A Histochemical Study of Cholinesterase Activity in Rabbit's Retinae

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ABSTRACT

In the present study the specific and nonspecific cholinesterase activities of the rabbit's retinae in the fetus, the neonatal, the light-isolated, and the reopened groups, which consisted of 65 healthy young rabbits, weighing about 300 to 500 gm, 33 rabbit's fetuses, and neonatal rabbits, were histochemically observed by means of the cholinesterase method recommended by Gerebtzoff (1953) and the embedding and sectioning method presented by Koele and Friedenwald (1950). Cholinesterase activity of the retinae in the 15 days fetuses was not present but began to develop in the 20 days fetuses. In the 1 week group after suturing the eyelids, the most remarkable activity of specific and nonspecific cholinesterase was observed in the posterior polar area. The nearer to the peripheral area of the retina the weaker the enzymatic activities became. In the 2 weeks group after suturing eyelids, the enzymatic activity was reduced. In the 4, and 8 weeks groups after suturing the eyelids, the enzymatic activities were remarkably reduced. In the 14 days after reopening eyelide, which group has previously been kept under the condition of light isolation for 4 weeks, enzymatic activities were fairly recovered and compared with the normal control group. Consequently it is histochemically deduced that the gradual change of specific cholinesterase activities in the rabbit's retinae was closely related to the visual function.

INTRODUCTION

Since early in the 19th century, much research on the relation between acetylcholine and nervous stimulation has been reported. Loewi (1913) reported that a stimulating factor in vagal transmission was found at the synapses and which was similar to acetylcholine in its physiological activity. Frank (1923) indicated that in all synapses of the autonomic system, the mediator stimulating involving acetylcholine was closely related with the nervous conduction. Brinkman and Van Dam (1922) found that following an experimental stimulation of the vagus nerves induced to secrete acetylcholine which appeared to be included in the irrigated fluid.

Engelhart (1931) noted that, when pupil narrowing occurred with strong light, considerable amounts of acetylcholine-like substance appeared in the aqueous humor of eye chambers.

Dale (1934) confirmed the fact that the activities of acetylcholine was suppressed by esterases, hydrolyzing enzymes of the ester. The existence of the high cholinesterase activity in the nerve synapses of skeletal muscles in amphibias, reptiles, and mammals has been biochemically demonstrated by Marnary and Nachmansohn (1938). They explained the reason why
these skeletal muscles having motor end plates or other nerve ending should show more strong cholinesterase activity than skeletal muscles without the nerve ending.

Since Gomori (1948) introduced a new method for detection of specific cholinesterase from nonspecific cholinesterase in the various sites (e.g., sympathetic ganglion, the brain). Koelle and Friedenwald (1949) designed a good method for the detection of cholinesterase using acetylthiocholine iodide or butyrylthiocholine iodide as a substrate in which the hydrolytic activities of specific or nonspecific cholinesterase in the brain, and autonomic ganglia could occur rapidly. Additionally Koelle (1955) developed more reliable histochemical method for acetylcholinesterase.

Recently much improvements in acetylcholinesterase determinations in mammalian tissues has been accomplished, especially those in the nervous system were notable. Hashimoto et al. (1963) reported that cholinesterase activities were demonstrated in the motor end plates, a number of sensory nerves and receptors, various noncornified circumferences of the capillaries, and nerve fiber plexus of the derma, sebaceous glands, and layers of the skin.

As mentioned above, a number of reports on the distribution of cholinesterase were made with subjects mainly related to the nervous and muscular systems, and the skin of vertebrates. Moreover, a few reports on cholinesterase activities of the retina of the eye were found. Koelle and Friedenwald (1950) reported that high cholinesterase activities appeared conspicuously in the inner and outer plexiform layers, and adjacent to the inner nuclear (or granular) layer in rats.

