Quantitative determination of choline acetylase, acetylcholine, and acetylcholinesterase in the developing rabbit cornea

Rufus O. Howard, William S. Wilson, and Brendan J. Dunn

Components of the cholinergic nervous system, acetylcholine (ACh), choline acetyltransferase (ChAc), and acetylcholinesterase (AChE) have been measured quantitatively in the developing rabbit cornea. ACh was detected initially approximately 10 days after birth, and subsequently increased in a nonlinear fashion to attain adult levels approximately 50 days after birth. ChAc was found initially about 12 days after birth, and increased in a linear fashion to reach adult levels approximately 56 days after birth. AChE activity was greater than adult levels just after birth, increased further to a peak activity 3 to 10 days after birth, then decreased to adult levels about 25 days after birth. The patterns of development of these three components in the rabbit cornea differ from that in the rabbit nervous system. It is inferred that a significant part of the corneal cholinergic components is unrelated to corneal innervation.

Key words: acetylcholine, choline acetyltransferase, acetylcholinesterase, developmental patterns, rabbit cornea, quantitative assay.

The mammalian cornea contains a very high concentration of acetylcholine (ACh), and correspondingly high activities of choline acetyltransferase (ChAc), the enzyme responsible for its synthesis, and acetylcholinesterase (AChE), the enzyme which hydrolyzes and inactivates ACh. Most of the ACh, ChAc, and AChE are concentrated in the epithelium where the contents are comparable to those found in the autonomic ganglia or central nervous system (CNS) of the same species. In the mammalian and avian species, ACh, ChAc, and AChE appear to be associated principally with nervous tissue. However, while nerve processes have been demonstrated in the corneal epithelium, they are found infrequently, and constitute a minor portion of the epithelial layer. Thus, unusually high levels of ACh, ChAc, and AChE would be required if these high concentrations were to be attributed only to cho-
linerigic nerve endings in the epithelium.

Using electron microscopy (EM) histochecmistry we have shown dense deposits of reaction product, indicating the presence of AChE, on the corneal epithelial cell surface; lesser staining occurs within epithelial cells. With EM histochemical techniques, nerves in the corneal stroma also stained for AChE, but nerve endings in the corneal epithelium were not identified. This EM study demonstrates a significant portion of AChE in the corneal epithelium is not directly related to nerve endings.

If the cornea is denervated by trigeminal nerve section or corneal limbal trephination, most but not all of the ACh and AChE disappear. It is difficult, if not impossible, to explain all the known information concerning corneal cholinergic components on the basis of a single function, namely mediation of sensory impulses, and only indirect evidence supports this interpretation.

The present study was undertaken to measure quantitatively ACh, ChAc, and AChE in the mammalian (rabbit) cornea, as a function of age, in the hope of clarifying the role of the cholinergic system in the cornea.

Methods

Adult albino female New Zealand rabbits and their progeny were used in all studies. They were killed with sodium pentobarbital administered through the marginal ear vein or by intracardiac injection.

The eyelids are fused in the fetal and newborn rabbits for approximately the first 12 days of life. So, for these animals, it was necessary to excise the eyelids carefully to avoid damaging the corneal epithelium. In most experiments, the corneal epithelium was removed by gently scraping the intact eye with a sharp scalpel. Care was taken not to mix corneal and conjunctival epithelium.

Acetylcholine. Corneal tissue for acetylcholine assay was placed directly into cold 5 per cent trichloracetic acid (TCA) in a microhomogenizer, weighed quickly, and homogenized, to make a suspension containing approximately 20 mg. tissue per milliliter TCA. The extract was centrifuged, the supernatant extracted with ether, adjusted to pH 6 to 7 with NaHCO₃, and stored at 20° C. until assayed for ACh. The precipitate was dissolved in 1 N NaOH, and protein estimated by the Lowry method.

ACh was determined by bioassay on the dorsal muscle of the leech as previously described.

