Laboratory Sciences

Cholinesterase in the Lens

Joseph Michon, Jr., MD, and Jin H. Kinoshita, PhD, Boston

Recent reports implicating anticholinesterase agents in the production of lens opacities have led us to look for the target of these agents, that is, the enzyme cholinesterase, in the lens. A form of cholinesterase, sensitive to low concentrations of eserine, has been found. This is true cholinesterase as indicated by inhibition of enzymatic activity with high substrate concentrations and by the enzyme's hydrolysis of acetylthiocholine more rapidly than the longer chain propionyl or butyryl analogues. The enzyme is localized near the lens surface, either within or closely bound to the lens capsule. The physiologic role of the enzyme is unknown.

MIOTICS, especially of the anticholinesterase type, have been increasingly implicated in the production of lens opacities. Initial case reports came from Muller and associates in 1956[1] and from Harrison in 1960.[2] Recently, surveys by Axelsson and Holmberg,[3] de Roetth,[4,5] and Shaffer and Hetherington[6] have suggested that isoflurophate (Floropryl), demecarium bromide (Humorsol), and echothiophate iodide (Phospholine Iodide) may be cataractogenic.

Experimental studies in this area have been sparse. Diamant[7] produced transient anterior lenticular changes in guinea pigs with the intracarotid injection of anticholinesterase agents. Muller and associates[1] reported a decrease in oxygen consumption in lenses incubated with 10^{-6}M diethyl p-nitrophenyl phosphate (Mintacol) or 10^{-3}M pilocarpine, but uncovered no basis for this even after an exhaustive search.

In light of the foregoing work it was of interest to determine whether or not the target-enzyme of these pharmacologic agents occurs in the lens. These drugs are concerned primarily with the inhibition of a group of enzymes known as cholinesterases.

Cholinesterases effect the rapid hydrolytic destruction of choline esters and are of two types. Specific or true cholinesterase hydrolyzes acetylcholine more rapidly than longer-chain analogues. This enzyme is found primarily in neural tissues such as autonomic ganglia, parasympathetic effector sites, and neuromuscular junctions. Its role here is well defined; the rapid degradation of the neurotransmitter acetylcholine in preparation for the next nervous impulse. True cholinesterase also occurs in nonneural tissue such as the erythrocyte where its role is speculative.

The second type of the enzyme is designated as nonspecific or pseudocholinesterase. This is found in such tissue as pancreas and plasma, and has no known function.

This study investigates the presence, type, and distribution of a cholinesterase in the lens.

Methods

Chemicals.— Those used were acetylthiocholine iodide (AcTCh); propionylthiocholine iodide (PrTCh); butyrylthiocholine iodide (BuTCh); and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), prepared for use by dissolving 39.6

Submitted for publication Feb 1, 1967.

From the Massachusetts Eye and Ear Infirmary and Harvard Medical School (Dr. Michon), and Howe Laboratory, Harvard Medical School (Dr. Kinoshita), Boston.

Read before the Third Ophthalmic Biochemistry Conference, Woods Hole, Mass, June 18, 1966.

Reprint requests to 243 Charles St, Boston 02114 (Dr. Michon).

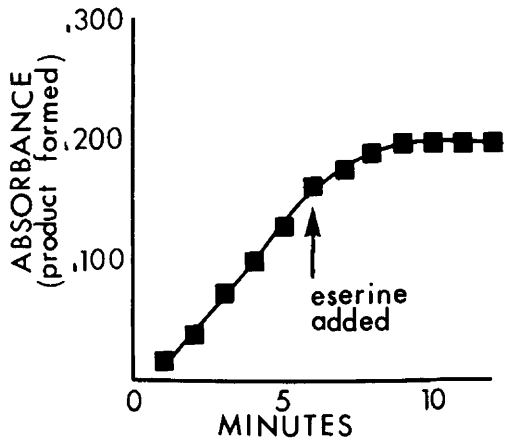


Fig 1.—Effect of 10^{-5}M eserine on lens esterase. Control, with buffer added, showed no inhibition.

mg in 10 ml of 0.1M phosphate buffer, pH 8, and adding 15 mg sodium bicarbonate.

