OCULAR ACETYLCHOLINE AND CHOLINE ACETYLTRANSFERASE

by

JOEL SIDNEY MINDEL

A dissertation submitted to the Graduate Faculty in Pharmacology of the School of Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1975

This manuscript has been read and accepted for the Graduate Faculty in Pharmacology of the School of Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor Philosophy.

May 27, 1975
date

Chairman of Examining Committee

May 27, 1975
date

Executive Officer

Supervisory Committee

The City University of New York

ABSTRACT

OCULAR ACETYLCHOLINE AND CHOLINE ACETYLTRANSFERASE

by

Joel S. Mindel

Adviser: Professor Thomas Mittag

1. Acetylcholine was consistently detectable in the corneas of rabbit, bovine, and human eyes using a qualitative pyrolysis-gas chromatography assay.

2. Feline eyes had no detectable corneal acetylcholine.

3. Average corneal epithelial choline acetyltransferase activities for these 4 different mammalian species were: rabbit, 20.6 nanomoles ACh formed/hr/mg protein; bovine, 46.5 nanomoles ACh formed/hr/mg protein; human, 11.2 nanomoles ACh formed/hr/mg protein; and feline, 0.1 nanomoles ACh formed/hr/mg protein.

4. Half the rabbit eyes assayed had detectable acetylcholine in their aqueous humors. There was no detectable acetylcholine in human aqueous humor even when pooled samples of 2.5 mℓ. were assayed.

5. Iris and ciliary body choline acetyltransferase activities of rabbit and feline eyes ranged from average values of 1.9 - 2.9 nanomoles ACh formed/hr/mg protein. Bovine ciliary body and human iris enzyme activities also fell within this range. Bovine iris and human ciliary body were exceptional in having higher enzyme activities of 10.8 and 16.8 nanomoles ACh formed/hr/mg protein, respectively.

6. Average retinal choline acetyltransferase activities for the 4 mammalian species studied were: rabbit, 15.9 nanomoles ACh formed/hr/mg protein; bovine, 13.0 nanomoles ACh formed/hr/mg protein; human, 6.1 nanomoles ACh formed/hr/mg protein; and feline, 0.7 nanomoles ACh formed/

hr/mg protein.

7. Pigment epithelium-choroid choline acetyltransferase activities of rabbit, bovine, and human eyes ranged from average values of 4.0 - 5.1 nanomoles ACh formed/hr/mg protein. Feline pigment epithelium - choroid enzyme activity was 0.7 nanomoles ACh formed/hr/mg protein.

8. A regional variation in rabbit corneal choline acetyltransferase activity was found: central > inferior or nasal > superior or temporal.

9. Corneal choline acetyltransferase activities in the two eyes of the same rabbit agreed, on the average, within 15% of each other. However, there could be a 25-fold range in the corneal enzyme activity of different rabbits.

10. The choline acetyltransferase activity of the tissues of the two eyes of rabbits differed less ($p < 0.01$) in highly inbred animals than in randomly chosen rabbits and siblings.

11. Corneal choline acetyltransferase activity was increased by lowering the temperature to which rabbit eyes were exposed. This effect was produced by lowering the room temperature from 30°C to 15°C for 17 days. Rabbits exposed to a 15°C room temperature for only 10 days did not exhibit an elevated enzyme activity.

12. Light deprivation, chronic light exposure, elevated atmospheric CO_2 and alteration of room humidity for periods of 7 days or more failed to change corneal choline acetyltransferase activity.

13. Unilateral tarsorrhaphy produced a fall in rabbit corneal choline acetyltransferase activity within 48 hours. The closed eye's enzyme activity fell, on the average, to a level of 58% that of the open eye; the decline was maximal by day 8. In rabbits unilaterally tarsorrhaphied 8 to 56 days, the corneal choline acetyltransferase activity fell, on

the average, to a level of 28% that of the open eye.

14. The decline in choline acetyltransferase activity produced by tarsorrhaphy appeared to be a specific response of the corneal epithelium to this condition because corneal lactic dehydrogenase activity and protein incorporation of amino acids were unaffected.

15. Tarsorrhaphy produced a small decrease in rabbit iris-ciliary body choline acetyltransferase activity that averaged 26%. It also lowered retinal choline acetyltransferase activity by an average value of 20% compared to that of the open eye.

16. Rabbit intra-ocular pressure did not vary when corneal, iris-ciliary body or retinal choline acetyltransferases were lowered by tarsorrhaphy. Unilateral closure of human eyelids for 4 to 5 day periods did not alter intra-ocular pressure. The enzyme activities of ocular tissues of patients with and without chronic simple glaucoma did not appear different. These data were interpreted as implying that the level of intra-ocular pressure was not dependent on choline acetyltransferase activity.

17. Application of pilocarpine nitrate 4%, ℓ-epinerphrine bitartrate 2%, diisopropylfluorophosphate 0.1%, and dexamethasone phosphate 0.1% eye drops three times a day for three weeks failed to alter rabbit ocular choline acetyltransferase activity.

18. Tissue cultures of rabbit corneal epithelium maintained for 7 days and rabbit corneas denervated by retrobulbar injections of ethanol retained their choline acetyltransferase activities. This was interpreted as indicating the enzyme resides in the corneal epithelial cells rather than in the sensory nerve endings.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

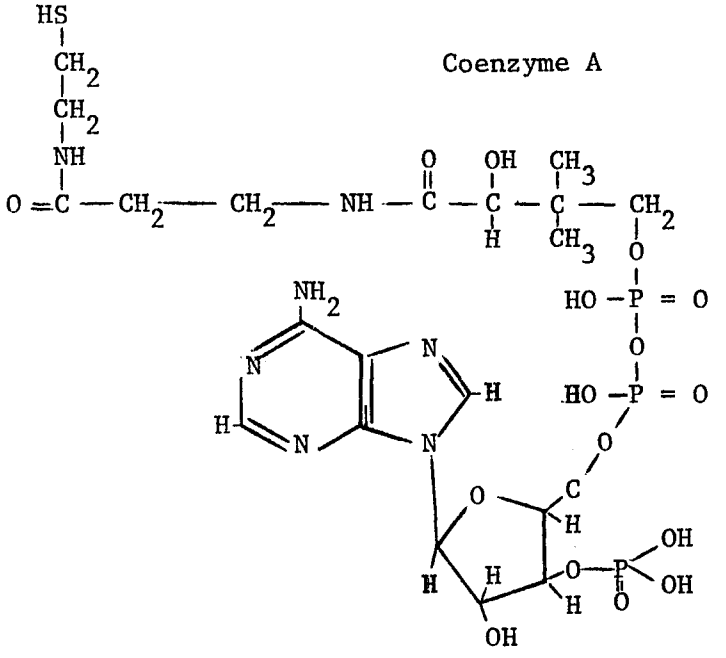
Introduction

This body of work is the result of studies into two aspects of choline acetyltransferase activity in ocular tissues. The first is the interspecies and intraspecies variations of enzyme activity in different tissues of mammalian eyes. The second is the investigation of those physiologic and pharmacologic factors that influence the level of choline acetyltransferase activity in the ocular tissues.

1. The Enzyme: Choline Acetyltransferase

Quastel, Tennenbaum, and Wheatley (1936) first showed that brain slices, under aerobic conditions and in a medium containing eserine, produced a substance having the chemical and pharmacological properties of a choline ester. Stedman and Stedman (1937) showed that the substance produced from minced ox brain was acetylcholine by isolating it as the chloroaurate. This material had the same melting point and gold content as contained in a known sample of acetylcholine chloroaurate. Having demonstrated brain synthesis of acetylcholine, the next step in the study of the enzymatic mechanism of acetylcholine formation required the use of cell-free extracts. It was Nachmansohn and Machado (1943) who reported the first cell-free synthesis of acetylcholine using homogenates of frog brain, rat brain, and eel electric organ. The early assumption that acetylcholine was produced by a simple enzyme reaction using choline and acetate as substrates and ATP as the energy source, i.e. choline + acetate $\xrightarrow{ATP}$ acetylcholine, soon was shown to be incorrect. For it was reported by Nachmansohn, John, and Waelsch (1943) that the enzyme rapidly become inactive on dialysis.

Acetate addition had no reactivating effect. Citrate addition partially reactivated this system in some instances (Feldberg and Mann, 1946). However, Nachmansohn and Weiss (1948) found citrate inhibitory under their experimental conditions. The inactivation produced by dialysis could be largely restored by adding a dialyzable heat-stable constituent of brain termed the "activator" (Feldberg, 1945; Feldberg and Mann, 1946). The preparation and chemical identification of the "activator" as coenzyme A followed (Nachmansohn and Berman, 1946; Lipmann, Kaplan, Novelli, Tuttle, and Guirard, 1947; Baddiley and Thain, 1951; Baddiley, Thain, Novelli, and Lipmann, 1953; Novelli, Schmetz, and Kaplan, 1954):



Lynen, Reichert, and Rueff (1951) showed that an acetate group could be conjugated to coenzyme A as the thioester and the work of Stern and Ochoa (1951), Ochoa, Stern and Schneider (1951), and Stern, Shapiro, Stadtman, and Ochoa (1951) showed that acetyl-coenzyme A was an active acetate donor in many enzymatic reactions. These authors (Stern, Shapiro, Stadtman, and Ochoa, 1951) gave credit to F. Lynen, who had notified them in a personal communications, for having first demonstrated that acetyl-coenzyme A was capable of acetylating choline in the presence choline acetyl-transferase. These same authors also demonstrated the reversibility of transacetylations involving coenzyme A.

If acetylcholine synthesis required acetyl-coenzyme A as the source of acetate, how could the activating effect of citrate, previously mentioned, be explained? Lipton and Barron (1946) proposed that citrate's activating effect might be due to its being enzymatically converted into an active form of acetate, i.e. acetyl-coenzyme A. Korey, Braganza, and Nachmansohn (1951) suggested that since this conversion required a second enzyme, the term choline acetyltransferase should be limited to the reaction: choline + acetyl-coenzyme A $\longrightarrow$ acetylcholine + coenzyme A.

Once the enzyme's substrates were shown to be acetyl-coenzyme A and choline, assay depended on having adequate concentrations of the two precursors. Choline represented no problem as it is easily obtained in large quantities. But acetyl-coenzyme A was a different matter. Two methods were available for providing acetyl-coenzyme A: a coupled enzyme system and synthetic molecules. Initially, the most satisfactory source was the coupled system wherein a phosphotransacetylase (Cohen, 1956) or acetyl-coenzyme A synthetase (Hebb, 1955) was used in the incubating mixture to

produce acetyl-coenzyme A. Synthetic acetyl-coenzyme A would have been more convenient to use, but early attempts to use synthetic substrate gave much lower rates of acetylcholine synthesis than coupled systems. Choline acetyltransferase activity with synthetic substrate was found to be about 50% that of coupled systems (Berman, Wilson, and Nachmansohn, 1953; Smallman, 1958; Morris and Tucek, 1966). It was assumed that coenzyme A, freed during the reaction, had an inhibitory effect and that this material was removed in coupled systems by recycling. However, Smallman (1958) pointed out that addition of ATP to provide energy for reacetylation of coenzyme A failed to improve the rate of acetylcholine synthesis using synthetic acetyl-coenzyme A in a coupled system. In 1965, McCaman and Hunt reported greater acetylcholine synthesis using synthetic acetyl-coenzyme A than prior workers. Morris, Hebb, and Bull (1966) found that McCaman and Hunt had omitted cysteine. High concentrations of cysteine had always been considered essential for full choline acetyltransferase activity (Balfour and Hebb, 1952). It was the added cysteine that was reducing the rate of acetylcholine synthesis. Stadtman (1952) had previously given evidence that non-enzymatic transfer of acetyl radicals from acetyl-coenzyme A to other mercaptans (e.g. glutathione) could occur. Morris (1967) showed that cysteine, as well as other sulfhydryl compounds could nonenzymatically thiolyse the acetyl groups from acetyl-coenzyme A. Thus, the added cysteine had been diverting acetyl-coenzyme A away from the choline acetyltransferase reaction and was preventing full activity of the enzyme. Smith and Weiskopf (1967) showed that cysteine at high concentrations was actually being acetylated to S-acetylcysteine. However the role of sulfhydryl compounds in choline acetyltransferase assays is not

simple. Subsequent work by Bull and Oderfeld-Nowak (1971) indicated that some protection of the enzyme against oxidative inactivation was obtained with low levels of sulfhydryl compounds that caused no appreciable thiolysis of acetyl-coenzyme A, e.g. cysteine, 3-6 mg/ml increased the activity of rabbit brain choline acetyltransferase.

A number of other factors have been found to influence the activity of choline acetyltransferase in the cell-free system. Reisberg (1954) found that ethylene diamine tetraacetate (EDTA) increased enzymatic activity, which she attributed to its preventing inhibition by traces of metal ions in the incubating medium. White and Cavallito (1970A) also found EDTA tended to stabilize activity, possibly by chelating inhibitory cations. Potter, Glover, and Saelens (1968) showed a broad pH optimum for purified rat brain enzyme of 7.0 to 7.7. Copper sulfate, 10μM, completely inhibited the enzyme. Fourteen salts were capable of activating the soluble enzyme: LiCl, NH_4Cl, KCl, $MgCl_2$, $CaCl_2$, $BaCl_2$, NaF, KBr, KI, CH_3COOK, $KHCO_3$, K_2SO_4, K glutamate, and K phosphate. This was consistent with work on rabbit brain (Morris and Tucek, 1966) where concentrations of Na, K, Mg, and Ca up to 100 mM stimulated enzyme activity; at higher concentrations Na and K continued to stimulate while Ca and Mg produced inhibition. Squid ganglia (Prince, 1967) and placental (Schuberth, 1966) enzyme were also found to be activated by some salts. However, in the latter two studies halide salts, with the exception of chloride (Schuberth, 1966), were found to inhibit. This contrasts with the rat brain enzyme where Potter, Glover, and Saelens (1968) found fluoride and, especially iodide could activate. White and Cavallito (1970A) found that dithiothreitol, 0.1 mM in extracts was not a requirement but did stabilize choline acetyltransferase activity and restored the activity of an aged extract. They also found that inhibition by

$CuSO_4$, 10 μM, or $HgCl_2$, 10 μM, could be reversed by 0.2 mM dithiothreitol. Reisberg (1974) found an 11-37% increase in choline acetyltransferase activity in the presence of 0.17 to 1.02 M ethanol. However, the denaturing effect of ethanol on enzyme activity could mask the stimulation when incubation times were longer than twenty minutes. Reisberg found it was possible to prevent the ethanol denaturation if 0.3 M NaCl were used in the incubation mixture.

Evidence exists for multiple forms of choline acetyltransferase. Not only are there different molecular forms in different species but isoenzymes have been found in the same species. Malthe-Sørenssen and Fonnum (1972) found that isoenzymes of the same species had the same choline affinity, indicating that the variations in amino acid sequence do not lie near the enzyme's active center. Fonnum and Malthe-Sørenssen (1973) used isoelectric focusing to show three different molecular forms of choline acetyltransferase in rat brain. White and Wu (1973) also separated multiple forms of the enzyme from human brain and sciatic nerve using isoelectric focusing. However, only a portion of the activity was found in the original position after re-electrofocusing which suggested that the different forms may represent, to some extent, denaturation products. Rabbit brain and sciatic nerve showed a single enzyme form on electrofocusing. Potter, Glover, and Saelens (1968) estimated the molecular weight of rat brain choline acetyltransferase by gel filtration and found it to be about 50,000 as compared to 67,000 for rabbit brain and 59,000 for human placenta (Bull, Feinstein, and Morris, 1964). Ox brain enzyme has been estimated to have a molecular weight of 65,000 (Glover and Potter, 1971). Chao and Wolfgram (1974) question the validity of the data indicating isoenzymes of choline acetyltransferase in a given species and also question the accuracy of the mole-

cular weights assigned to the enzyme in different species. They have found that the extraction procedures for choline acetyltransferase used by prior workers required ammonium sulfate. But ammonium sulfate causes aggregation of the enzyme. There was only one molecular species of choline acetyltransferase in their preparations of bovine brain obtained without the use of ammonium sulfate.