Pak and Choi (1964) indicated that there was a distinct difference of cholinesterase activity between neonatal rabbit's retinae whose palpebrae were still fused and young rabbit's which were able to see. On the other hand, the change of cholinesterase activity of various organs associated with the development of the mammalian fetus has been observed by several workers. Kupfer and Koelle (1951) compared the cholinesterase activity of forelimb muscles in rat fetuses with in neonatal ones. Engel (1961) studied cholinesterase activity by tissue culture of the skeletal muscle cells of the chick fetuses. Hashimoto, et al. (1963) compared and discussed cholinesterase activities demonstrated in various structures of the fetal skin with the adult ones.

However, a number of histochemical works on cholinesterase activities of various organs and tissues have been reported. But it is known that not only few histochemical reports on the retinae of the vertebrate are reported, but also practically nothing on the functional relations between the retinae and cholinesterase is to be found.

The present study attempts to observe 1) the normal localities of cholinesterase activity in normal retinae, of the rabbit's fetuses and neonatal, 2) the gradual enzymatic changes in the light-isolated animals, and 3) the gradual recovering status of the enzymatic activity in the reopened eyes.

**MATERIALS AND METHODS**

65 healthy young rabbits weighing 300 to 500 gm, 33 rabbit's fetuses, and 5 neonatal rabbits with closed eyes were used in this study. In order to observe cholinesterase activity of the retinae in the fetuses and neonatal, the rabbit's fetuses (15th, 17th, and 20th day of fetal age) obtained from the pregnant rabbits by mean of cesarean section, and neonatal were used respectively. In order to observe the gradual changes in the light-isolated ones, young rabbit's palpebrae (the upper and lower palpebrae) were bilaterally sutured by means of the continuous mattress suture, and then were kept in the dark for 1, 2, 4, and 8 weeks respectively. In order to observe the gradual recovering status, some of the above animals eyes were re-
opened by pulling out the silk, and then they were kept in a bright place for 3, 7, and 14 days respectively.

The animals were sacrificed by means of the intravenous injection of air. Immediately the enucleated eyeballs were opened. The excised retinæ were fixed in cold formalin-sucrose ammonia fluid (Pearson, 1963) for 24 hours and after rinsing in distilled water briefly, the retinal tissues were incubated at 37°C in the substrate containing acetylthiocholine iodide for 2 hours as recommended by Gerebtzoff (1953). Some of the material from the animals was incubated for 6 hours or 12 hours. After treatment of the pieces with 1% ammonium sulfide solution for 1~3 minutes, the retinal tissues were embedded in paraffin by the method of Koelle and Friedenwald (1950), and sectioned at 5 micra.

In order to differentiate the specificity of the cholinesterase activity, some of the retinal tissues were treated with $10^{-7}$ M or $10^{-8}$ M solutions of diisopropylfluorophosphate (DFP, an irreversible cholinesterase inhibitor) for 30 minutes before incubation. The typical dark brown pigmented substances which would prove cholinesterase sites were not observed in the cases treated with $10^{-7}$ M solution of DFP and with $10^{-8}$ M solution of eserine. The positive reaction of the cholinesterase of the retinae treated with $10^{-6}$ M solution of DFP was considerable as the specific cholinesterase. Some of the preparations were stained with hematoxylin for counterstain. All of the specimens were observed with the light microscope. In order to compare those enzymatic activities, the retina was conveniently divided in into three areas; the posterior polar, the equatorial and the peripheral areas.

**RESULTS**

**A. Cholinesterase Activity in Fetal and Neonatal Retinæ of Rabbits**

1. The 15-day fetus group:

   It was clear that the retinal structures were not fully differentiated within the 15-day fetuses. Incidentally, specific and nonspecific cholinesterase activities were not observed in this group.

2. The 17-day fetus group:

   The histological structures of the retinæ in this group were differentiated more than the former group. However, the activities of these enzymes were relatively similar to the former group.

3. The 20-day fetus group:

   The retinal structure was fairly differentiated; the ganglion cell layer and the inner plexiform layer of the retinae began to show slight cholinesterase activities.

4. The neonatal group:

   Although the neonatal rabbit's retinæ of the closed eyes could not exhibit their roles in the visual function, after 2 hours of incubation the nerve fiber layer of the retina showed a weak enzymatic activity and the ganglion cell layer a moderate one. The other retinal layers hardly showed the activity even in cases of prolonged incubation.