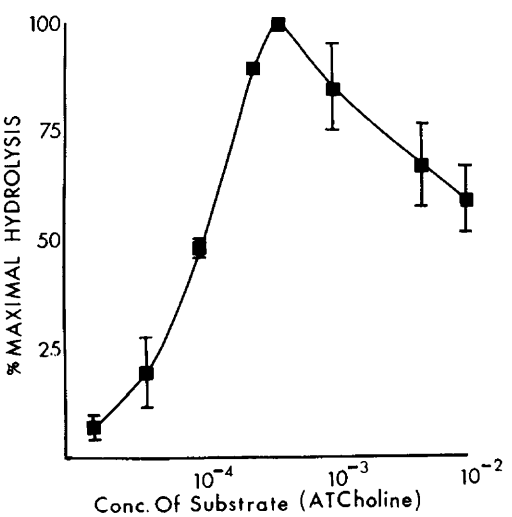
Method of Assay.—The photometric method of Ellman et al[8] was used for the in vitro assay of cholinesterase activity. This method measures the rate of hydrolysis of thiocholine esters by the enzyme. The thiocholine released reacts with DTNB to form the yellow anion, 5-thio-2-nitrobenzoic acid:

$$\text{Acetylthiocholine} \xrightarrow{\text{enzyme}} \text{thiocholine} + \text{acetate}$$

$$\text{Thiocholine} + \text{DTNB} \longrightarrow \text{5-thio-2-nitrobenzoic acid}$$

The rate of color production, in terms of absorbence per unit of time, is measured at 412μ in a spectrophotometer. Ellman et al[8] showed that the rate of hydrolysis of acetylthiocholine is not significantly different from that of acetylcholine.

Fig 2.—Effect of substrate concentration on rate of hydrolysis by lens cholinesterase. Brackets represent average deviation.



Calf eyes were obtained shortly after death of the animal; the lenses were removed and freed from adherent vitreous, iris, and zonules. The tissue was partitioned into surface, (capsule with adherent epithelium), cortex, and nucleus; and homogenized in 0.1M phosphate buffer, pH 8, (10%-20% weight/volume). The mixture was centrifuged at 13,400 × gravity for 15 minutes in a refrigerated centrifuge. The supernatant fluid containing the enzyme was dialyzed for 2 to 3 hours against 0.1M phosphate buffer. Dialysis was found necessary to remove sulfhydryl compounds which give a high blank reading in the assay system. The dialysate was quick-frozen in a mixture of ethanol and solid carbon dioxide, and stored at 0 C until time of assay. The activity of purified cholinesterase was unaltered when treated in a similar manner.

Mechanical scraping with a spatula was used to obtain preparations of capsule freed from adherent epithelium. The effectiveness of this method was ascertained by microscopic examination of preparations stained with methylene blue.

The reaction mixture usually contained 0.4 ml of the enzyme preparation; 0.2 ml of DTNB; 2.5 ml of phosphate buffer (0.1M; pH 8.0); and 0.1 ml of substrate, measured to give a final concentration of 0.6×10^{-3} M. The assay was made at room temperature. Optical density was recorded each minute and rate of hydrolysis calculated from the slope between 20 and 25 minutes. Controls consisted in a mixture lacking the enzyme and in a mixture lacking the substrate. An alternative control, often used, consisted in waiting until the endogenous tissue sulfhydryl had completely reacted with the DTNB and then resetting the photometer to 0.

Results

It was found that the lens does catalyze the hydrolysis of AcTCh (Table 1). Activity in terms of the whole lens is slight, 0.6 mg AcTCh hydrolyzed per hour per gram tissue. However, the enzyme is concentrated at the surface, that is, the capsule with adherent epithelium, and no activity is detected in the cortex and nucleus. When the activity is assayed in terms of the capsule and epithelium, a significant value of 27.5 mg AcTCh hydrolyzed per hour per gram of tissue is observed.