Invertebrate choline acetyltransferase differs from that of vertebrates. Stephenson and Rowatt (1947) studied the choline acetylating enzyme of Lactobacillus plantarum, and White and Cavallito (1970A) found it to be more heat stable than that of calf brain. The rate of acetylcholine production is significantly increased for the mammalian enzyme by raising the temperature from 5°C to 37°C in contrast to the bacterial enzyme. Emson, Malthe-Sørenssen, and Fonnum (1974) studied snail, cockroach, and horseshoe crab enzyme. The three had specific activities similar to mammalian choline acetyltransferase and were also similar in their activation by NaCl and KCl. However, the enzymes were not sensitive to inhibition by styrylpyridines in contradistinction to vertebrates.

It was assumed that choline acetyltransferase used acetyl-coenzyme A exclusively as a substrate until propionylcholine was demonstrated physiologically and chromatographically in ox spleen (Banister, Whittaker, and Wijesundera, 1953; Gardiner and Whittaker, 1954) and extracts of squid ganglia were shown capable of synthesizing propionylcholine if propionyl-coenzyme A were added to the incubate instead of acetyl-coenzyme A (Berman, Wilson, and Nachmansohn, 1953). Similarly, the enzyme's specificity for choline alone was questioned after squid head ganglia were shown able to acetylate dimethylethanolamine as fast as choline (Korey, deBraganza, and Nachmansohn, 1951); ethanolamine, monomethylethanolamine,

and diethylethanolamine were not acetylated at all. Burgen, Burke, and Desbarato-Schonbaum (1956) demonstrated that many analogues of choline can be acetylated. Often, replacing the methyl groups of choline with longer acyl groups did not lower the derivative's affinity for the enzyme. Thus, during a 30 minute incubation, 11.1 millimolar ethyldimethylethanolamine or methyldiethylethanolamine was acetylated more completely than 11.1 millimolar choline. Replacement of one methyl group of choline by increasingly longer chains had relatively little effect until the hexyl derivative was used; this was not acetylated. Hemsworth and Morris (1964) gave evidence that at least two methyl groups are necessary on the choline molecule to enable enzymatic acetylation.

Michaelis constants have been determined for the acetyltransferase reaction:

Author	Tissue	Km (μM) choline	Km (μM) acetyl-coenzyme A
McCaman and Hunt (1965)	rat brain	500	25
Potter, Glover, and Saelens (1968)	rat brain	39	19
Kaita and Goldberg (1969)	ox brain	770	--
Glover and Potter (1971)	ox brain	750	10
White and Cavallito (1970A)	calf brain	800	16
White and Wu (1973)	human brain	510	11
White and Cavallito (1970A)	Lactobacillus plantarum	440	8
Emson, Malthe-Sørenssen, and Fonnum (1973)	snail	370	51

Author	Tissue	choline	acetyl-coenzyme A
Emson, Malthe-Sørenssen, and Fonnum (1973)	cockroach	550	16
Emson, Malthe-Sørenssen, and Fonnum (1973)	horseshoe crab	2700	68

Several mechanisms have been proposed for the enzyme's interaction with substrates. Schuberth (1966), working on human placental enzyme proposed the following:

(1) Enzyme + acetyl-coenzyme A $\rightleftharpoons$ Enzyme - acetyl-coenzyme A

(2) Enzyme - acetyl-coenzyme A $\rightleftharpoons$ acetyl - Enzyme + coenzyme A

(3) acetyl - Enzyme + choline $\rightleftharpoons$ Enzyme - acetylcholine

(4) Enzyme - acetylcholine $\rightleftharpoons$ Enzyme + acetylcholine

However, Morris and Grewaal (1971), working with placental enzyme also, found that acetylcholine could be converted to acetyl - [^{14}C] choline in the presence of [^{14}C] choline only if coenzyme A were present. They proposed the following sequence, which has also been advocated by White and Cavallito (1970A), Morris, Maneckje, and Hebb (1971), and White and Wu (1973):

(1) Enzyme + acetyl-coenzyme A $\rightleftharpoons$ Enzyme - acetyl-coenzyme A

(2) Enzyme - acetyl-coenzyme A + choline $\rightleftharpoons$ Enzyme - acetyl-coenzyme A-choline

(3) Enzyme - acetyl-coenzyme A - choline $\rightleftharpoons$ acetylcholine + Enzyme--coenzyme A

(4) Enzyme - coenzyme A $\rightleftharpoons$ Enzyme + coenzyme A

Roskoski (1973, 1974) has found evidence that the stable intermediate complex Enzyme - acetyl-coenzyme A - choline exists.

The development of simple, accurate assay techniques for choline acetyltransferase activity progressed in several stages. Two of these

have already been mentioned, namely: realization that acetyl-coenzyme A was the source of acetate and successful replacement of coupled enzyme systems with synthetic acetyl-coenzyme A. The next step was to simplify and improve the separation and quantitative determination of the reaction product, acetylcholine. Bioassay has been used to measure acetylcholine, and the technique has been constantly perfected beginning with Chang and Gaddum (1933) who systematically tested different tissues for contractile sensitivity until the present method of Hsu and Gerald (1973) who described a bioassay technique sensitive to femtomole (10^{-15}M) levels of acetylcholine. While bioassay is tedious, it was the only sensitive method available until 1963 when Schuberth published a radioisotope technique for choline acetyltransferase activity assay. ^{14}C labelled acetyl-coenzyme A was the substrate and [^{14}C] acetylcholine was the product. The disadvantage of this system was that labelled acetyl-coenzyme A was a contaminent of the product that was being measured in a gas flow counter and the blanks were high. Refinements of the radiometric assay have been developed by Fonnum (1966) and Morris and Grewaal (1971). Fonnum destroyed any [^{14}C] acetyl-coenzyme A remaining at the end of the reaction by adding hydroxylamine; the labelled acetylcholine was precipitated with sodium tetraphenylborate, then dissolved in acetonitrile-benzyl alcohol, and measured by scintillation counting. Morris and Grewaal used radio-labelled choline. Both choline and acetylcholine were precipitated as the reineckate salt and then separated by electrophoresis. The acetylcholine spot was cut out and measured with a scintillation counter.

McCaman and Hunt (1965) noted that while 0.5 mM [^{14}C] acetyl-coenzyme A produced maximal enzyme activity, 0.05 mM concentrations of the substrate

gave 90% of the value and resulted in much lower blanks. They separated the labelled acetylcholine from the labelled acetyl-coenzyme A by precipitating it as the Reineckate, as did Alpert, Kisliuk, and Shuster (1966). Goldberg, Kaita, and McCaman (1969) believed that the low toluene solubility of the reineckate salts limited these methods to measuring small amounts of acetylcholine, and, in addition, the blanks were still quite high, being approximately 20% of the samples. They found that precipitation with iodine-iodide gave blanks of only 5%. Fonnum (1966, 1969) used tetraphenylboron in his precipitation method. These precipitation methods were quite specific and sensitive, but also quite tedious. The earlier methods of product isolation by electrophoresis (Potter, Glover, and Saelens, 1968; Geller and Schwartz, 1968) and gas chromatography (Stavinoha and Ryan, 1965) were no less cumbersome.

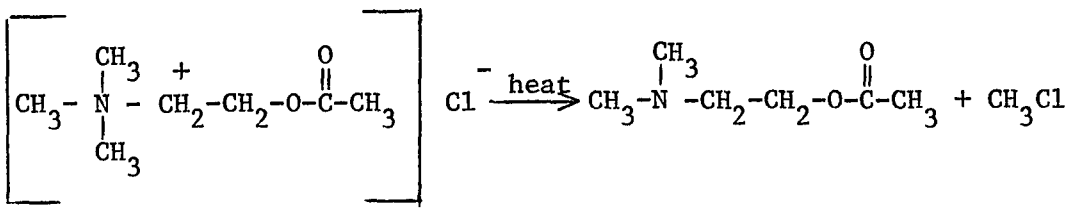
A major improvement in separating the radioisotope labelled substrate from the radioisotope labelled product was the introduction of anion exchange resins by Schrier and Shuster (1967). The method takes advantage of the ^{14}C labelled acetyl moiety being transferred from an anionic substrate, acetyl-coenzyme A, to a cationic product, acetylcholine. The anion exchange resin only permits the cationic product to pass through and this is collected at the end of the column. The radioactivity of the effluent is then determined by scintillation counting. Schrier and Shuster stressed the importance of using preparations of synthetic acetyl-coenzyme A that were nearly completely acetylated since coenzyme A inhibited the reaction. The method gave specific activities for choline acetyltransferase that were similar to the reineckate precipitation technique. In addition, the results were reproducible to within 2%, and less than 1% of

the acetyl-coenzyme A or 0.02% of the sodium acetate placed in the incubate passed through the column. However, acetylcarnitine, a contaminant formed from acetyl-coenzyme A by carnitine acetyltransferase, passed through the anion exchange column. But it is not hydrolyzed by acetylcholinesterase and Hamprecht and Amano (1974) made use of this. They assayed with an acetylcholinesterase inhibitor in one tube and eel acetylcholinesterase in a second tube. The latter is subtracted from the former and the difference is assumed to be the choline acetyltransferase activity. Depending on the cell type, overestimates of choline acetyltransferase activity ranged from 2-8% in tissues rich in choline acetyltransferase to 650% in tissues low in activity.

While radioisotope assays of acetylcholine have been useful for in vitro studies of choline acetyltransferase activity, there are limitations to their use in vivo. Exogenously supplied labelled substrates may not reach the enzyme in physiologic concentrations or by the pathways normally used. Radioisotopes are expensive and their use in human studies by the potential damage they can produce. In vivo studies would be aided by a sensitive and specific chemical assay of acetylcholine that did not rely on labelled materials. Hanin (1974) has edited a text describing the different chemical assay techniques for acetylcholine. The recurring problems in these methods are their complexity, their inability to distinguish different choline esters, and their lack of sensitivity at the nanogram level.

Perhaps the simplest technique, combining specificity with sensitivity in the 1-10 nanogram range, is that of pyrolysis-gas chromatography (Szilagyi, Schmidt, and Green, 1968; Schmidt, Szilagyi, Alkon, and Green,

1969; Schmidt, Szilagyi, Alkon, and Green, 1970; Szilagyi, Green, Brown, and Margölis, 1972). The nonvolatile quanternary compound, acetylcholine, is converted to a volatile tertiary amine by pyrolysis provided it is in the form of a halide salt, e.g.:



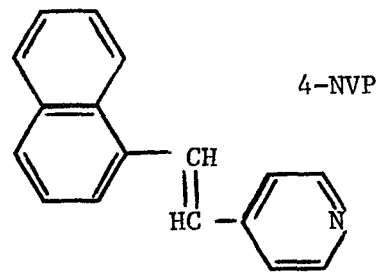
Pyrolysis takes place in a chamber attached to a gas chromatograph and the reaction products are swept immediately onto the column by a stream of nitrogen. The components are resolved as they move along the column and then measured using a flame-ionization detector connected to an electrometer and recorder. The different choline esters have different retention times and can therefore be identified. The method has been used to measure quantities as small as 2 nanograms in nerve effluents.

Specific inhibitors of choline acetyltransferase have been developed. This represents a step forward since they permit the quantification of substrates diverted into other pathways. Chase and Tubbs (1966) found bromoacetyl-coenzyme A to be a potent inhibitor of choline acetyltransferase but, unfortunately, it inhibitied carnitine acetyltransferase as well. The inhibition is irreversible and enhanced by choline. Morris and Grewaal (1969) studied halogenated derivatives of acetylcholine. They found chloro-, bromo-, and iodoacetylcholine to be potent, reversible inhibitors; the most effective inhibitor was chloracetylcholine. It gave complete inhibition at 6.2×10^{-5}M. The inhibition is terminated by hydrolysis of the halogenated acetylcholine. Unfortunately,

acetylcholinesterase is also inhibited by these halogenated compounds (Chiou and Rama Sastry, 1968).

Persson, Larsson, Schuberth, and Sorbo (1967) synthesized an alkylating compound structurally related to choline. This was 3-bromoacetonyltrimethylammonium bromide (BAT), $[(CH_3)_3NCH_2COCH_2Br]^+Br^-$. It was an irreversible inhibitor of 95% of the enzyme's activity at 5×10^{-3}M concentration. Steinberg and Cramer (1970) found BAT to irreversibly inhibit 35% of acetylcholinesterase activity at 5×10^{-4}M concentration within 30 minutes. Acryloylcholine (Malthe-Sørenssen, Andersen, and Fonnum, 1974), an unsaturated derivative of propionylcholine, is a potent inhibitor but it too has anti-acetylcholinesterase properties.

Smith, Cavallito, and Foldes (1967) studied a group of styrylpyridine analogues that inhibited choline acetyltransferase. The most potent of these with little anti-acetylcholinesterase activity was trans-4-(1-naphthylvinyl) - pyridine (4-NVP):



4-NVP

It produced 95% inhibition of choline acetyltransferase activity at 6×10^{-4}M with only 4% inhibition of acetylcholinesterase at 10^{-3}M. Subsequent work (Cavallito, Yun, Smith, and Foldes, 1969) indicated that some quaternary derivatives of this compound, by virtue of the cationic charge they carried, were even more potent inhibitors while retaining their minimal anti-cholineresterase activity. Trans-N-methyl-4-(1-naphthylvinyl) pyridinium iodide was ten times as potent as 4-NVP as a

choline acetyltransferase inhibitor and had an $I_{50} = 2 \times 10^{-4}$M for acetylcholinesterase. Trans-N-hydroxyethyl-4-(1-naphthylvinyl) pyridinium iodide is a more water soluble quaternary derivative (White and Cavallito, 1970B). It inhibited choline acetyltransferase with $I_{50} = 3.8 \times 10^{-7}$M; at 4.5×10^{-6}M only 5% of acetylcholinesterase activity was inhibited (Cavallito, Yun, Kaplan, Smith, and Foldes, 1970). Trans-N-hydroxyethyl-4-(1-naphthylvinyl) pyridinium bromide had an $I_{50} = 6 \times 10^{-7}$M for choline acetyltransferase in dark but in a flourescent light illuminated area the potency rapidly dropped to $I_{50} = 1 \times 10^{-6}$M. The I_{50} of acetylcholinesterase in dark or light was 1.4×10^{-3}M. White and Cavallito (1970B) found that the inhibition was non-competitive with both acetyl-coenzyme A and choline. The inhibition was completely reversible after thirty minutes of incubation at 5°C if the trans-N-methyl-4-(1-naphthylvinyl) pyridinium iodide was removed; however, at 37°C, only 70% activity returned after a thirty minute incubation. The inhibition was neither prevented nor reversed by dithiothreitol; this contrasts to inhibition by 10 um $CuSO_4$ and $HgCl_2$, which could be reversed by 0.2mM dithiothreitol. White and Cavallito (1970C) studied the partial inactivation of trans-N-methyl-4-(1-naphthylvinyl) pyridinium iodide by light. They found that the trans form is more active than the cis form and that light converts the former to the latter. With acetylcholinesterase, either configuration was equally potent. For N-methyl-4-(1-naphthylvinyl) pyridinium iodide, it was estimated spectrophotometrically that at equilbrium in room light, solutions may still contain as much as 20% of the trans isomer. The specificity of the styrylpyridines has been questioned by Goldberg, Salama, and Blum (1971) who found that a hexobarbital metabolizing enzyme system of microsomes was inhibited by 4-NVP at 1/17 the concentration needed to inhibit choline acetyltransferase.