Choline acetyltransferase. Tissue for ChAc assay was placed directly in a ground-glass microhomonogizer and weighed. The sample was ground in 50 mM. Tris (trishydroxymethylamino microhomonogizer) buffer (pH 7.4) containing 150 mM. NaCl, at a concentration of approximately 20 mg. tissue per milliliter buffer. The tissue was then dispersed more finely with a Hamilton microsyringe, and aliquots taken for ChAc and protein assay.

ChAc activity was determined by a modification of the radiometric method of Schrier and Schuster, in which labeled ACh is synthesized by incubating [acetyl-14C] CoA with choline in the presence of the enzyme. In the present work, an attempt was made to activate any possible membrane-occluded ChAc by adding two drops of ether to the incubation medium. Each value of ChAc reported represents the mean of at least three determinations.

Acetylcholinesterase. Corneal tissue for AChE assay was homogenized immediately in 0.1 M. phosphate buffer.

AChE activity was determined by the rapid colorimetric method of Ellman and co-workers. The assay is based on the hydrolysis of acetylthiocholine by AChE: the released thiol reacts with 5,5 dithiobis-2-nitrobenzoate (DTNB) to produce a yellow color, which is measured at 412 nm. A Cary Model 16 spectrophotometer recorded changes in absorption at 27° C. Separate control experiments were performed without substrate and without enzyme. In some experiments, enzyme was inhibited with 10⁻⁵M. eserine or 10⁻⁶M. diisopropylfluorophosphate (DFP). Each assay was followed for 10 to 20 minutes, and a value representing (absorbance change per minute per milligram protein) was calculated.

Absolute enzyme activity of the individual samples was calculated from standard curves in which the rate of hydrolysis of acetylthiocholine was measured for known concentrations of bovine erythrocyte esterase (true ChE, Sigma Chemical Co., St. Louis, Mo.), and horse serum esterase (pseudo-ChE, Sigma).

For histochemical determination of ChE activity, corneal tissue was embedded in Tissue Tek OCT compound at -30° C. Ten to fourteen micron thick sections of whole cornea were cut with a cryostat microtome, and were stained for true and pseudo ChE using a modification of the Koelle-Friedenwald histochemical technique.

Control experiments were run omitting the acetyl or butyryl thiocholine substrate. Pseudocholinesterase was inhibited with 10⁻⁵M. DFP and both enzymes were inhibited with 10⁻⁶M. eserine.
Table I. ACh content, ChAc activity, and AChE activity of the corneal epithelium from 6 mother rabbits and 51 young rabbits of various ages

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<th>Family</th>
<th>Code</th>
<th>Age (days)</th>
<th>No. of animals pooled</th>
<th>(ACh) (pmoles/mg. protein)</th>
<th>ChAc (nmoles ACh formed/hr./mg. protein)</th>
<th>AChE (units of AChE activity/mg. protein)</th>
<th>Per cent of value for mother</th>
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Results

The adult rabbit cornea is differentiated into a well-defined epithelium, a relatively acellular stroma, Descemet's layer, and endothelium. In contrast, at the time of late fetal life and the first few days after birth, the stroma is highly cellular. The corneal epithelium alone can be scraped directly and reproducibly from the adult eye. However, when the superficial cornea of a fetal or neonatal rabbit was removed by a similar technique, variable amounts of a highly cellular superficial stroma were removed. This was apparent by the variable sample weights. Histologic slides of the cornea indicated epithelium alone could be removed at eight days after birth, and thereafter. Consequently, the results in this report refer to epithelium alone for the animals whose age was greater than 8 to 10 days after birth, while at earlier ages, the results refer to epithelium plus a variable amount of a highly cellular superficial stroma.

Corneal tissue taken from one eye of a rabbit was used for ACh assay. The tissue sample from the contralateral eye was used for ChAc activity; any remaining sample was examined for AChE activity.

The comparison of the ACh value from one corneal epithelium with the ChAc activity from the other cornea of the same animal was assumed to be valid since it has been shown that there is no significant difference between the corneal ACh levels of the left and right eyes (in rabbits).

The newborn and young rabbit eye is very small, and therefore it was sometimes necessary to pool samples for ACh, ChAc, and AChE assay.
**ACh content.** The ACh content of the superficial cornea is listed in Table I as a function of the age of the rabbit.