A characteristic of cholinesterases is their sensitivity to low concentrations of eserine,

Table 2.—*Distribution of Cholinesterase Activity at the Lens Surface

	Activity Per Lens	Activity Per mg Protein
Anterior capsule and epithelium	4.00 ± 1.02	8.60 ± 2.44
Anterior capsule	3.76 ± 1.28	15.70 ± 1.68
Posterior capsule	0.59 ± 0.21	

* Activity expressed as change in absorbance per min × 10^{-3}. Mean ± standard deviation listed.

and this fact is useful in distinguishing cholinesterase from other esterases.[9] It has been shown that eserine at 10^{-5}M causes complete inhibition of cholinesterases while being without effect on other tissue esterases. When added to the lens reaction mixture, eserine (10^{-5}M) effected complete inhibition of the AcTCh hydrolysis (Fig 1). This finding indicates that all of the activity uncovered in the lens is due to the action of cholinesterases.

The types of cholinesterase can be distinguished one from another by the use of selective inhibitors, selective substrates, or by differences in enzyme kinetics. Two types of study were done to determine the nature of the lens cholinesterase.

A distinguishing feature of true cholinesterase is that it is susceptible to substrate inhibition, while increasing substrate concentration does not affect pseudocholinesterase activity.[9] The cholinesterase preparation from lens capsule and epithelium does exhibit substrate inhibition. The activity reaches a maximum at 0.6 × 10^{-3}M of AcTCh and shows a definite decline with higher concentrations, suggestive of a true cholinesterase (Fig 2).

Substrate specificity is another property used to distinguish the two types of cholinesterase. True cholinesterase hydrolyzes AcTCh more rapidly than PrTCh; and PrTCh more rapidly than BuTCh. Pseudocholinesterase, on the other hand, hydrolyzes either PrTCh or BuTCh more rapidly than AcTCh.[10] The lens cholinesterase hydrolyzes AcTCh twice as rapidly as PrTCh, and ten times as rapidly as BuTCh (Fig 3). The results of these experiments indicate the lens enzyme to be a specific, or true cholinesterase similar to that observed in nervous tissue and erythrocytes.

In an effort further to define the location of true cholinesterase, the lens surface was partitioned into: (1) anterior capsule with adherent epithelium; (2) anterior capsule alone; and (3) posterior capsule. Each segment was then assayed separately for cholinesterase activity. Significant activity occurs in the preparation of anterior capsule with adherent epithelium (Table 2). Scraping away the bulk of the epithelium, yielding a preparation of pure anterior capsule, causes no loss of activity. In fact, when viewed in terms of activity per milligram of protein, activity actually increases with removal of the epithelium, suggesting a dilution effect by epithelial protein. The cholinesterase is either within the capsule or at least closely bound to it.

Table 1.—*Cholinesterase Activity in Calf Lens*

Lens Component	Activity*
Total lens	0.6 ± 0.1
Surface	27.5 ± 3.4
Cortex	0.0
Nucleus	0.0

* All activities expressed as mg acetylthiocholine hydrolyzed per hr per gm tissue. Mean ± standard deviation listed.

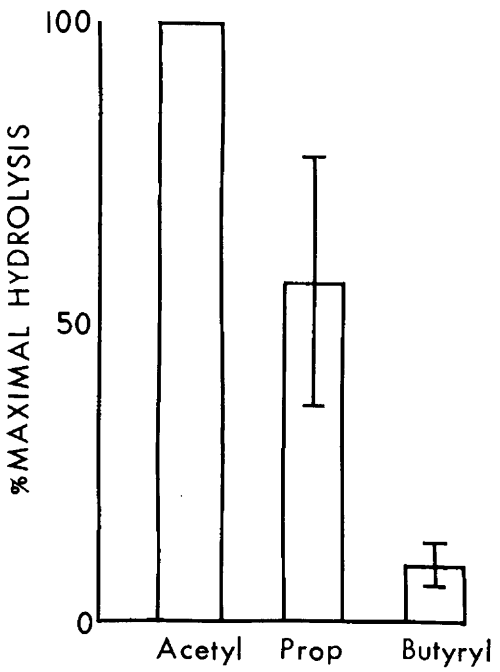


Fig 3.—Relative rates of hydrolysis of acetyl, propionyl, and butyryl esters of thiocholine. Bars represent standard deviation.