Barker and Mittag (1973) have shown that choline uptake by synaptosomes is blocked by styrylpyridines; the mechanism appears unrelated to their action on choline acetyltransferase. The I_{50} for choline uptake, both high and low affinity, was 50 uM using trans-N-hydroxyethyl-4-(1-naphthyl-vinyl) pyridinium bromide, while the I_{50} for choline acetyltransferase activity was 1 uM. Domino, Mohrman, Wilson, and Haarstad (1973) found that a deriviative of hemicholinium-3, a drug known for its ability to block choline uptake, also inhibited choline acetyltransferase. The compound was acetylseco hemicholinium-3 and its $I_{50} = 10^{-5}$M; it is a competitive inhibitor of choline and a mixed inhibitor of acetyl coenzyme A.

Choline acetyltransferase is found in both the soluble cytoplasm of cells and attached to acetylcholine containing vesicles. The earliest attempts to localize intracellular choline acetyltransferase used differential centrifugation (Hebb and Smallman, 1956) and seemed to indicate that 52-69% of the enzyme was in the mitochondria. The rest was in the supernatant, differentiating a soluble and particle fixed enzyme. Hebb and Whittaker (1958) expressed doubt that the enzyme activity was really within the mitochondria because the same procedures releasing the enzyme also released acetylcholine. Gray and Whittaker (1962) subsequently showed that the layer containing mitochondria also contained synaptosomes and these were the source of enzyme activity. Fonnum (1967) studied synaptosomes of rabbit, rat, pigeon, and guinea-pig and found the enzyme in either a bound or soluble form depending on the conditions of isolation. In the bound form, the enzyme appeared associated with the larger membrane fragments rather than the synaptic vesicles. The bound form predominated at sightly acid pH and low ionic strength. The soluble form predominated at the more physiologic alkaline pH's and higher ionic strengths. NaCl,

KCl, $MgCl_2$, and $CaCl_2$ were equally effective in solubilizing the enzyme. Fonnum believed that the enzyme was soluble in the in vivo state but that low osmotic pressures caused the enzyme to bind nonspecifically to membranes. This explanation seemed to clear up a controversy: de Robertis, de Lores Arnaiz, Salganicoff, Iraldi, and Zieher (1963) had found that choline acetyltransferase was located in the vesicle fraction of synaptosomes firmly bound to the vesicular membrane. They considered the soluble enzyme as representing that fraction not yet incorporated into vesicles. Whittaker, Michaelson, and Kirkland (1964) found that choline acetyltransferase, unlike acetylcholine, was not a constituent of synaptic vesicles but was localized in the soluble cytoplasm. An explanation of the differences had been offered by McCaman, de Lores Arnaiz, and de Robertis (1965) who suggested that species differences could account for the discrepancy. Tucek (1966A) found the quantity of synaptosome bound enzyme to vary in different species, and concluded (Tucek, 1966B) that this binding was not within the vesicle. The binding of choline acetyltransferase to membranes is reversible, therefore, and presumably due to ionic attraction between positively charged choline acetyltransferase and negatively charged membrane (Fonnum, 1970). The species variations are explained as being due to differences in the amount of charge. As the enzyme is active in both the soluble and membrane bound forms, the active site must not be involved in the binding (Fonnum, 1968).

Hebb (1972) believes that the ability of cysteine to increase enzyme activity of tissue homogenates is due to more enzyme becoming available rather than stimulation of enzyme activity. She reasons that the amount of cysteine resulting in a 50-75% increase in acetylcholine production is only effective when added to tissue extracts and not when added to incub-

ation media containing relatively purified enzyme. This would be similar to the release of "occluded" or "sequestered" choline acetyltransferase by treatment with ether, acetone, or chloroform (Hebb and Smallman, 1956), detergents (Fonnum, 1966), or hyperosmotic solutions (McCaman and Hunt, 1965). Hebb (1972) finds ether extraction most effective in this regard, but only slightly more so than the simpler procedure Fonnum (1966) favored using Triton-X or Nonex.

The active site has generally been considered to contain an -SH group essential for activity. Potter, Glover, and Saelens (1968) attribute the inhibitory effect of copper ions to reaction with this sulfhydryl group. Reisberg (1954) showed that choline acetyltransferase prepared from squid was inhibited by the thiol reagents iodoacetate and p-chloromercuribenzoate. Roskoski (1973, 1974), working with thiol reagents, has added further support for this concept and has also provided evidence that the acetyl-enzyme bond is a thio ester. However, White and Cavallito (1970B) favor the view that an imidazole group is of prime importance. They found that the addition of imidazole stimulated enzymatic synthesis of acetylcholine from acetyl-coenzyme A and choline. Non-enzymatic acetylcholine formation was not observed. However, Burt and Silver (1973) found a pH dependent non-enzymatic hydrolysis of acetyl-coenzyme A to acetate and coenzyme A, and a nonenzymatic synthesis of acetylcholine, by imidazole. As pH or imidazole concentration was increased, non-enzymatic acetylcholine production increased. In sixty minutes, 36 mM imidazole in a 220 µl volume at pH 7.5 produced 4 nanomoles of acetylcholine. They caution against the use of imidazole in the buffer as an activator.

Because choline acetyltransferase accumulates proximal to a nerve ligature, it has been assumed that the enzyme is transported by axonal flow

from nerve cell body toward nerve terminals (Hebb and Waites, 1956; Hebb and Silver, 1961; Frizell, Hasselgren, and Sjostrand, 1970; Kasa, Mann, Karscu, Toth, and Jordan, 1973). However, Fonnum, Frizell, and Sjostrand (1973), using double ligatures on rabbit vagal and hypyglossal nerves, found evidence for a distal-to-proximal flow of enzyme as well as proximal-to-distal. Furthermore, only 5-20% of the enzyme was mobile. The cell bodies contained only 2% of the choline acetyltransferase activity; 42% was in the nerve trunk and 56% in the pre-terminal axon and axon terminal.

Another approach to understanding the cellular localization of choline acetyltransferase has been the use of histochemistry and fluorescent antibody techniques. The histochemical methods are based on the precipitation by lead of the coenzyme A released after enzymatic transfer of the acetyl group. The technique is not specific for choline acetyltransferase since acetyl-coenzyme A is a substrate for other enzymes. In addition, any molecule containing a free sylfhydryl group will be precipitated by the lead. Burt (1969) first developed the technique which is similar to that for carnitine acetyltransferase (Barnett and Higgins, 1968; Higgins and Barnett, 1970). Its specificity for choline acetyltransferase only resides in (1) the choice, by the histochemist, of tissues rich in the enzyme and (2) the ability to localize other enzymes hydrolyzing acetyl-coenzyme A. The latter is achieved by using either copper ions (to inhibit choline acetyltransferase) or 0.1M KNO_3 in the incubation medium (to solubilize and remove choline acetyltransferase). There are many difficulties with the technique: 1) Burt (1970) estimates that despite formaldehyde fixation prior to assay, over 50% of the enzyme is lost due to its solubility; 2) the formaldehyde fixation interferes to some degree with enzyme activity; 3) acetyl-coenzyme A can be precipitated by lead; 4) copper either does not

prevent choline acetyltransferase from hydrolyzing acetyl-coenzyme A, although it does prevent acetylcholine production, or it stimulates an acetyl-coenzyme A hydrolase; 5) histochemical identification of choline acetyltransferase activity is occasionally lacking where biochemical assay identifies it as being present (Burt, 1970; Goldberg and McCaman, 1967); and 6) the nonsoluble enzyme which is found attached to membranes by histochemistry may represent nonspecific artifactual binding rather than revealing the enzyme's true location in the intact cell. The major contribution of choline acetyltransferase histochemistry to date is its failure to show staining inside vesicles. This indicates that acetylcholine production probably does not occur within these structures.

Burt (1970) found that when choline was not available as a substrate, the amount of coenzyme A formed from acetyl-coenzyme A was doubled. He explained this as representing hydrolysis of acetyl-coenzyme A by choline acetyltransferase in the absence of choline. Kasa (1970) and Kasa, Mann, and Hebb (1970A; 1970B) suggested that the acetate represented acetylcholinesterase hydrolysis of acetylcholine. However, eserine did not completely circumvent the problem and this explanation did not explain the increased precipitation found on histochemistry. The alternative suggestion, that acetyl-coenzyme A was being hydrolyzed by other esterases, seemed more logical. DFP, 10^{-3}M, has been found to decrease the nonspecific hydrolysis of acetyl-coenzyme A by 90%. As choline acetyltransferase is not affected by DFP at this concentration, Kasa, Mann and Hebb (1970B) used it for electron microscopic histochemical studies. They found lead precipitate in unexpected places, e.g. synaptic clefts and postsynaptic membranes. They acknowledged that the soluble enzyme could adhere to membranes and be found in abnormal locations.

Flourescent antibody techniques potentially offer a specific and sensitive tool for intracellular localization of the enzyme. Malthe-Sørenssen, Eskeland, and Fonnum (1973) have purified rat brain choline acetyltransferase, placed it in Freund's adjuvant, and injected in into rabbits to produce antibodies. However, they were unable to detect any antibody in the rabbit sera using immuno-precipitation techniques. They concluded that choline acetyltransferase is not a very antigenic compound. Rossier, Bauman, and Benda (1973) produced precipitating antibodies to rat brain choline acetyltransferase by injecting rabbits with the enzyme in a Freund's adjuvant emulsion. However, only one rabbit produced the antibody and not until a year after the start of immunizaton. Carnitine acetyltransferase was not precipitated by the antibody. There was no apparent species specificity as the antibody reacted with mouse, human, sheep, ox, hamster, and guinea pig brain extracts. However, there was no reactivity with frog brain extract and only limited activity with cat and pigeon brain extracts. Eng, Uyeda, Chao, and Wolgram (1974) have reported production in guinea pigs of a precipitating antibody to bovine choline acetyltransferase in Freund's adjuvant. They have used it in immuno-fluorescent studies of the central nervous system. The antibody could be formed consistently and only required thirty-five days from antigen injection till antibody isolation from sera.

2. Ocular Choline Acetyltransferase

In 1946, two papers appeared that gave the first estimates of choline acetyltransferase levels in ocular tissues. Feldberg and Mann (1946) reported specific activities of 0-15 micrograms acetylcholine formed per hour per gram acetone dried powder (μgACh/hr/gm powder) for canine optic nerve, 400 μg ACh/hr/gm powder for canine retina, and 1170 μg ACh/hr/gm powder for porcine retina. Nachmansohn and Berman (1946) reported that

rabbit optic nerve produced 13-21 micrograms acetylcholine per hour per gram whole tissue (μg ACh/hr/gm tissue). While the levels of choline acetyltransferase activity were low in optic nerve compared to other tissues, Nachmansohn and Berman expressed some surprise that any had been found, since Loewi and Hellauer (1938) had been unable to detect any acetylcholine in optic nerve. de Roetth (1951) found the specific activities of horse and rabbit optic nerve choline acetyltransferases to range from 100-300 μg ACh/hr/gm powder. Cohen (1955) reported rabbit optic nerve activity to be 15-20 μg ACh/hr/gm tissue. While some of the variation in these values was explained by acetone dried powders generally giving specific activities 10-15 times those of whole tissues, Hebb (1955) pointed out that all these assays used a coupled enzyme system. Perhaps optimum concentrations of acetyl-coenzyme A were not being produced. She advised that liver acetyl kinase be added to the incubate to insure saturating levels of acetyl-coenzyme A. Using this technique herself, she found the following values:

	μg ACh/hr/gm powder	
Species	Retina	Optic Nerve
Dog	600-800	0-25
Rabbit	4000-4800	0-12.5
Chicken	22000-32000	0-25
Pigeon	18000-22000	300-425

The rabbit optic nerve values were so much less than those of de Roetth (1951) that she repeated these experiments six times. In three experiments there was no evidence of acetylcholine by bioassay, but in the other three, there was a substance capable of producing muscle contractions which did not appear to be acetylcholine. This substance produced a single vigorous

contraction in guinea pig ileum but was subsequently completely refractory to it. Yet, the ileum remained normally responsive to acetylcholine. In addition, the sensitivity of frog rectus abdominis muscle to this material could vary by as much as 1000% while its response to acetylcholine was stable. Since guinea pig ileum only responded once to this substance while acetylcholine sensitivity remained, she discarded the data obtained on initially applying the test solution and used only the subsequent results. Another problem Hebb acknowledged was that Gardiner and Whittaker (1954) had found pigeon brain capable of synthesizing propionylcholine. The conditions of the in vitro assay for choline acetyltransferase activity might also assay for a propionyltransferase.

On a per gram weight basis, Hebb (1955) found that the central half of the pigeon retina had two-thirds the enzyme activity of the peripheral half. This she attributed to myelinization of the nerve fiber layer lying adjacent to the optic nerve head. Neither the vitreous nor the aqueous humors were found to contain choline acetyltransferase.

de Roetth (1950A) was the first to assay choline acetyltransferase activity in iris and ciliary body. He studied these tissues, as well as retina, in cat and rabbit eyes:

	micrograms ACh formed/hr/gram powder			
	Iris	Ciliary Body	Iris and Ciliary Body	Retina
Cat	158	375	---	420
Rabbit	---	---	105	625

More recently, attention has been turned to the different neuronal components forming the retina. Lam (1972) has shown that nonmammals, such as turtles, have retinal photoreceptors capable of synthesizing acetylcholine from radioactive precursors. Ross and McDougal (1974) have studied

mammalian retinas and found the inner plexiform layer contains the highest level of enzyme activity. In the rat, the specific activities were 114 $\pm$ 16 nanomoles ACh formed/hr/gm dry weight in the inner plexiform layer, 30 $\pm$ 6 nanomoles ACh formed/hr/mg dry weight in the inner nuclear layer, 56 $\pm$ 8 nanomoles ACh formed/hr/mg dry weight in the ganglion cell layer, and less that 1 nanomole in the photoreceptor layer, outer plexiform layer, and optic nerve. A similar distribution was found in mouse and monkey retinas.

The most intensively studied ocular tissue has been the corneal epithelium. Brucke, Hellauer, and Umrath (1949) found that bovine and rabbit corneal epithelium contained 100-200 micrograms acetylcholine per gram of tissue and stated this was the highest concentration reported for mammalian tissues. van Alphen (1957), assaying acetone dried powder of rabbit corneal epithelium, found levels of choline acetyltransferase activity capable of synthesizing 7 micromoles of acetylcholine/hr/gram powder. Williams and Cooper (1965), using bovine corneal epithelium, found the level of activity to be 33 micromoles ACh formed/hr/gram tissue. Corneal stroma only produced 0.7 micromoles ACh/hr/gram tissue. Howard and Wilson (1972) and Howard, Wilson, and Dunn (1973) studied the developing choline acetyltransferase activity in newborn rabbits. It was not until twelve days after birth that the enzyme was first detectable; maximum activities, i.e. adult levels, were attained at about fifty-six days.