**B. Cholinesterase Activity in the Young Rabbits**

1. The control group of normal young rabbits:

   In the specific cholinesterase activity, the peripheral area near the ora serrata revealed a weak enzymatic reaction in general. However, the ganglion cell layer and the inner plexiform layer showed a weak and distinct reaction. In the equatorial area near the equatorial portion, the ganglion cell, inner plexiform, and inner nuclear layers showed marked activities. In the ganglion cell layer of the posterior polar area, a considerable enzymatic activity was observed and compared with the other layers. The cytoplasm of the ganglion cells of this posterior polar area showed the most strong activity, which was probably in the intracellular site, among those of the other retinal cells. Considerable specific cho-
linesterase activities were observed not only on the inside of the inner plexiform layer and the inner nuclear layer but also in the outer plexiform layer (Fig. 1).

The activity of the nonspecific cholinesterase throughout the whole three areas in this group was not considered as remarkable.

2. The 1 week group after suturing the eyelids:
In the peripheral area of the retina, the inner plexiform layer showed moderate enzymatic activity of the specific cholinesterase, and the ganglion cell layer slight activity. In the equatorial area, the ganglion cell layer was more marked than of the peripheral one. In the posterior polar area. The ganglion cell layer was the most remarkable in activity. Comparing with the normal retina, the enzymatic activities in this group were less than those of the normal retina. A wide distribution pattern of the enzymatic activity even to the outer plexiform layer was also seen in this group as in the normal retina.

3. The 2 weeks group after suturing the eyelids:
The specific cholinesterase activities of the retinal layers were fairly reduced compared with the 1 week group. But the three retinal layers were clearly distinguishable in all of the preparations.

4. The 4 weeks group after suturing the eyelids:
All of the retinal layers were distinguishable but the enzymatic activities of all of the layers was markedly reduced. Only several ganglion cells showed weak enzymatic reaction.

5. The 8 weeks group after suturing the eyelids:
All of the retinal layers were still distinguishable. Enzymatic activities in this group were similar to those of the former group. In the posterior polar area, the ganglion cell layer and the inside of the inner plexiform layer showed slight activity. Additionally nonspecific cholinesterase in these experimental group showed less significance throughout the whole retinal areas.

6. The 3 days group after reopening eyes:
In the peripheral area of the retina, the specific cholinesterase activity of the ganglion cell layer began to show recovery of the enzymatic activity and the outside of the inner nuclear layer showed weak activity. In the equatorial area, the activity of the ganglion cell layer and the inner nuclear layer was less than those of the peripheral areas. In the posterior polar area, the ganglion cell layer showed more enzymatic activity than that of the other retinal areas.

7. The 7 days group after reopening eyes:
In the retinal areas and layers, the cholinesterase activities had recovered and were similar to those of the normal retina.

8. The 14 days group after reopening eyes:
In the retinal areas and layers, the enzymatic activities were almost recovered and observed as seen in the normal retina (Fig. 3).

DISCUSSION

In the enzymatic activities of cholinesterase of the embryonic tissues, Youngstrom (1941) reported that the increase in cholinesterase concentration coincided with the development of functional activity and sharp rise in enzymatic activity followed in the central nervous system of the human fetuses. Kupfer and Koelle (1951) found that the cholinesterase activity was chiefly localized on the surface of the nuclei of the muscle fibers in the 16 days rabbit’s fetuses even though they saw no evidence for the establishment of motor endings within the muscle fibers which usually do not appear until the 21st to the 22nd day of the prenatal life and discussed the relationship between the 1st appearance of the muscular contraction and the enzymatic activity in the fetuses of albino rats. Shen et al. (1956) presented in the retinæ of the chick embryos, the early specific cholinesterase activity
was recognized at the 4th and 5th day of incubation especially in the ganglion cell layer and then extended to the outer layers of the retinae. The authors assessed that in the fetuses of rabbits the earliest cholinesterase activity was observed in the ganglion cell layer of the retinae at the 20th day of the pregnancy with the incubation time applied by us.