Cholinesterase can also be detected at the posterior capsule, though the relative significance of activity here cannot be stated because of difficulties in obtaining valid measurements here on a weight or protein basis.

Comment

This study demonstrates the presence of true cholinesterase in the capsule of the calf lens. This species was chosen for detailed study because of the ready availability of large numbers of calf eyes. Less extensive studies in this laboratory have demonstrated a cholinesterase in the rabbit lens. Data presented by Glick[11] indicate a cholinesterase in the lens of the swine and de Roetth[4,5] states that he has found a cholinesterase at the anterior subcapsular region of both the rabbit and human lens. The indication is that, at least in the mammal, cholinesterase is a normal constituent of the lens.

A strict comparison between the amount of lens cholinesterase and the literature values for cholinesterase in other ocular tissue is not possible, because of inherent differences in the methods from one study to the next. However, a general notion of the relative magnitude of cholinesterase activity from one ocular tissue to the next is possible. Activity in the mammalian retina ranges from 125 to 160 cholinesterase units, that is, 125 to 160 mg ACh hydrolyzed per hour per gram tissue.[12] Activity in the mammalian iris ranges from 20 to 56 cholinesterase units.[12] In comparison, the value of 0.6 cholinesterase units for the total lens is not remarkable, while the activity of 27.5 cholinesterase units at the surface of the lens appears significant.

As demonstrated, the lens enzyme is a true cholinesterase similar to that found in nervous tissue and erythrocytes. Like the cholinesterase of erythrocytes, the function of lens cholinesterase is unknown. Several possibilities exist.

First, the enzyme could be concerned with regulation of cation transport through modification of membrane permeability or active transport. Certainly the acetylcholine-cholinesterase system functions at sites of nervous excitation by modification of membrane permeability. A similar role has been proposed for cholinesterase in nonneural tissue such as the erythrocyte and amphibian skin, based on experiments in which pharmacologic agents modifying the acetylcholine-cholinesterase system caused shifts in ionic distribution.[13-15] In view of Becker and Cotlier's study[16] indicating that the capsule does not participate in cation transport, such a role seems remote in the lens, in light of the enzyme's localization. Furthermore, before such a role was seriously considered, the demonstration also of choline acetylase and acetylcholine in the lens would seem necessary.

Protection against the action of environmental acetylcholine would seem a possible function of lens cholinesterase. Studies suggest that acetylcholine can modify not only membranes of neural tissue, but also the erythrocyte membrane and, in fact, other lipid-aqueous interfaces.[13,17,18] The lens is situated in the immediate vicinity of the iris and ciliary apparatus, active sources of acetylcholine. Cholinesterase at the anterior surface of the lens would seem reasonable as a defensive maneuver against a constant insult to lens membranes by acetylcholine. This role would be appropriate in tissue required to maintain strict cation and water balance for purposes of optical clarity. The role of cholinesterase in protecting against membrane insult by acetylcholine has already been suggested to explain the peculiar, presynaptic location of cholinesterase in autonomic ganglia.[19]

The lens may represent a site at which cholinesterase had a former function, subsequently lost in phylogenetic development. This has been suggested for another type of esterase, the aliesterases, since their inhibition in rats is without discernible effect while inhibition in bacteria, seedlings, and malignant cell cultures results in altered growth rates.[20] Furthermore, lens cholinesterase may have only a transient role occurring in the course of embryonic development.

This work was supported by Public Health Service grants 2-101 NB 05142, NB 06090, and S-K3-17082; and US Atomic Energy Commission Contract AT (30-1)1368.

Lorenzo O. Merola and Bill Tung gave technical assistance.