Supportive evidence for the presence of choline acetyltransferase in ocular tissues would be the detection of acetylcholine or the finding of clear vesicles by electron microscopy. Acetylcholinesterase activity is not considered a reliable indicator of nearby choline acetyltransferase because the degradative enzyme is found in all mammalian cells. Many

investigators have shown significant levels of acetylcholinesterase in corneal epithelium and lesser amounts in stroma (Petersen, Lee, and Donn, 1965; Williams and Cooper, 1965; Robertson and Winkelmann, 1970). Oer (1961) found high activities in cattle, rabbit, and cat corneal epithelium, but not in dog. Fresh primary aqueous and vitreous humors contain virtually no cholinesterase activity, but secondary aqueous or aqueous removed after eyes have been refrigerated five to six hours do contain increased cholinesterase, mainly butyrylcholinesterase (de Roetth, 1951). The increase in activity in secondary aqueous is attributed to a breakdown of the blood-aqueous barrier and subsequent entrance of butyrylcholinesterase from the serum into the anterior chamber. The increase in cholinesterase activity on storage of the eyes is attributed to postmortum cellular autolysis of tissues bordering the ocular fluids. de Roetth (1950B) reported that the highest cholinesterase activities of intraocular tissues of rabbit, cat, kitten, dog, pigeon, rooster, pig, ox and horse were in the iris, ciliary body, and retina. The retina showed approximately the same enzyme activity in different species, but there was considerable interspecies variation for iris and ciliary body. This degree of cholinesterase activity in iris and ciliary body correlated well with the amount of musculature and parasympathetic innervation. Human iris and ciliary body also contained significant amounts of acetylcholinesterase. These results have been confirmed by Koelle, Wolfand, Friedenwald, and Allen (1951) in cats, Csillik and Halasz (1968) in rats, Dardenne, Leyhecker and Helferich (1957) in man, and Laties and Jackobowitz (1964) in rabbit.

Koelle, Wolfand, Friedenwald, and Allen (1951) showed histochemically that most acetylcholinesterase activity was confined to the amacrine cells of cat retinas. Nichols and Koelle (1967, 1968) also localized the

degradative enzyme to these cells in the inner plexiform layer of pigeon, squirrel, rabbit, rat, and cat.

Low concentrations of acetylcholinesterase, limited to the anterior subcapsular region, have been found in rabbit and human lenses (de Roetth, 1966) and calf lenses (Michon and Kinoshita, 1967).

Acetylcholine, the product of choline acetyltransferase activity, has been assayed in a number of ocular tissues. Brucke (1938) was the first to realize that the cornea is rich in acetylcholine. Using a bio-assay, Brucke, Hellauer, and Umrath (1949) found that rabbit and bovine corneas contained 13 micrograms acetylcholine per gram of tissue. The great majority of this was localized to the corneal epithelium which contained 100-200 micrograms acetylcholine per gram tissue. Hellauer (1950) found the following levels of corneal acetylcholine:

	µg acetylcholine/gm tissue
Dog	0.12
Cat	0.16
Guinea pig, old	13.5
Guinea pig, young	21.8
Rat	1.2
Horse	3.9
Toad, old	0.8
Toad, young	2.5
Man	3.0
Pig	10.8
Rabbit	13.3
Bovine	21.9

He found that the central half of bovine and human corneas contained

twice the acetylcholine of the periphery. The stroma of guinea pigs, on a per gram weight basis, contained less than 4% of the acetylcholine found in the epithelium. Williams and Cooper (1965) reported bovine corneal epithelium contained 38 $\pm$ 2.2 micrograms ACh/gram tissue while stroma and endothelium combined contained less that 0.5% of this amount. The authors had no explanation as to why their level of corneal epithelial acetylcholine was so much lower than that of Brucke, Hellauer, and Umrath (1949). Fitzgerald and Cooper (1971) found the two corneas of the same albino rabbit to have a similar acetylcholine content. The ratio of acetylcholine in the right eye compared to the left was 1.03:1.00 with a standard deviation of 0.089. Although the variation between eyes of the same animal was minimal, the variation in the acetylcholine content between different rabbits was large. The left eyes had a mean value of 253 nanograms ACh/mg protein with a standard deviation of $\pm$160 nanograms. The right eyes had a mean value of 263 nanograms ACh/mg protein with a standard deviation of $\pm$160 nanograms. Howard and Wilson (1972) and Howard, Wilson, and Dunn (1973) also found large interanimal variations in corneal acetylcholine though these differences were smaller in litter mates. At twelve days after birth the corneal acetylcholine was 3% that of the mother; 100% was reached at about fifty days. However, the variation between maternal and sibling corneal epithelial acetylcholine content could be very large. In one of two litters studied at age seventy-five days, the average acetylcholine content of the offspring was 235% that of the mother's. In another litter, the acetylcholine content at age thirty-five days was 307% that of the mother.

The acetylcholine content of the aqueous humor has also been assayed. Since choline acetyltransferase activity has not been detected in this acellular fluid, it is assumed any acetylcholine found would derive from

the tissues surrounding it. These are the corneal endothelium, filtration angle, iris, lens, ciliary epithelium, and vitreous face. Velhagen (1930) could not consistently find acetylcholine in mammalian aqueous humor. Engelhart (1931) found the same inconsistency in aqueous humor of rabbits and cats. However, if rabbits were given physostigmine and placed in light they did demonstrate "vagus-like" activity in their aqueous humors. Similar manipulations did not produce detectable acetylcholine in the cat. Engelhart assumed that the source of the acetylcholine was the parasympathetic innervation of the iris and ciliary body. He mentioned that Loewi, in unpublished experiments, had been unable to demonstrate aqueous humor acetylcholine in rabbits exposed to light but not given physostigmine. Presumably, any acetylcholine released by ciliary ganglion activity had been hydrolyzed by cholinesterase. Luco and Lissak (1938) confirmed Engelhart's results. Bloomfield (1947) found that the aqueous humor of 85% of nonglaucomatous human eyes had detectable parasympathomimetic activity; 75% of eyes with chronic simple glaucoma did not. He proposed that deficient parasympathetic nervous activity caused both the glaucoma and the absence of aqueous humor acetylcholine. Schumacher and Ehl (1974) have used a photometric technique to detect acetylcholine in the aqueous humor of cats and rabbits. The level of acetylcholine was too low to detect in individual eyes, despite the addition of a cholinesterase inhibitor to the puncturing syringe. Samples were pooled and rabbits were found to contain 690 nanograms ACh per ml of aqueous humor. For cats, the value was 360 nanograms.

The iris and ciliary body (Velhagen, 1930, 1936) were also shown to contain an acetylcholine-like substance. Engelhart (1931) found that electrical stimulation of the oculomotor nerve of rabbits and cats

increased the level of acetylcholine in these tissues, but severing the nerve caused the material to disappear within fourteen days. The source of the acetylcholine was therefore presumed neuronal. Ehinger, Falck, Persson, Rosengren, and Sporrong (1970) found a mean value of 4.6 micrograms ACh per gram of cat iris. Sympathetic denervation of the iris did not alter the acetylcholine level while parasympathetic denervation led to its disappearance. The choroid, which, along with the iris and ciliary body, forms the uveal tract, has been studied by Kovacik, Jezek, and Mrazova (1973). They found 2-6 nanograms of acetylcholine per milligram of bovine choroidal tissue. Interestingly, the level of choroidal acetylcholine was higher than that found in the retina (Kovacik, Jezek, and Mrazova, 1972, 1973).

The ocular neuroepithelial tissues, retina and pigment epithelium, have been examined for the presence of acetylcholine. Velhagen (1932, 1936) investigated the retinas of cats and Kovacik, Jezek, and Mrazova (1972) studied bovine retinas. Both species were found to contain retinal acetylcholine. Kovacik (1974) assayed the pigment epithelium and obtained values of 30 nanograms ACh per 50 mg tissue. Dowling and Boycott (1966) and Curtis (1969) have observed vesicles in retina, using electron microscopy, that are similar to those associated with acetylcholine storage.

An intriguing problem has been the role played by corneal epithelial choline acetyltransferase in ocular function. Classically, acetylcholine has been considered the chemical transmitter released by neurons at synapses, myoneural junctions, and parasympathetically innervated smooth muscle. None of these structures is present in corneal epithelium. The only significant nerve supply to the cornea consists of sensory fibers from the ophthalmic division of the trigeminal nerve

(Duke-Elder, 1961). This division gives off the nasociliary branch, which in turn gives off the two long posterior ciliary nerves. These travel toward the globe and penetrate the posterior sclera slightly anterior to the short ciliary nerves. The long posterior ciliary nerves run between sclera and choroid in the suprachoroidal space accompanied by autonomic fibers and the long posterior ciliary arteries. In the region of the ciliary muscle a plexus is formed, branches of which pass to sclera, episclera, and conjunctiva. Fibers from these regions join again and form a series of pericorneal plexuses in the limbal region. Zander and Weddell (1951) found a variable number of nerve bundles supplied the cornea, depending on the species: 22-29 in frog, 61-68 in rabbits, and 76-84 in man. The bundles enter the corneal stroma, form a plexus, and penetrate Bowman's membrane almost perpendicularly to reach the epithelium. At the limbus a few fibers enter the corneal epithelium from the conjunctiva. On entering the corneal epithelium, the axons no longer have Schwann cells and run their course naked. These axons subdivide in a radial fashion and run between the epithelial cells. Many axons terminate in the plane of the basal layer of the epithelium. The majority of axons terminate freely without interconnections. Thickenings, called beads, are found along the course of the axons. The terminal bead is commonly somewhat larger than the other beads. Zander and Weddell found that cervical sympathectomies in rabbits did not diminish the number of nerve fibers in the cornea. However, they do not rule out the presence of a rare autonomic fiber. Bell, Kirby, and Rodger (1952) performed intracranial transection of the trigeminal or oculomotor nerve in cats. Cervical sympathectomies were performed on another group. Corneal degeneration occurred only after trigeminal neurectomy. Rodger (1953) repeated these

experiments performing histologic studies as well. These showed complete degeneration of corneal neurons following trigeminal nerve transection; no degeneration followed severing the nerve supply from the oculomotor nerve and cervical sympathetic ganglia. Ehinger (1966) reported that adrenergic neurons could be identified by fluorescent techniques in embryonic corneas of rabbits, dog, cats, guinea pigs, and man, but the intra-epithelial fibers disappeared shortly after birth. Wolter (1964) stated that the Krause end bulbs, which serve the sensation of cold and are found at the corneal limbus, seemed to have an innervation that morphologically appeared to be autonomic fibers. If autonomic nerve fibers are found in the corneal epithelium, they are few in number and confined to the limbal area. While Zander and Weddell believe the sensory nerve endings lie exclusively between epithelial cells, Scharenberg (1955) described terminations intra-cellularly both to cytoplasm and nuclei.

Brucke (1938) and Brucke, Hellauer, and Umrath (1949) initially stressed a trophic effect for corneal acetylcholine. They noted that severing the trigeminal nerve produced degeneration of the epithelial cells. Clinically, topical application of acetylcholine appeared to improve patients with neuro-paralytic keratitis. The assumption was that the choline acetyltransferase resided in the sensory nerve endings and the acetylcholine produced stimulated epithelial cell growth. After severing the trigeminal nerve in rabbits, however, only a 35-50% decrease in acetylcholine was produced in the desensitized cornea. Another possible role for corneal acetylcholine was investigated by Hellauer (1950): Perhaps acetylcholine was involved in corneal sensation and was synthesized not in the neurons of the trigeminal nerve but rather in epithelial sensory

receptors. Physical stimulation of the cornea mechanically, as by touch, would cause receptor release of acetylcholine. The acetylcholine would then serve as a receptor-to-neuron transmitter. This concept meant that acetylcholine was being produced in non-neuronal tissue. Brucke, Hellauer, and Umrath (1949) had found that topical anesthetics diminished corneal epithelial acetylcholine, which seemed consistent with the theory. Hellauer (1950) measured the threshold of corneal sensory stimulation using von Frey hairs and correlated the results with the corneal acetylcholine level:

Animal	Averaged Value	
	ACh µg/gram cornea	Threshold Pressure(mg)
Dog	0.12	540
Cat	0.16	380
Guinea pig, old	13.5	310
Guinea pig, young	21.8	310
Rat	1.2	100
Horse	3.9	50
Toad, old	0.8	47
Toad, young	2.5	30
Man	3.0	41
Rabbit	13.3	37
Bovine	21.9	18

Hellauer interpreted these results as indicating that the lower the corneal acetylcholine content, the lower the corneal sensitivity. The exceptions, toads and guinea pigs, were vaguely explained as probably being due to "differing corneal sizes and other structural differences." Hellauer's finding that central corneal acetylcholine content was twice

that of the periphery both in cattle and man would imply, according to his theory, that corneal sensation was greater centrally than peripherally. Work by Boberg-Ans (1955) indicates that this is so. He found human central corneal sensitivity to be somewhat greater than peripheral. Umrath and Mussbichler (1951) found that atropine sulfate, given topically to rabbits, raised the corneal touch threshold so that it approximated that of less sensitive species. That atropine did not completely block corneal sensation was attributed to the ability of the free nerve endings themselves to respond to the stronger stimuli. These authors believed only sensitivity above this baseline required the chemical transmitter, acetylcholine. van Alphen (1957) states, however, that in rabbits and humans, he found no diminution of pain or touch sensations after applying topical atropine or cholinesterase solutions. Further, physostigmine did not enhance these sensations. Oer (1961) found that the cholinergic blocking agents atropine and oxyephedrine decreased rabbit corneal sensitivity while curare and hexamethonium did not. Yet eserine did not increase corneal sensitivity when applied topically. A solution of 0.1% acetylcholine produced corneal pain. Interestingly, lower concentrations of propionylcholine (0.001%) and of butyrylcholine (0.01%) were more effective in producing pain. If atropine or oxyephedrine were applied before the choline esters, 1% acetylcholine had no pain producing effect and sensitivities to propionylcholine and butyrylcholine were decreased by factors of 1000 and 100 respectively. Surprisingly, sensitivity to the choline esters was also decreased by prior pretreatment with hexamethonium. Acetylcholine and butyrylcholine were effective in lower concentrations if pre-treated with eserine. Oer argued that the decreased sensitivity produced by atropine could not be explained, as Witzleb (1959) had proposed, by an anesthetic property of the drug. He

cited Umrath and Mussbichler (1951) who had shown that while the rabbit corneal touch sensitivity can be decreased to the level of dogs and cats, the touch sensitivity of canine and feline subjects could not be further lowered by atropine. Oer (1961) also found that epinephrine, 0.1%, while not having any effect of its own did increase corneal sensitivity to the choline esters by two log units. While these results defied a coherent interpretation, his study of interspecies sensitivity to acetylcholine solutions was more suggestive:

	Average Threshold Pressure Producing Blink (mg) (Hellauer)	Threshold Concentration ACh Producing Blink (molar)
Dog	540	10^{-5}
Cat	380	10^{-5} to 10^{-6}
Guinea pig, old	310	10^{-4}
Guinea pig, young	100	10^{-3} to 10^{-4}
Rat	100	10^{-4}
Horse	50	10^{-3} to 10^{-4}
Toad	47	10^{-2}
Man	41	10^{-4}
Rabbit	37	10^{-3} to 10^{-4}
Bovine	18	10^{-3} to 10^{-4}

He concluded that the lower the corneal sensitivity (using Hellauer's data), the greater that species' sensitivity to exogenous solutions of acetylcholine. This was a rather surprising result since it implied that species with low endogenous corneal acetylcholine need not have low corneal sensitivity. Yet Hellauer's data had indicated that low corneal acetylcholine correlated well with low corneal sensitivity. Oer wished to rule

out acetylcholinesterase as the explanation, i.e. increased corneal acetylcholine meant increased corneal acetylcholinesterase and it might be the latter that diminished the effectiveness of exogenous acetylcholine. To do so he tested with arecoline, which is not hydrolyzed by acetylcholinesterase. The sensitivity to arecoline paralleled that of acetylcholine except in guinea pigs and rabbits where concentrations up to and including 1% had no effect. Unable to use acetylcholinesterase to explain his results, Oer was forced to interpret his data as indicating acetylcholine was not directly involved in receptor-to-nerve ending transmission.