About the relationships between the function of the cholinesterase and the site of the enzyme, Dale (1934) and his co-workers suggested the theory of the chemical mechanism of motor nerve impulses to voluntary muscle and Brown et al. (1936) described that the acetylcholine liberated at the nerve endings must be removed in the very brief limits of the refractory period.

Marnary and Nachmansohn (1938) supported the works of Brown et al. (1936) by means of the biochemical hydrolysis of acetylcholine by the nerveless pelvic end of a frog’s sartorius muscle compared with that of the parts containing nerve endings in which 100 mg. of the nerve free ground muscle tissue split only 0.13 mg. of acetylcholine where as 100 mg. of the same muscle containing nerve endings split 0.4 to 0.8 mg., according to the richness in nerve endings. Nachmansohn (1930, 1939) reported that the acetylcholine system is responsible for the electric currents with propagate impulses in conducting tissue and believed that in nerve and skeletal muscle the Na-K mechanism depends on an underlying acetylcholinesterase mechanism. Shanes (1958) considered that most, if not all form of action potential passage along cell surface membrane, are due to a sodium-potassium mechanism. Petersen et al. (1965) discussed the possible significance of the ion transport by the acetylcholinesterase system for the high activity of acetylcholinesterase in the corneal epithelium (especially the basal layer) and the endothelium of the rabbits histochemically demonstrated. They also reported the biochemical data of cholinesterase contents of different ocular tissues which were 28 units for iris and ciliary body, 130 units for retina and 80~100 units for brain in the rabbit.

In the relationship between the function and histochemically demonstrable cholinesterase activity of the retina considering the high activity of cholinesterase content by the Petersen’s result, the histochemical localization of cholinesterase activity appeared to be greatest in the inner nuclear layer and the immediately adjacent portion of the plexiform layers of rabbit’s retina reported by Koelle and Friedenwald (1950), Pak and Choi (1964) studied the histochemical changes of cholinesterase of the neonatal and young rabbit’s retina during the early postnatal development. In the neonatal rabbit’s retinae that were probably unable to function because of fusion of both palpebrae, the weak enzymatic activity was mostly localized in the ganglion cell layer of the retina while in the young rabbits which were able to see the weak or moderate enzymatic activity was diversely distributed in the inner nuclear layer, inner plexiform layer, and the ganglion cells of the retina.

The authors also confirmed the developmental changes of the cholinesterase activity associated with the functional differentiation in the early neonatal and postnatal young rabbits presented by Pak and Choi (1964).

Additionally the authors observed the histochecmical decrease of the cholinesterase activity in the retina of the young rabbit that was light-isolated by means of surgical fusion of both palpebrae for 8 weeks and the recovery of the enzymatic activity in the retina in two weeks after reopening palpebrae.

REFERENCES

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Fig. 1. Normal young rabbit’s retina.
Posterior polar portion showed marked specific cholinesterase activity in the ganglion cell layer and inner nuclear layer. Gerbstoff’s recommended method without counterstain, ×100.

Fig. 2. Normal young rabbit’s retina.
Magnified picture of Fig. 1. The enzymatic activity in the ganglion cell and inner nuclear layers was evident, ×400.

Fig. 3. 4 weeks light-isolated young rabbit’s retina.
Posterior polar portion showed slight enzymatic activity in the ganglion cell layer. Gerbstoff’s recommended method without counterstain, ×100.

Fig. 4. 4 weeks light-isolated young rabbit’s retina.
Posterior polar portion of Fig. 3 in magnification. Reduced enzymatic activities were observed in the retinal layers, ×400.

Fig. 5. 2 weeks rabbit’s retina after reopened eyelids.
Posterior polar portion showed fairly recovered enzymatic activity in the ganglion cell, inner plexiform and nuclear layers. Gerbstoff’s recommended method without counterstain, ×100.

Fig. 6. 2 weeks rabbit’s retina after reopened eyelids.
Magnified picture of Fig. 5. Fairly recovered enzymatic activity was evident in the inner nuclear and ganglion cell layers, ×400.