Generic and Trade Names of Drugs

Isoflurophate—*Floropryl.*
Demecarium bromide—*Humorsol.*
Echothiophate iodide—*Phospholine Iodide.*
Pilocarpine—*Isopto-Carpine, Pilocar, Pilocel, Pilovisc.*

References

1. Muller, H.K., et al: Der Einfluss von Pilocarpin und Mintacol auf den Stoffwechsel der Linse, *Ber Deutsch Ophth Ges* **60**:115-120, 1956.

2. Harrison, R.: Bilateral Lens Opacities Associated With Use of Diisopropyl Fluorophosphate Eye Drops, *Amer J Ophthal* **50**:153-154 (July) 1960.

3. Axelsson, U., and Holmberg, A.: The Frequency of Cataract After Miotic Therapy, *Acta Ophthal* **44**:421-429, 1966.

4. de Roetth, A., Jr.: Lenticular Opacities in Glaucoma Patients Receiving Echothiophate Iodide Therapy, *JAMA* **195**:664-666 (Feb 21) 1966.

5. de Roetth, A. Jr.: Lens Opacities in Glaucoma Patients on Phospholine Iodine Therapy, *Amer J Ophthal* **62**:619-628 (Oct) 1966.

6. Shaffer, R.N., and Hetherington, J., Jr.: Anticholinesterase Drugs and Cataracts, *Amer J Ophthal* **62**:613-618 (Oct) 1966.

7. Diamant, H.: Cataract Due To Cholinesterase Inhibitors in the Guinea-Pig, *Acta Ophthal* **32**:357-361, 1954.

8. Ellman, G., et al: A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity, *Biochem Pharmacol* **7**:88-95, 1961.

9. Augustinsson, K.: "Butyryl- and Propionyl-Cholinesterases and Related Types of Eserine-Sensitive Esterases," in Boyer, P.; Lardy, H.; and Myrback, K. (eds.): *The Enzymes*, London: Academic Press, 1960, vol 4, pp 521-540.

10. Leopold, I.H.: Ocular Cholinesterase and Cholinesterase Inhibitors: The Friedenwald Memorial Lecture, *Amer J Ophthal* **51**:885-919 (May) 1961.

11. Glick. D.; Lewin, A.; and Antopol, W.: Occurrence of Cholinesterase in the Swine, *Proc Soc Exp Biol Med* **40**:28-32 (Jan) 1939.

12. de Roetth, A., Jr.: Cholinesterase Activity in Ocular Tissues and Fluids, *Arch Ophthal* **43**:1004-1025 (June) 1950.

13. Holland, W.C., and Grieg, M.E.: Studies on the Permeability of Erythrocytes: III. The Effect of Physostigmine and Acetylcholine on the Permeability of Dog, Cat, and Rabbit Erythrocytes to Sodium and Potassium, *Amer J Physiol* **162**:610-615 (Sept) 1950.

14. Thompson, E.H., and Whittaker, V.P.: Cholinesterase Activity and Sodium Transport in the Human Red Blood Cell, *Biochem Biophys Acta* **9**:700-701, 1952.

15. Kirschner, L.B.: Effect of Cholinesterase Inhibitors and Atropine on Active Sodium Transport Across Frog Skin, *Nature* **172**:348-349 (Aug 22) 1953.

16. Becker, B., and Cotlier, E.: The Efflux of ^{86}Rubidium From the Rabbit Lens, *Invest Ophthal* **4**:117-121 (Feb) 1965.

17. Franklin, J.E., and Asakri, A.: Effect of Quaternary Ammonium Ions on Oil-Water Phase Boundary Potentials, *Fed Proc* **25**:290 (March-April) 1966.

18. Hyman, E.S.: Cation Penetration of a Lipid Membrane, *Fed Proc* **25**:567 (March-April) 1966.

19. Koelle, W.A., and Koelle, G.B.: The Localization of External or Functional Acetylcholinesterase at the Synapses of Autonomic Ganglia, *J Pharmacol Exp Ther* **126**:1-8 (May) 1959.

20. Mendel, B., et al: Ali-Esterase Inhibitors and Growth, *Brit J Pharmacol* **8**:217-224 (June) 1953.