Fitzgerald and Cooper (1971) excised superficial cornea from rabbits in circular bands 2 mm. wide and 0.3 mm. deep in an attempt to transect the sensory nerve supply to this area. The beds of keratectomized tissue were 5 to 7 mm. from the corneal centers. To their surprise, instead of an immediate anesthesia occurring, it took ten days for the corneal touch-blink reflex to disappear. By then corneal acetylcholine had decreased 87-100% when compared to the unoperated eye. This was interpreted as indicating a neuronal, rather than epithelial, location of the acetylcholine. They next injected hemicholinium subconjunctivally or intra-ocularly to inhibit acetylcholine synthesis. When acetylcholine levels fell to about 40% that of the control eye, corneal anesthesia was present. In 4-6 hours the acetylcholine content had returned to at least 60% of the control eye value and the touch-blink reflex was intact. This, they concluded, was suggestive of a role for acetylcholine as a sensory mediator. Stevenson and Wilson (1973), however, found that intra-ocular injection of trans-4-(1-naphthylvinyl) pyridinium hydrochloride, a choline acetyltransferase inhibitor, resulted in a 62% decrease in corneal acetylcholine without

abolishing the corneal reflex. This indicated that depletion of acetylcholine levels by 60% or more does not per se abolish corneal sensitivity.

Howard and Wilson (1972) and Howard, Wilson, and Dunn (1973) found that newborn rabbits did not have detectable corneal acetylcholine or choline acetyltransferase activity until their eyelids opened at about age ten days. Adult levels were not attained until about age fifty days, yet the corneal blink reflex to touch was intact by age twelve days. Acetylcholinesterase levels were greater than adult levels shortly after birth, increased further to a peak activity at day 3-10, and then decreased to adult levels about twenty-five days after birth. The low level of acetylcholine content and choline acetyltransferase activity at age twelve days when the blink reflex is intact indicates that little, if any, acetylcholine is involved in corneal sensation. Gnadinger, Heimann, and Markstein (1973) and Gnadinger, Walz, Hahn, and Grun (1967) have given supportive evidence for the belief that choline acetyltransferase activity resides in the corneal epithelial cells. They found that rabbit corneal epithelial cells cultured 6-9 days maintained a choline acetyltransferase activity about 50% that of freshly scraped tissue; the cholinesterase activity was greater in the cultured cells than in the fresh tissue.

Other roles than sensation have been suggested for corneal acetylcholine. Williams and Cooper (1965) proposed that acetylcholine may be involved in regulation of water and ion transport. They incubated the entire bovine eye at temperatures of 4°C and 37°C. A correlation was made of corneal swelling at 4°C, or deturgescence at 37°C, with the formation of acetylcholine. They found that hypothermic corneal swelling was associated with a fall in epithelial acetylcholine. Raising the temperature to 37°C

after hypothermia caused a return of acetylcholine content to control levels (the controls being kept at 37°C continuously). However, there was only a 50% deturgescence of the excess fluid. Placing a metabolic inhibitor, iodoacetate, in the aqueous humor did not, at 37°C, affect the level of corneal acetylcholine. However, the corneas did swell significantly. At 4°C, the iodacetate treated eyes had levels of acetylcholine half that of eyes without iodoacetate but the degree of swelling was no greater. Elevating the temperature to 37°C produced a return to control levels of acetylcholine without any deturgescence. They concluded that corneal acetylcholine content and corneal hydration varied independently of each other.

Petersen, Lee, and Sonn (1965) and Gnadinger, Heimann, and Markstein (1973) hypothesized that acetylcholine might increase the permeability of basal epithelial cells to the sodium of tear fluid just as it increases the permeability of nerve fibers to sodium from the extracellular fluid. No experiments were performed by these groups to support this theory.

Fitzgerald and Cooper (1971) covered one eye of rabbits for 2.5 to 4 hours in a lighted room. No correlation could be detected between corneal exposure to light and acetylcholine content of the epithelium. Howard, Wilson, and Dunn (1973), who found a correlation of lid opening in new born rabbits with the presence of acetylcholine and choline acetyltransferase, state that, "....it is possible that part of the acetylcholine system in the superficial cornea represents some adaptive developmental response of the cornea to the new environments of the atmosphere and light."

3. Areas of Investigation

The studies described in the ensuing pages deal with ocular choline acetyltransferase activity and its product, acetylcholine. The initial approach was to evaluate, tissue by tissue, the enzyme activity in the eyes of four different mammalian species: human, rabbit, bovine, and feline. The *in vitro* assay technique used synthetic [C^{14}] acetyl-coenzyme A as a substrate; an ion exchange resin was employed to separate this substrate from the labelled product. Use of a styrylpyridine inhibitor of choline acetyltransferase permitted identification of this enzyme's activity as opposed to that of other acyl transferases. Endogenous tissue acetylcholine was identified by pyrolysis-gas chromatography.

The ocular tissues with significant choline acetyltransferase activity included the iris, ciliary body, retina, and pigment epithelium-choroid. However, emphasis was placed on the corneal epithelium. The confirmation that large quantities of choline acetyltransferase activity are present in this tissue led to investigations aimed at determining whether the enzyme was located in the sensory nerve endings of the ophthalmic branch of the trigeminal nerve or in the squamous epithelial cells of the cornea. Another interesting aspect of corneal epithelial choline acetyltransferase was the marked inter-animal variation found in enzyme activity. Investigations were also directed, therefore, towards elucidating those factors that might produce this large animal-to-animal difference in corneal choline acetyltransferase activity. The effects of inbreeding, tarsorrhaphy, and drugs applied topically to the cornea were investigated to learn if they could be used to manipulate the level of enzyme activity.

MATERIALS AND METHODS

1. Ocular tissue

   A. Rabbit: Female Dutch Belt rabbits, 1.5 - 2.5 kg., were used almost exclusively. Several experiments required litter-mates or highly inbred Dutch Belt rabbits[1] (inbreeding co-efficient= 82-85%). Albino rabbits were used rarely because of a desire to test normally pigmented eyes. Rabbits were killed using intravenous or intraperitoneal pentobarbital or secobarbital; in some comatose animals, death was hastened with intravenous air.

   B. Bovine: Cattle eyes were enucleated at an abattoir and delivered within 5 hours of death.

   C. Feline: Female mongrel cats were killed with intraperitoneal pentobarbital.

   D. Human: Aqueous humor and intraocular lenses were obtained[2] hours to days after death; the eyes had been kept at room temperature and 4°C for variable lengths of time.

Corneas were dissected by either scraping off the epithelium with a blade or removing a 9 mm. diameter full thickness corneal button with a trephine. The iris and ciliary body were removed by excising the 4 mm. of sclera posterior to the corneal limbus. A 360 degree incision was made at the iris base so that this tissue could be removed. A second circular incision was then made at the exposed ora serrata and the ciliary body was dissected free.

---

1. Jackson Laboratory; Bar Harbor, Maine.
2. Eye-Bank for Sight Restoration; New York, New York.

In rabbits, the ciliary body is poorly developed and both it and the iris were excised and assayed together. The vitreous was removed by gently squeezing the remaining scleral coat from posterior to anterior. A stream of saline was directed at the edge of retina freed by the previous ora serrata incision in order to float the sensory neuro-epithelium away from the underlying pigmented layer. The dissection of retina was completed by severing its only attachment at the optic nerve. The thin and fragile retina was removed in pieces rather than as a single entity. The pigment epithelium and choroid were removed together using blunt dissection to break the latter's attachments to the sclera.

Eyes to be assayed for acetylcholine content were dissected rapidly and the tissues placed in acetonitrile. If choline acetyltransferase activity were to be measured, the tissue was placed in the pH 7.4 buffer solution used in that assay and stored at -5^{o} C. or -94^{o} C.

2. Acetylcholine assay -

The pyrolysis-gas chromatography technique used to measure acetylcholine was based on the method originally described by Szilagyi, Schmidt, and Green (1968).

A. Initial method:

1. The ocular tissue is placed immediately after dissection into 3 ml acetonitrile containing an appropriate amount of internal standards: 50-500 nanograms each of propionylcholine and valerylcholine. The tissue is homogenized.

2. Centrifuge for 20 minutes at room temperature and 6000

r.p.m. Transfer the supernatant with a Pasteur pipet to a conical centrifuge tube.

3. With solid samples, add 3.0 more ml.acetonitrile to the precipitate and repeat the preceding step. Save the precipitate to determine tissue protein content using the method of Lowry, Rosebrough, Farr, and Randall (1951).

4. Add 0.5 ml. aqueous solution of 5% sodium sulfate (anhydrous basis) and 0.11% anhydrous calcium chloride.

5. Add 2 ml. toluene for every ml. acetonitrile. Mix well and centrifuge 5 minutes at room temperature and 2500 r.p.m. Aspirate and discard the top organic layer.

6. Add 2 ml. hexane for each ml. of remaining aqueous phase. Mix well and centrifuge 5 minutes at room temperature and 2500 r.p.m. Aspirate and discard the top organic layer.

7. Direct a nitrogen gas stream at the surface of the water phase until the odor of any remaining hexane has disappeared.

8. Adjust the volume to 1 ml. and pH 4.3 with HCl. Transfer the solution to a 3 ml. test tube.

9. Add 0.2 ml. of a solution containing 31.4% iodine and 40% potassium iodide. Place on ice 20 minutes and centrifuge 20 minutes at 2500 r.p.m.

10. Aspirate and discard the water layer, being careful to avoid the small amount of precipitate.

11. Prepare micro ion exchange columns:

    a) cut in half capillary melting point tubing, 150 mm. x 10 mm.

internal diameter,

b) force a small piece of polyethylene tubing, 1.1 mm. outside diameter, into one end to form a connection for a #25 hypodermic needle,

c) using a needle and syringe, draw a few particles of Rexyn[3] RG-3 resin, 10-50 mesh, chloride form, into the tube.

d) fill the rest of the tube with AG-3 resin,[4] 100-200 mesh, chloride form. (Resins are suspended in water to facilitate being drawn into tubing.)

e) store micro ion exchange columns in water until use.

12. Add 100 microliters of a solution of 85 ml. acetonitrile and 15 ml. water (85/15) to dissolve the precipitate.

13. Rinse the micro ion exchange column by slowly drawing through it 200 microliters of the 85/15.

14. Draw the sample through column slowly to exchange chloride ions for iodide and tri-iodide ions.

15. Add another 100 microliters of 85/15 to the test tube and draw it through the resin.

16. Place an appropriate aliquot of the 200 microliter sample on a platinum pyrolysis ribbon heated by a hot plate to approximately 120° C.

17. Place the dried ribbon in the pyrolysis chamber connected to a gas chromatograph containing a column of:

---

3. Fisher Scientific Company; Pittsburgh, Pennsylvania.

4. Bio-Rad Laboratories; Richmond, California.

a) 2.5 meters long aluminum tubing, 3.18 mm. outside diameter.

b) 2% Ucon 50 HB 660 on Cerabeads, 80-100 mesh, unsilanized.[5]

c) temperature 87° C, N_2 flow rate approximately 80 ml/minute at 70 psig.

18. Pyrolize the sample at a temperature and for a length of time previously determined to give maximum conversion of acetylcholine to dimethylaminoethylacetate.

19. Measure the peak areas on the chromatogram for dimethylaminoethylacetate and dimethylaminoethylpropionate. Their ratio is used to compute the acetylcholine content using a standard curve.

B. Improved method:

1. Is same as preceding method through step 8 except:

a) valerylcholine deleted as internal standard.

b) 1.0 ml. HCl, pH 4.3, added as part of step 4.

2. Cover the mouth of the conical centrifuge tube with gauze held in place by a rubber band. Place the sample in a freezer until solid.

3. Lyophilize 24-48 hours till dry. Place the sample in a desiccator until it returns to room temperature.

4. Remove the gauze and add acetonitrile, 1.0 ml. Gently agitate.

5. Centrifuge at room temperature 5 minutes at 2500 r.p.m.

6. Transfer the clear supernatant into a test tube, 75 x 100 mm., using a Pasteur pipet.

7. Add 1.0 ml. acetonitrile to the conical test tube and repeat the preceding two steps.

8. Direct a nitrogen gas stream at the 2 ml. acetonitrile

---

5. Analabs; North Haven, Connecticut.

sample until the volume is reduced by evaporation to approximately 50 microliters.

9. Place an appropriate aliquot of the sample on a platinum pyrolysis ribbon heated by a hot plate to approximately 120°C.

10. Add to the dried sample 1.5 micrograms butyrylcholine in 1.5 microliters acetonitrile.

11. After the sample has dried, place the platinum ribbon in a pyrolysis chamber connected to a gas chromatograph whose column consists of:

a) 3.7 meters long aluminum tubing, 3.18 mm. outside diameter,

b) 10% Poly A-135 on Gas-Chrom Q, 100-120 mesh[6].

c) temperature 115° C, N_2 flow rate approximately 100 ml/minute at 85 psig.

12. Pyrolyze the sample and calculate the acetylcholine content as in preceding method.

3. Choline acetyltransferase activity assay -

The assay is based on the methods of McCaman and Hunt (1965) and Schrier and Shuster (1967) as modified by Mittag (Glick, Mittag, and Green, 1973; Barker and Mittag, in press).

A. The tissue is homogenized on ice in 2-15 ml. of a pH 7.4 buffer a solution made up so as to give a final incubation medium containing

1. a tissue concentration less than 10% wt/volume.
2. 0.5% Triton-X.
3. 10 mM disodium ethylenediamine tetra-acetate (EDTA).
4. 300 mM sodium chloride.
5. 150 mM potassium phosphate, monobasic.

---

6. Applied Science Laboratories, Inc.; State College, Pennsylvania.

B. Each tissue assay requires 4 tubes, 6 x 50 mm., containing 200 microliters of homogenate-pH 7.4 buffer solution.

C. The tubes are kept on ice. Solutions are added to give a 220 microliter incubation volume and the following final concentrations:

1. Tube 1 - 2 mM dithiothreitol, 0.1 mM physostigmine, and 0.2 mM acetyl-coenzyme A[7] containing sufficient acetyl-1-C^{14}-coenzyme A[8] to give approximately 1 count per minute per picomole.

2. Tubes 2 and 3 - same as tube 1 with the addition of 5 mM choline chloride.

3. Tube 4 - same as Tube 1 with the addition of 0.1 mM naphthylvinylpyridinium hydroxyethyl bromide.

D. The tubes are incubated 1 hour at 37.5° C. The reaction is terminated with 1 drop of $CuCl_2$, 2.5%, per tube.

E. The content of each tube is quantitatively transferred to an ion exchange column made by plugging a Pasteur pipet with glass wool and filling it with Bio-Rad[9] AG 1-X8, 200-400 mesh, chloride form ion exchange resin in water.

F. The sides of the tubes are washed 5 times with 0.2 ml. distilled water and the water is then transferred to the resin column after each wash.

G. Effluents are collected directly in scintillation vials. Brays solution, 10 ml.is then added to each vial.

---

7. Sigma Chemical Company; St. Louis, Missouri.
8. New England Nuclear; Boston, Massachusetts.
9. Bio-Rad Laboratories; Richmond, California.

