epithelium shows the greatest density of formazan. As the lens matures, most of the precipitate is noted in the anterior epithelium, indicating that most of the enzyme activity is present there. A recent report by Becker and Cotlier demonstrates active transport of tagged rubidium by the lens epithelium, as manifest by the increased concentration of the tagged cation in that part of the lens stroma closest to the epithelium. This data correlate well with the histochemical demonstration of greatest enzyme activity in the lens epithelium.

The iris and ciliary body demonstrate the adult histochemical pattern at 10 days of age post partum, several days before the rat's eyes are open. In the development of these structures, the parts derived from ectoderm and mesoderm can be detected by differential density of formazan precipitation.

The authors are indebted to Mrs. Delores Rytel for the excellent preparation of the sections used in this study.

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