A large variation in the ACh content was observed among the different families studied. This could be explained by the variability in sampling of the superficial cornea; however, variability was also present in the older animals; and a hereditary variation in corneal ACh content may exist. This variation was reduced in Table I by expressing ACh content as a percentage of the ACh content of the mother's corneal epithelium.

In a few preliminary experiments, attempts were made to assay ACh in the whole cornea of 30-day rabbit embryos. The amount of ACh in embryonic cornea and in the first few days after birth was too low to measure by this technique. At approximately 10 days after birth, ACh can be demonstrated regularly, and thereafter the amount of ACh in the superficial cornea increases, in a nonlinear fashion, to adult levels approximately 50 days after birth.

The data fit a nonlinear curve of the form:

\[ \text{Age in days} = 1.4 + 24.2 \log (\% \text{ ACh}) \]

Pearson's correlation coefficient \( r \) was 0.72.

**ChAc activity.** The ChAc activity in the superficial corneal tissue is shown in Table I as a function of the age of the rabbit. As with the ACh content, a considerable variation was found in the ChAc activity among the different families studied.

In preliminary experiments on whole embryonic cornea the ChAc activity was below the limit for the assay (about 0.1 nmol. per hour per milligram protein). ChAc activity remained too low to measure until the animals were a few days old. In Fig. 2, ChAc activity shows a linear increase with age. The intercept on the horizontal axis indicates that ChAc activity first appears in the superficial rabbit cornea approximately 12 days after birth. Adult levels on enzyme activity are attained approximately 56 days after birth.

The data fit a linear curve of the form:

\[ \text{Age in days} = 12.6 + 0.44 (\% \text{ ChAc activity}) \]

Pearson's coefficient of correlation \( r \) was 0.89.

**Relation of ACh to ChAc.** In Fig. 3, the ACh content of superficial corneal tissue of rabbits of different ages is compared to the ChAc activity in the same individual animals. At low ACh content and low ChAc activity, the relationship is approximately linear; however, as ChAc activity increases, ACh content appears to increase more rapidly. The data can be expressed by the curve:
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4000 ACh CONTENT vs ChAc ACTIVITY
rabbit corneal epithelium

Fig. 3. The relationship between ACh and ChAc in the rabbit is not linear. The adult cornea has the ability to synthesize large quantities of ACh.

\[ \log(\text{ACh}) = 2.05 + 0.0114 \times (\text{ChAc activity}) \]

Pearson’s correlation coefficient (r) was 0.72.

**AChE activity.** AChE activity in Table I was measured on any residual sample of superficial cornea used for ACh and ChAc assay. Thus, all results for ACh, ChAc, and AChE in Table I refer to corneal tissue from the same animals.

Limited information was available from the above experiments, so additional experiments were performed. As with ACh and ChAc, a considerable variation in AChE activity was measured in the different families studied.

In the youngest litter studied, 28 days after mating, a deliberate attempt was made to obtain a sample of epithelium plus a variable amount of stroma. After the superficial cornea was removed, the eyes were placed in buffered formalin, and histologic slides of the remaining corneas were prepared and examined. In general, the samples of lower weight represented more superficial corneal cells and had a higher activity per unit weight than the larger samples which contained more stromal cells.

The AChE activity is plotted in Fig. 4 as a function of the age of the rabbit. Each point represents one animal. The AChE activity is high in the rabbit superficial cornea just before birth, and increases after birth to a peak approximately 3 to 10 days after birth. The activity then decreases, leveling off at the adult value around 25 days after birth.

**AChE localization in the cornea.** Histochernical staining of the cornea for true ChE by light microscopy was similar at all ages. Enzyme staining was not identified in the endothelium. True ChE staining was identified in the stroma only in the corneal nerves, which were present between the midstroma and basal epithelium. Nerves were identified in the stroma at all ages examined, from 20 days gestation to adult. Focal enzyme staining was sometimes seen in the region of the basal epithelium. Staining for true ChE was constantly demonstrated in the epithelium. The appearance of the staining in the epithelium, however, was different from that in the stroma: a characteristic linear, beaded staining was present in all layers between the basal and surface cells, both centrally and near the limbus.