H. In a fifth vial, place an aliquot of the acetyl-coenzyme A solution in Brays solution, 10 ml.

I. Count the beta irradiation emissions of the 5 vials using a liquid scintillation counter. The results are calculated by subtracting Tube 4's value from that of Tube 1; this indicates the degree of acetylation of endogenous tissue choline. Subtracting Tube 4's value from the averaged value of Tubes 2 and 3 indicates the total acetylation of endogenous and exogenous choline.

The physostigmine solution is made up daily. The naphthylvinyl-pyridinium hydroxyethyl bromide, dithiothreitol, choline chloride, and acetyl-coenzyme A solutions are made up weekly and stored frozen.

4. Amino acid incorporation into corneas -

A. The corneas are excised at the limbus and placed in a 5 ml Ringer's solution, pH 7.48, containing 10 mM leucine with 10 μCi H^3-leucine and 10 mM alanine with 10 μCi C^{14}-alanine.

B. Incubate 6 hours at 37.5° C with 99% O_2 - 1% CO_2 bubbling through the media.

C. Remove the corneas and wash them with distilled water. Place them in conical centrifugation tubes.

D. Add 5 ml. trichloroacetic acid, 10%, and sonicate to break up the corneal epithelium.

E. Leave the samples at 4° C for 12 hours.

F. Centrifuge at 5000 r.p.m. for 15 minutes. Aspirate most of the trichloroacetic acid and discard being careful not to disturb the precipitate.

G. Wash the precipitate 5 times by adding 5 ml. trichloroacetic acid, 10% and repeating the centrifugation.

H. Dissolve the corneal material in 3 ml. Soluene.[10]

I. Add 15 ml of scintillation fluid cocktail made up by dissolving 4 gm. 2,5-diphenyloxazole (PPO) and 50 mg. 1,4-bis-(5-phenyloxazol-2-yl) benzene (POPOP) in 1 liter toluene.

J. Transfer to counting vials and place in liquid scintillation counter.

5. Lactic dehydrogenase assay -

A. The corneas are excised at the limbus and placed in a tissue grinder tube containing 2 ml. saline, 0.9%.

B. The corneas are homogenized and then centrifuged 15 minutes at 10,000 r.p.m.

C. The supernatant is removed and stored frozen.

D. The assay is performed using a commercial preparation[11] of lactate and NAD (Enztrate) as the enclosed brochure describes.

E. NADH formed during the conversion of lactate to pyruvate is measured spectrophotometrically at 340 nm.

6. Tissue culture of corneal epithelium -

Rabbit corneal epithelium was cultured using a method based on that described by Gnadinger, Heimann, and Markstein (1973).

A. The corneal epithelium is removed, along with some underlying stroma, by lamellar keratectomy using a Gill knife.

B. Place each corneal sample in a separate Petri dish, stroma side down and cut it into 4 pieces. The Petri dish bottom has been pre-moistened with growth media consisting of:

---

10. Packard Instrument Company, Inc.; Downers Grove, Illinois.
11. Beckman Instruments Company; Fullerton, California.

1. Hanks balanced salt solution with L-glutamine but without $NaHCO_3$ (10 XM - 199)[12], 40 ml.

2. 5% $NaHCO_3$, 5.2 ml.

3. Streptomycin, 40 mg.

4. Potassium penicillin G, 40,000 units.

5. Preheated (to 56° C for 30 min before storage) fetal calf serum[12], 80 ml.

6. Water, 314.8 ml.

C. The Petri dishes are kept at 37.5° C in air for 1 hour.

D. Growth media, 5-10 ml. is added sufficient to immerse the explants.

E. Incubate 7 days at 37.5° C in air with 5% CO_2.

F. Remove and replace the media every third day.

G. To terminate the incubation, remove the media and add 10 ml phosphate buffer, pH 7, 37.5° C. Aspirate and discard this phosphate buffer; repeat twice more to remove any remaining serum protein.

H. The original explants are removed and discarded.

I. 3 ml. of the pH 7.4 buffer solution used in the choline acetyltransferase activity assay is added to the Petri dishes.

J. The dishes are incubated 10-15 minutes at 37.5° C to allow the Triton-X time to disrupt any cellular adhesion to the glass.

K. The contents of the Petri dishes are transfered to homogenizing tubes, homogenized and then assayed for choline acetyltransferase activity.

---

12. Microbiological Associates; Bethesda, Maryland.

7. Measurement of intraocular pressure-

A. Rabbit: A Schiotz tonometer with 5.5 gram weight is placed on the eye of a rabbit held so that the anterior-posterior axis of globe is vertical. The eye has been anesthetized seconds before with the topical anaesthetic proparacaine, 0.5%.[13]. Measurements are recorded as the scale units of the tonometer.

B. Humans: A drop of proparacaine is instilled into the conjunctival sac bilaterally. A paper strip containing fluorescein dye is placed on the conjunctiva of each lower lid. The intraocular pressure is measured using a Goldmann tonometer.

---

13. Allergan Pharmaceuticals; Irvine, California.

RESULTS

1. Development of an improved acetylcholine assay:

Two faults were detected in the initial method of pyrolysis gas chromatographic assay of acetylcholine. One was that small amounts of AG-3 ion exchange resin were contaminating the samples and giving extraneous peaks. Either pieces of resin were being drawn through the micro ion exchange columns or a contaminant was being solubilized by the acetonitrile-water solution. Figure 1 shows the extraneous peaks resulting from pyrolysis of a 2 microliter aliquot of acetonitrile-water that had been drawn through a micro ion exchange column containing AG-3 resin. Changing to different resins gave different contamination peaks but did not prevent them. Prior resin washing with acetonitrile-water did not solve the problem. Interposition of a 0.22 μ millipore filter between the syringe and ion exchange column was also unsuccessful, indicating the problem was a soluble impurity. Ultimately the extraction procedure had to be altered so as not to require an ion exchange resin at all.

The second problem was that if one pyrolyzed aliquots of a solution of acetylcholine containing less than a microgram of the neurotransmitter, the peak areas of dimethylaminoethylacetate on the chromatograms varied considerably. In effect, it meant that there was poor reproducibility of the assay in the nanogram range. The problem appeared to be either that pyrolysis of samples was incomplete or that for some unknown reason, not all the pyrolysis products were passing out of the chamber and through the chromatograph column. While the exact cause of the problem could not be determined, it could be prevented by increasing the size of the sample on the ribbon. For

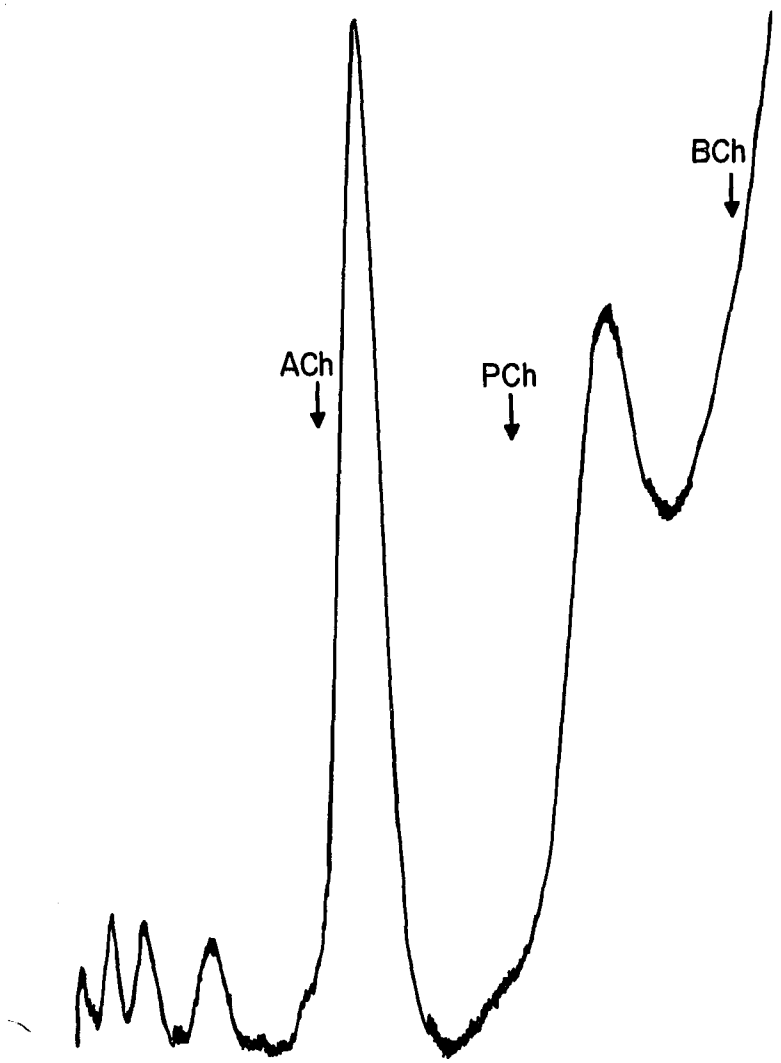


Figure 1. Extraneous peaks resulting from pyrolysis of a 2 microliter aliquot of acetonitrile - water drawn through an ion exchange column containing AG-3 resin. Arrows indicate the retention times of dimethylaminoethylacetate (ACh), dimethylaminoethylpropionate (PCh), and dimethylaminoethylbutyrate (BCh). Attenuation 1 X 10^2.

example, if 1.5 micrograms of choline, butyrylcholine, or valerylcholine were added to the platinum ribbon prior to pyrolysis, both the sensitivity and reproducibility of the method were improved. Figure 2 shows that, by increasing the amount of quaternary ammonium compounds pyrolyzed, the peak area representing 100 nanograms acetylcholine increases from undetectable at 1 X 10^2 attenuation to easily detectable at 4 X 10^2 attenuation. Figure 3 shows the same phenomenon in graph form and indicates that the optimum maximizing effect on the peak area representing 100 nanograms acetylcholine is attained with 1200 nanograms of choline. One could consistently obtain chromatograms giving measureable dimethylaminoethylacetate peaks from as little as 5 nanograms of acetylcholine (Figure 4). Two nanograms of acetylcholine were detectable although the chromatograph peaks often could not be accurately measured.

2. Acetylcholine content of ocular tissues:

Using the improved method of acetylcholine assay (Szilagyi, Mindel, Goldberg, Green, and Blaiklock), the various ocular tissues were examined in different species. Acetylcholine was detected (Figure 5) in albino rabbit cornea, aqueous humor, iris-ciliary body, and retina. Vitreous humor contained a substance(s) that was not removed by the extraction procedure and which gave large extraneous interfering peaks on pyrolysis; the chromatograms were uninterpretable. Lens capsule and nucleus contained no detectable acetylcholine.

A pooled sample of the corneal epithelium of 40 bovine eyes was stored in a container without acetonitrile at -17°C. Aliquots were removed and assayed on days 1, 7 (Figure 6), and 42. Table 1 shows the progressive fall in acetylcholine content with time. Residual levels of acetylcholine were also present 13-17 hours after death in human

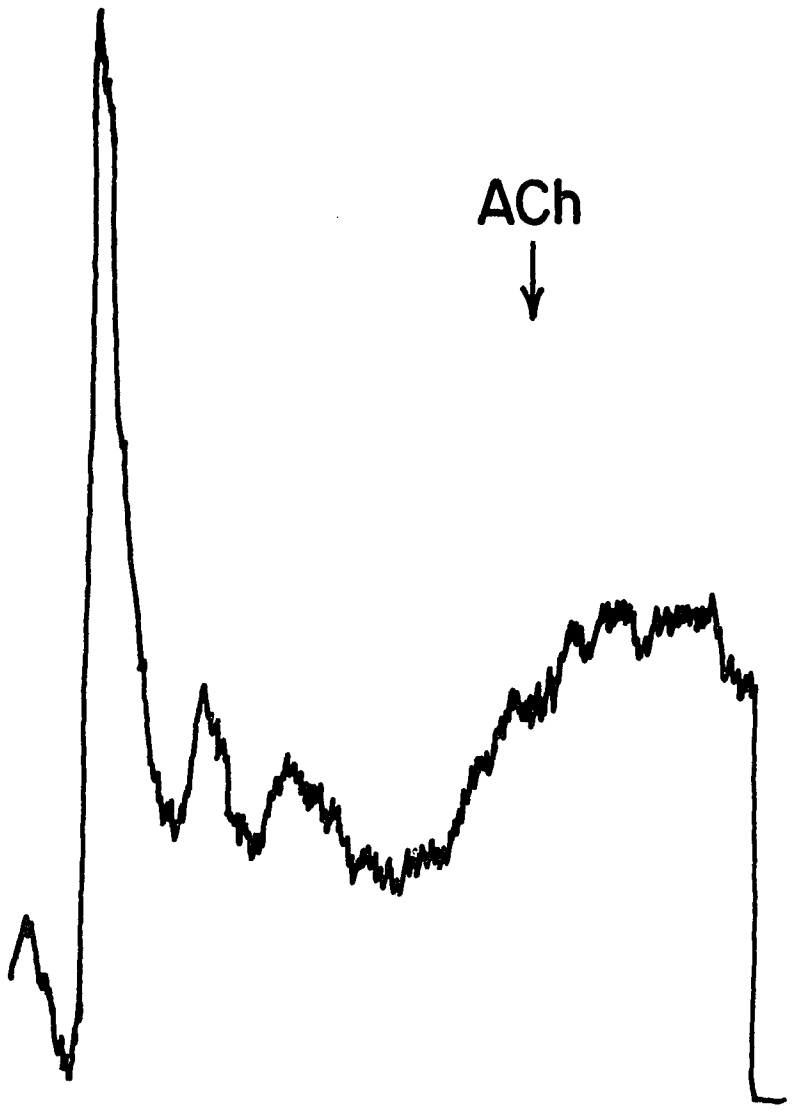


Figure 2. Increased peak size of dimethylaminoethylacetate (ACh) produced from 100 ng acetylcholine as the amount of choline added to the pyrolysis ribbon is increased: a) no added choline. Attenuation 1 X 10^2.

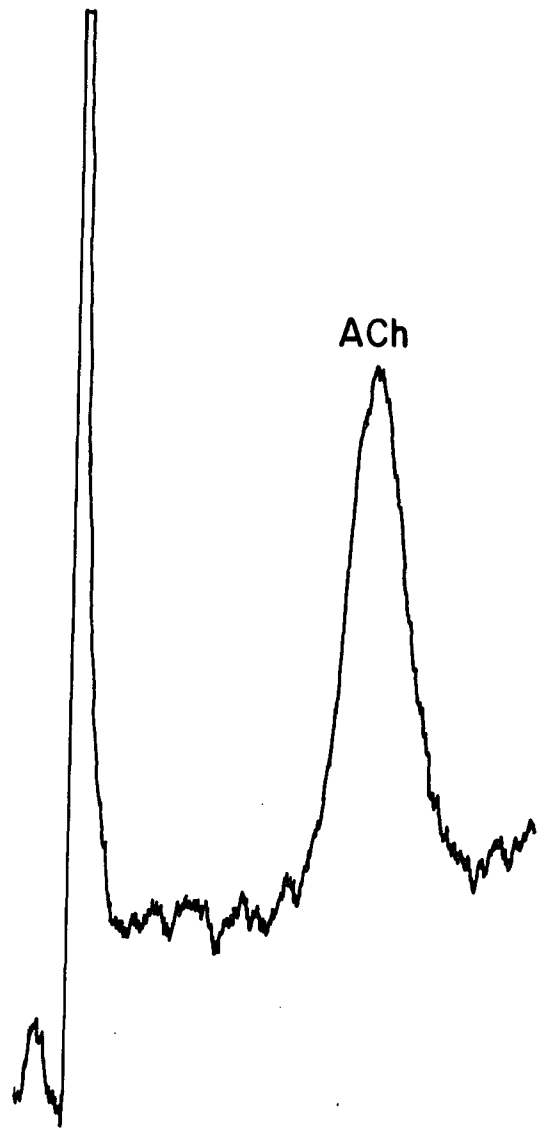


Figure 2. Increased peak size of dimethylaminoethylacetate (ACh) produced from 100 ng acetylcholine as the amount of choline added to the pyrolysis ribbon is increased: b) 200 ng choline added. Attenuation 1 X 10^2.

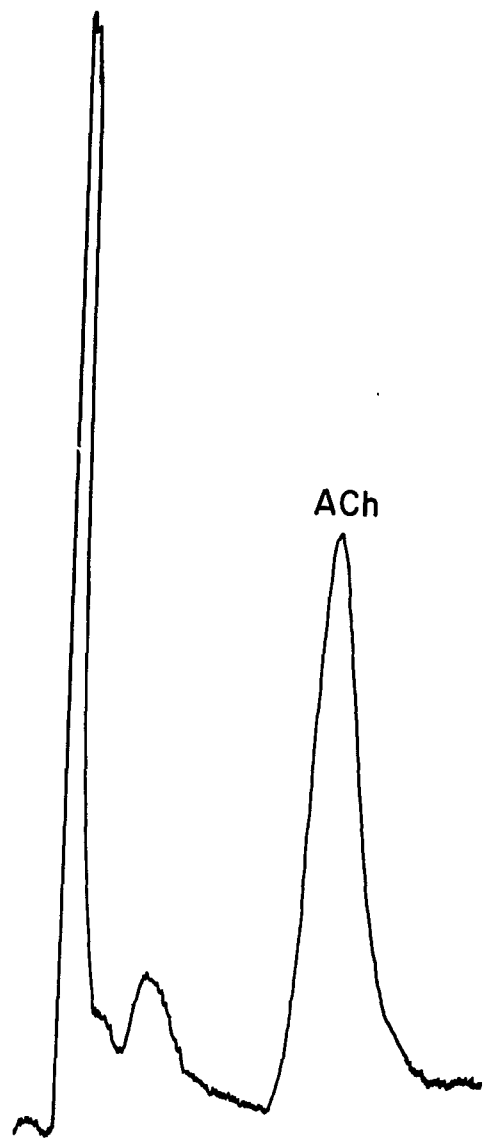


Figure 2. Increased peak size of dimethylaminoethylacetate (ACh) produced from 100 ng acetylcholine as the amount of choline added to the pyrolysis ribbon is increased: c) 1600 ng choline added. Attenuation 4 X 10^2.

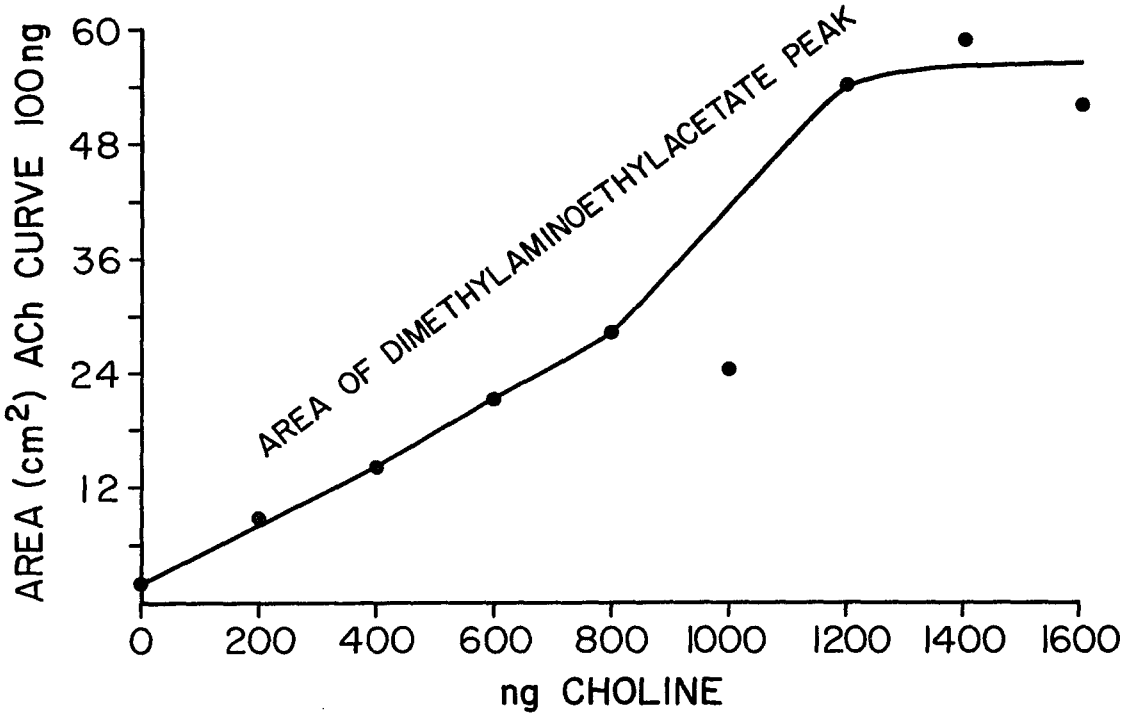


Figure 3. Increased peak size of dimethylaminoethylacetate (ACh) produced from 100 ng acetylcholine as the amount of choline added to the pyrolysis ribbon is increased.

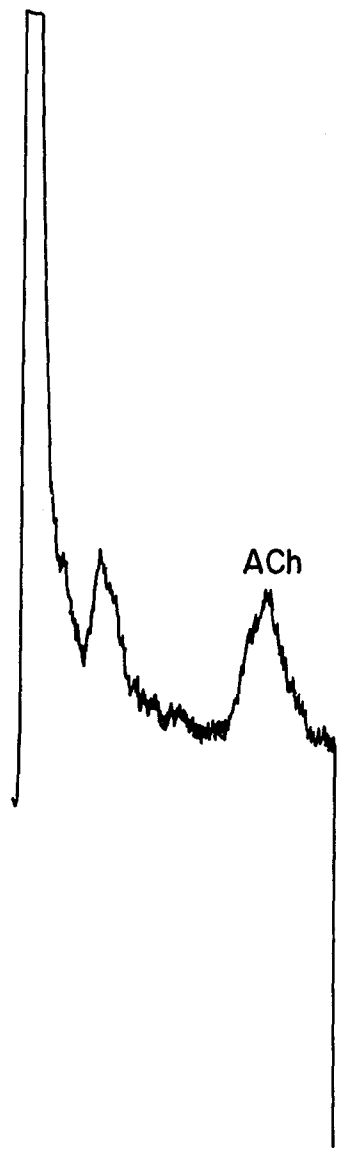


Figure 4. Dimethylaminoethylacetate (ACh) peak resulting from the pyrolysis of 5 ng acetylcholine and 2.4 µg choline. Attenuation 1×10^2.

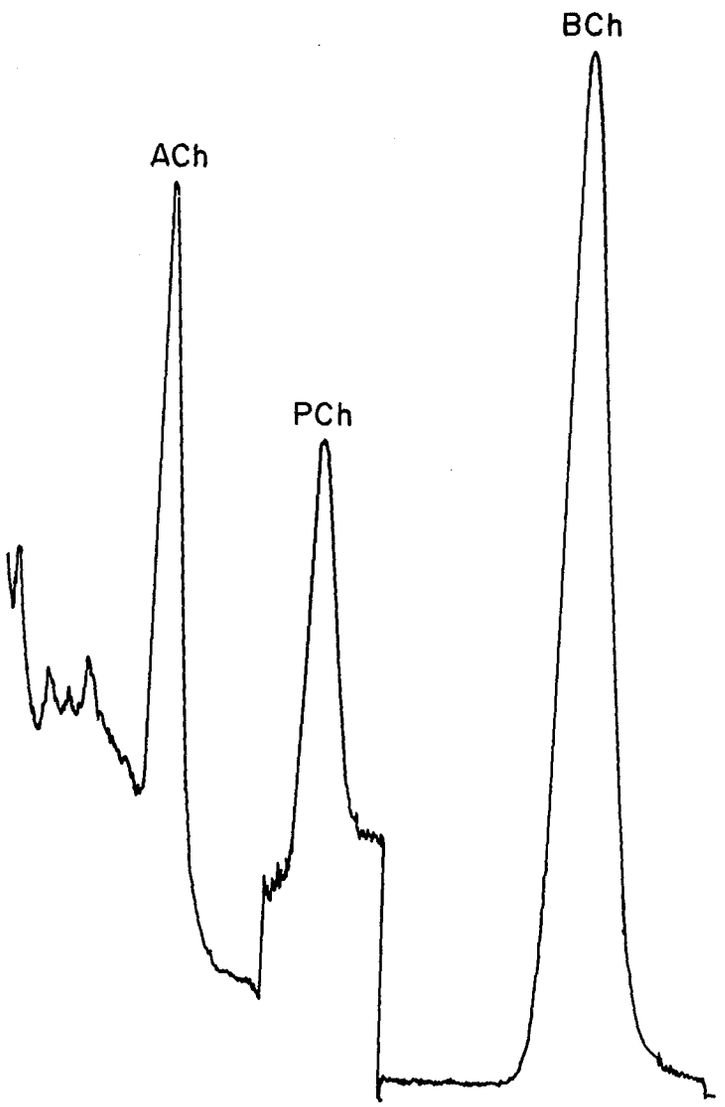


Figure 5. Chromatograms from the tissues of one albino rabbit eye. Propionylcholine, 50 ng, added as internal standard and butyrylcholine, 1.5 µg, placed on the pyrolysis ribbon. Attenuation of the dimethylaminoethylacetate (ACh) and dimethylaminoethylpropionate (PCh) peaks is 1 X 64 except for the iris-ciliary body chromatogram where the PCh peak sensitivity is 1 X 512. Attenuation of the dimethylaminoethylbutyrate peak is 32 X 10^2. a) cornea.

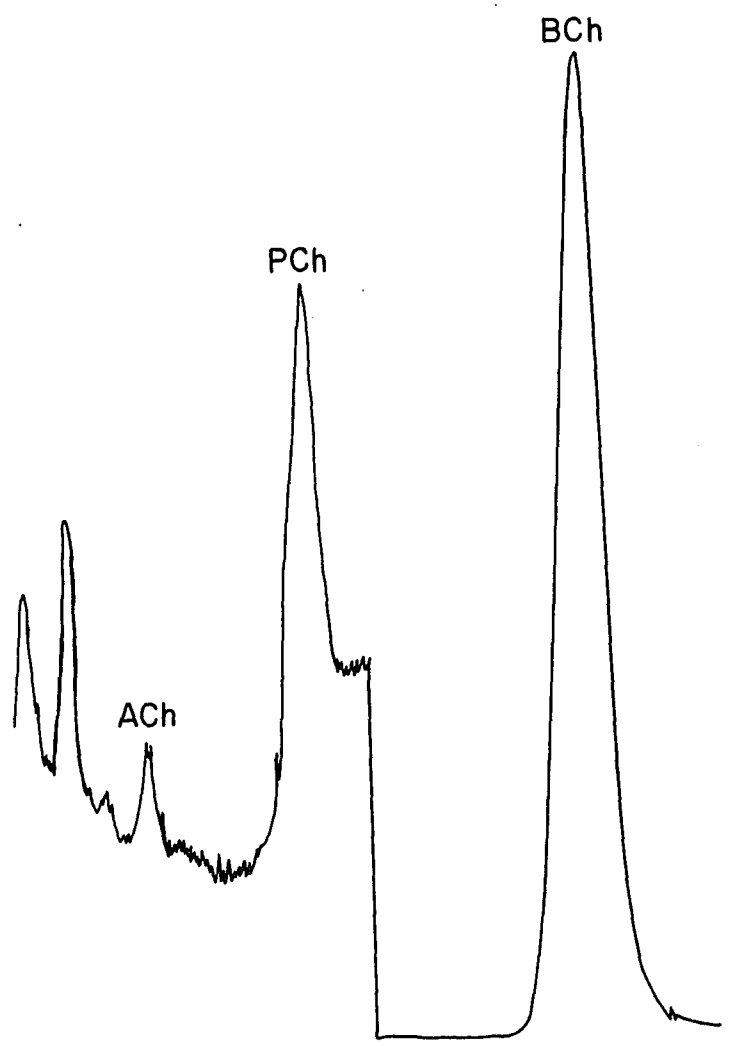


Figure 5. Chromatograms from the tissues of one albino rabbit eye. Propionylcholine, 50 ng, added as internal standard and butyrylcholine, 1.5 µg, placed on the pyrolysis ribbon. Attenuation of the dimethylaminoethylacetate (ACh) and dimethylaminoethylpropionate (PCh) peaks is 1 X 64 except for the iris-ciliary body chromatogram where the PCh peak sensitivity is 1 X 512. Attenuation of the dimethylaminoethylbutyrate peak is 32 X 10^2. b) aqueous humor, 380 microliters.

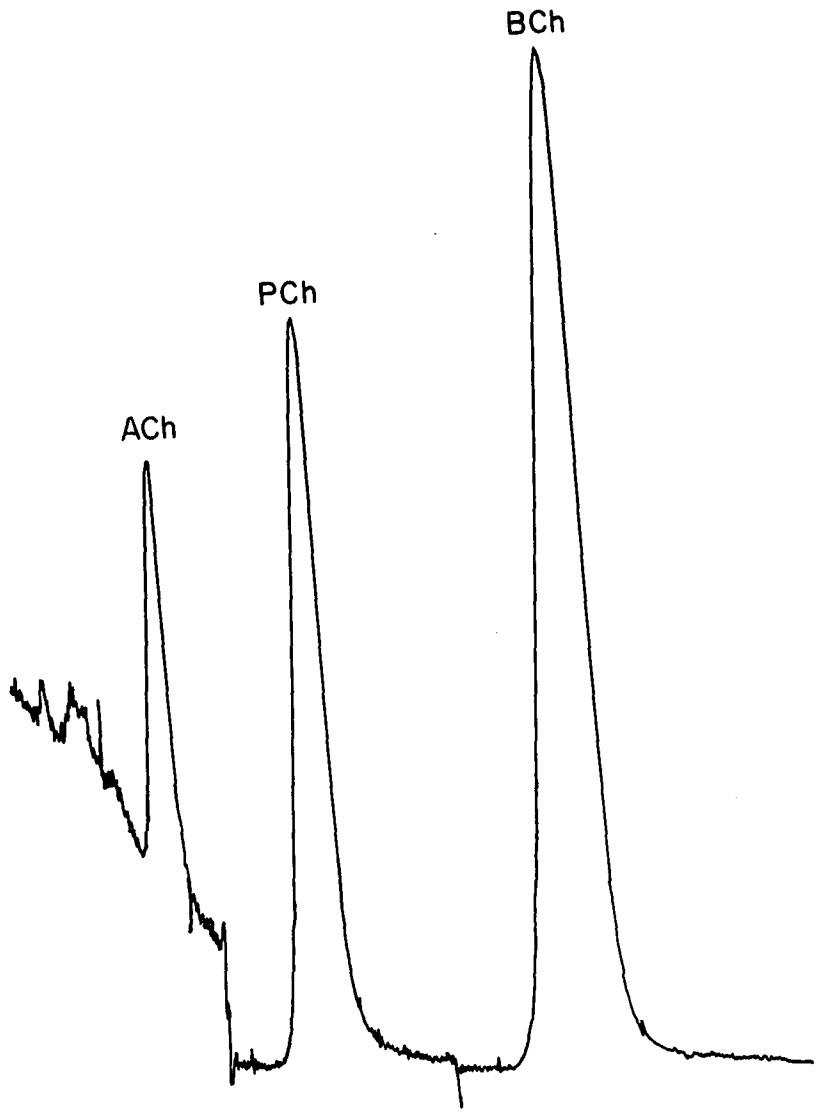


Figure 5. Chromatograms from the tissues of one albino rabbit eye. Propionylcholine, 50 ng, added as internal standard and butyrylcholine, 1.5 µg, placed on the pyrolysis ribbon. Attenuation of the dimethylaminoethylacetate (ACh) and dimethylaminoethylpropionate (PCh) peaks is 1 X 64 except for the iris-ciliary body chromatogram where the PCh peak sensitivity is 1 X 512. Attenuation of the dimethylaminoethylbutyrate peak is 32 X 10^2. c) iris and ciliary body.