**Pseudo ChE staining** was also present in the stromal nerves between the midstroma and the basal epithelium. In the adult rabbit corneas, pseudo ChE was observed rarely, if at all, in the corneal epithelium. However, in eyes removed just before or after birth, fine linear staining was present in the epithelium. Focal staining was present in the subepithelial region at all ages examined. Pseudo ChE was not identified in the endothelium at any age. These histochemical observations are compatible with quantitative measurements of ChE activity; pseudo ChE activity was present in embryonic corneal epithelium, diminished in neonatal epithelium, and consistently absent in adult epithelium.

Relationship of AChE to ACh and ChAc.
AChE was present in high concentration before significant quantities of either ACh or ChAc could be measured in the developing rabbit cornea. A peak in AChE activity occurred just prior to the time when significant amounts of ACh and ChAc can be measured. The relationship between AChE and either ACh or ChAc cannot be expressed by any simple curve.

**Development of the corneal reflex.** Corneal sensation was present consistently 24 to 48 hours after the eyelids opened spontaneously; that is, about 12 days after birth. The rabbit eyelids closed when the cornea was touched with a wisp of cotton.

**Discussion**

The activity of the enzymes AChE and ChAc increases during the development of the cholinergic system in the CNS of amphibians, birds, and mammals. The cerebrum of the rabbit is relatively undeveloped at birth. Patterns of development of ChAc and AChE have been characterized in the rabbit cerebrum and cerebellum.\(^{13-15}\) ChAc activity is demonstrated initially in the rabbit cerebrum by embryo day 18 (approximately midgestation). The ChAc activity increases by birth to one-quarter adult level and attains the adult level approximately 60 days following birth (Fig. 5). In the rabbit cerebellum, activity is detected initially at embryo age 21 days. A peak of activity, approximately three times adult level, occurs 14 to 16 days later, and drops to adult level 30 days after birth.

While AChE activity has been demonstrated with EM histochemistry as early as the eleventh embryonic day in the rabbit hypothalamus,\(^{10}\) AChE in the CNS is low at birth and increases to high values during the first three weeks after birth, concomitantly with the morphologic maturation of the cortical centers (Fig. 6). The rabbit electroencephalogram attains the adult pattern within three weeks of birth.

The patterns of AChE and ChAc enzyme development in the rabbit CNS are different from those demonstrated in the rabbit cornea in this study. It is possible to
interpret these findings as representing a nonneural function for a significant portion of the AChE and ChAc in the corneal epithelium.

Observations in bovine tissue\textsuperscript{17} indicate that here, too, ACh develops in the corneal epithelium sometime after the eye seems to be relatively mature; at 2 to 3 weeks after birth, ACh is still only 25 per cent of the adult level.

Thus, it is clear that the bulk of the ACh and ChAc in the rabbit cornea is absent until about the time the eyelids open (10 to 12 days after birth), i.e., an appreciable time after the sensory nerves and AChE have appeared in the tissue, and after corneal sensation can be demonstrated. Although nerves were identified in the corneal stroma at 20 days gestation in the rabbit, the age at which they first appear in the epithelium was not determined. The corneal cholinergic system may have a role in the mediation of sensory impulses in the corneal epithelium. This interpretation has been made because most of the ACh and AChE disappear from the cornea following denervation; and, because corneal sensitivity decreases following treatment of the cornea with hemicholinium,\textsuperscript{4} a drug which inhibits the synthesis of ACh. The present data suggest, however, that a significant portion of the cholinergic system does not subserve such a role.

It will be recalled that the newborn rabbit eyelids are closed. Approximately 12 days following birth, the eyelids separate and the cornea is exposed to a new environment. Because of the striking changes that occur in the levels of ACh, ChAc, and AChE, related in time to the opening of the eyelids, it is possible that part of the ACh system in the superficial cornea represents some adaptive developmental response of the cornea to the new environments of the atmosphere and light.

REFERENCES