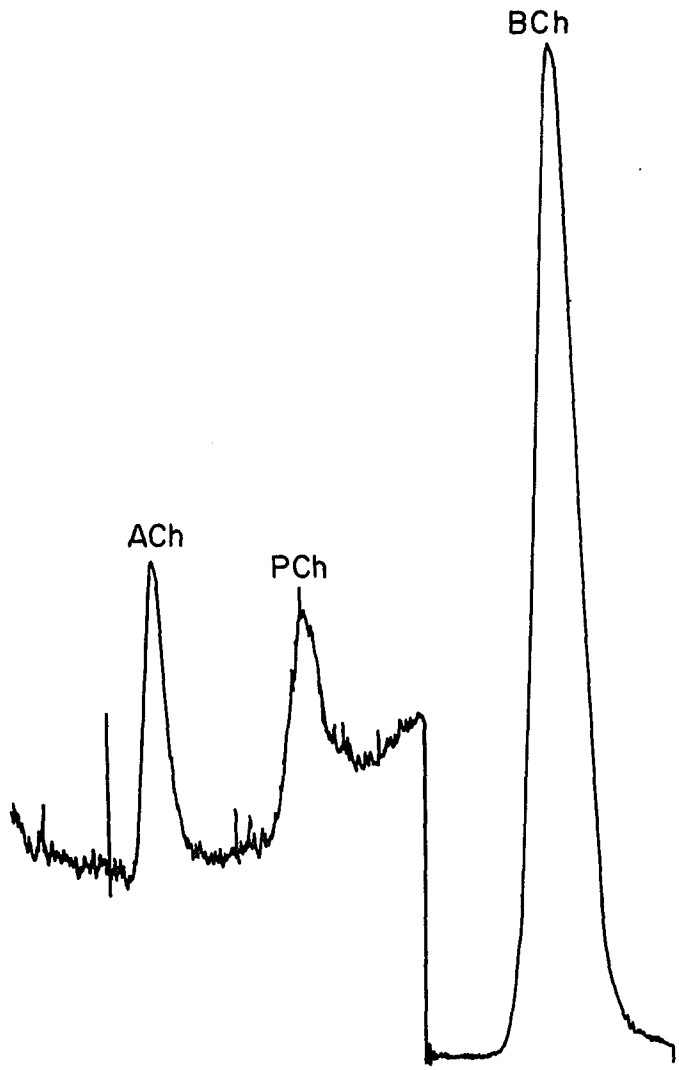


Figure 5. Chromatograms from the tissues of one albino rabbit eye. Propionylcholine, 50 ng, added as internal standard and butyrylcholine, 1.5 µg, placed on the pyrolysis ribbon. Attenuation of the dimethylaminoethylacetate (ACh) and dimethylaminoethylpropionate (PCh) peaks is 1 X 64 except for the iris-ciliary body chromatogram where the PCh peak sensitivity is 1 X 512. Attenuation of the dimethylaminoethylbutyrate peak is 32 X 10^2. d) retina.

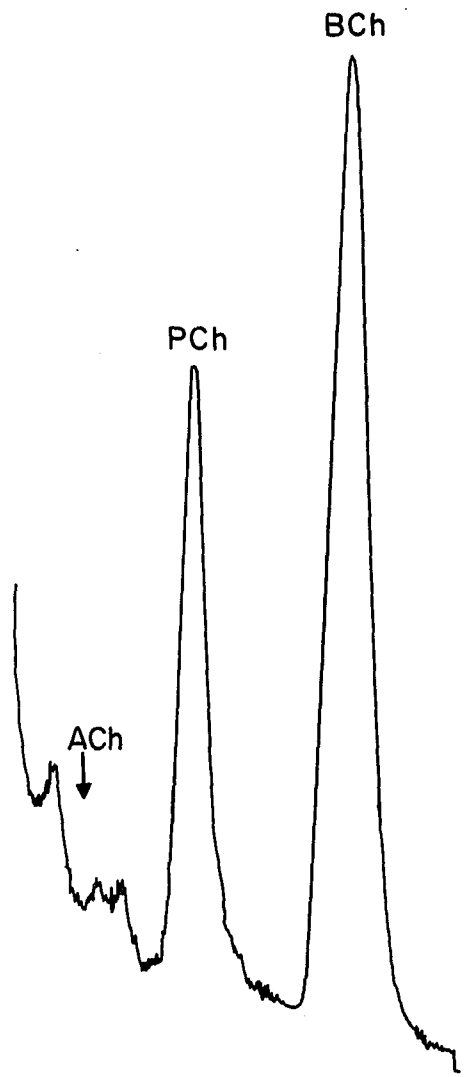


Figure 5. Chromatograms from the tissues of one albino rabbit eye. Propionylcholine, 50 ng, added as internal standard and butyrylcholine, 1.5 µg, placed on the pyrolysis ribbon. Attenuation of the dimethylaminoethylacetate (ACh) and dimethylaminoethylpropionate (PCh) peaks is 1 X 64 except for the iris-ciliary body chromatogram where the PCh peak sensitivity is 1 X 512. Attenuation of the dimethylaminoethylbutyrate peak is 32 X 10^2. e) lens.

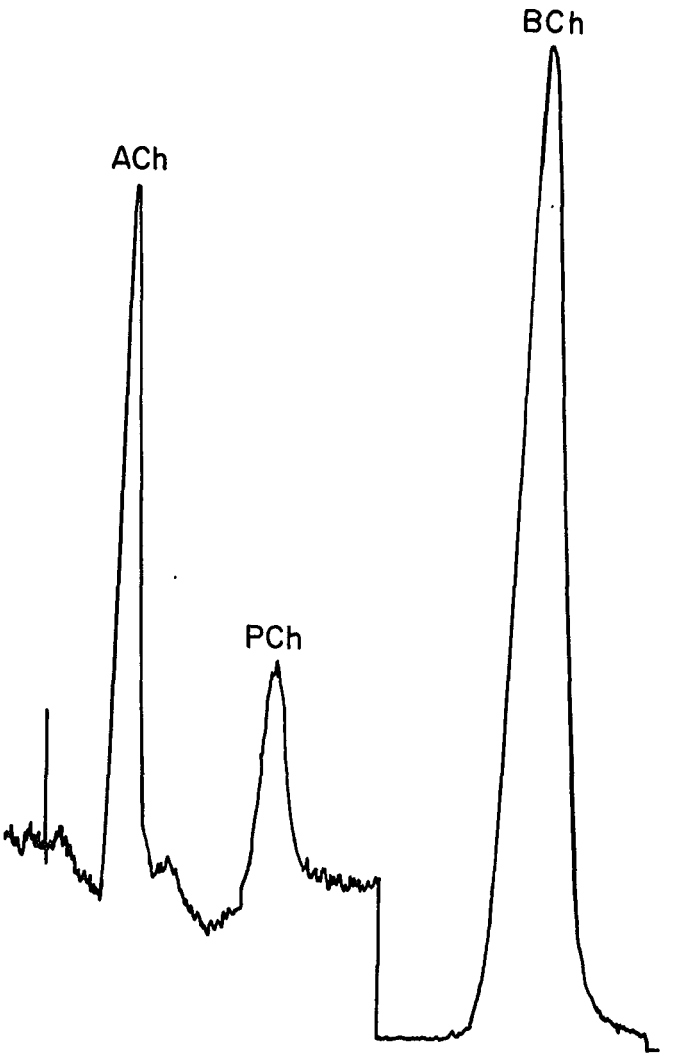


Figure 6. Chromatogram of an aliquot of bovine corneal epithelium stored 7 days at -17°C. Attenuation of dimethylaminoethylacetate (ACh) and dimethylaminoethylpropionate (PCh) peaks is 1 X 64. Attenuation of dimethylaminoethylbutyrate (BCh) peak is 32 X 10^2.

Table 1. Time course of acetylcholine disappearance in a pooled corneal epithelium sample from 40 bovine eyes maintained at -17°C.

Days after death	nanograms ACh/mg corneal epithelium
<1	35.5
7	20.5
42	1.1

ocular tissues such as cornea, iris, ciliary body, retina, and pigment epithelium-choroid.

Attempts to quantitate the amount of acetylcholine in samples of individual ocular tissues failed. While pooled samples of tissues rich in acetylcholine gave reproducible chromatogram values, the same was not true for samples where only small quantities of acetylcholine were present. Reproducible standard curves could not be obtained for ocular tissues to which as much as 50 nanograms acetylcholine had been added. Since reproducible standard curves could be obtained for known quantities of acetylcholine if no tissue were present, the source of error was the tissue. The extraction procedure was unable to exclude some unknown tissue components that altered the ratio of the acetylcholine and propionylcholine peaks. As a result the data from individual ocular samples was considered qualitative in nature rather than quantitative.

3. Species variation in acetylcholine content of ocular tissues:

Acetylcholine was detectable not only in bovine corneal epithelium but in each assay of 20 different rabbit corneas and of 4 different human corneas. However there was no detectable acetylcholine in the corneas of 8 feline eyes. Acetylcholine was also present in 12 of 12 rabbit iris-ciliary body samples, 7 of 7 retinal samples, 6 of 7 pigment epithelium-choroid samples and 0 of 4 lens samples. A similar distribution was found in man in that 6 of 7 iris samples, 7 of 7 ciliary body samples, 5 of 7 retinal samples, 10 of 10 pigment epithelium-choroid samples, and 0 of 4 lens samples had detectable acetylcholine.

Aqueous humor was collected from 18 rabbit eyes and each sample was assayed individually; 9 of the 18 contained detectable acetylcholine. Aqueous humor samples were assayed individually from 17 patients requiring cataract surgery. Most of these gave a small chromatogram peak near, but not at, the correct retention time for dimethylaminoethylacetate. None had detectable acetylcholine. Pooled samples of human aqueous humor, 1.5-2.5 ml, also were without detectable acetylcholine. Pre-operative application of diisopropylfluorophosphate 0.1% or echothiophate 0.25% eye drops did not permit detection of acetylcholine in human aqueous humor.

4. Acetylcholine content in aqueous humor in the presence of intraocular inflammation:

Intraocular inflammation is accompanied by a decrease in the pupil's diameter. Perhaps this is associated with, or caused by, an increased release of acetylcholine from the parasympathetic nerve endings of the iris sphincter muscle. To test this hypothesis, intraocular inflammation was produced by placing prostaglandins on the cornea or withdrawing aqueous humor from the anterior chamber of rabbits. Topical application of PGE_1, 10μg, followed by aspiration of the aqueous humor 30-60 minutes later gave samples only 2 of 8 of which contained acetylcholine. If PGE_2, 10μg, were used instead, only 2 of 6 samples contained acetylcholine. All 6 samples assayed after $PGF_{2\alpha}$, 10μg or 100μg, contained acetylcholine. However the overall rate of detection of acetylcholine in this prostaglandin treated group was 50%, which is the same rate found in untreated eyes.

In rabbits whose eyes were inflammed by removal of aqueous humor, the secondary aqueous humor, aspirated 1-3 days later, contained

detectable acetylcholine in only 4 of 8 eyes. Interestingly, those eyes containing detectable acetylcholine before aspiration were the same ones having detectable acetylcholine after aspiration. Those eyes without detectable acetylcholine in the primary aqueous humor also had none detectable in secondary aqueous.

5. Interspecies variation in ocular choline acetyltransferase activity:

The choline acetyltransferase activity of rabbit, cat, cattle, and human eyes was assayed. Values were calculated on a per mg protein basis (Tables 2-5). In addition, enzyme activities of iris, ciliary body, retina, and pigment epithelium-choroid were calculated on a per whole tissue bases (Tables 6-9). The corneal epithelium of cats, unlike the other three mammalian species had little or no detectable choline acetyltransferase activity. Rabbit, bovine, and human corneal epithelia had high levels of enzyme activity. The corneal stroma and endothelium of all four species had little or no choline acetyltransferase activity. The irides of man, rabbit, and cat contained, on the average, enough enzyme to form approximately 2 nanomoles ACh/hr/mg protein. However, the average bovine iris contained 4-5 times this level of activity. The ciliary bodies of cattle, rabbits, and cats formed 2-3 nanomoles ACh/hr/mg protein while the average human ciliary body had 5-8 times this level of activity. The vitreous and aqueous humors (Table 10) contained small amounts of choline acetyltransferase activity which may have resulted from autolysis of adjacent solid tissues since the pattern of enzyme activity (man> bovine> rabbit and cats) correlated well with the length of time between the animal's death and the dissection of its ocular tissues (man>bovine>rabbit

Table 2. Distribution of choline acetyltransferase activity in the ocular tissues of Dutch Belt rabbits (per mg protein).

Tissue	nanomoles ACh formed/hr/mg protein			
	Average	Range	Std Dev.	n
Conjunctiva	0.3	---	0.0	3
Cornea - epithelium	20.6	3.9-41.2	13.6	21
stroma-endothelium	0.0(1)	---	---	8
Iris-ciliary body	1.9	0.2-4.9	---	34
Lens	0.0(2)	---	1.2	13
Vitreous	0.5	0.0-2.1	---	8
Retina	15.9	5.8-30.3	0.7	26
Pigment epithelium-choroid	4.0	0.2-12.4	6.8	30
Optic nerve	0.0	0.0-0.1	3.7	8
Sclera	0.0(3)	---	0.03	8

(1) $\leq$0.036 nanomoles ACh/formed/hr/mg protein

(2) $\leq$0.003 nanomoles ACh formed/hr/mg protein

(3) $\leq$0.010 nanomoles ACh formed/hr/mg protein

Table 3. Distribution of choline acetyltransferase activity in ocular tissues of cats (per mg protein).

Tissue	nanomoles ACh formed/hr/mg protein			
	Average	Range	Std Dev	n
Cornea-epithelium	0.1	0.0-0.6	0.2	8
stroma-endothelium	0.0(1)	---	0.004	4
full thickness	0.0	0.0-0.3	0.1	12
Iris	2.0	0.3-5.5	1.5	16
Ciliary body	2.9	0.5-6.6	2.2	16
Lens	0.0(2)	---	0.01	4
Vitreous	0.0(3)	---	0.006	4
Retina	0.7	0.4-1.2	0.3	12
Pigment epithelium-choroid	0.5	0.2-0.7	0.2	6

(1) $\leq$0.010 nanomoles ACh formed/hr/mg protein

(2) $\leq$0.029 nanomoles ACh formed/hr/mg protein

(3) $\leq$0.013 nanomoles ACh formed/hr/mg protein

Table 4. Distribution of choline acetyltransferase activity in the ocular tissues of cattle (per mg protein).

Tissue	nanomoles ACh formed/hr/mg protein			
	Average	Range	Std Dev	n
Cornea-epithelium	46.5	11.6-89.2	27.5	8
stroma-endothelium	0.4	0.0-1.4	0.6	8
Iris	10.8	1.6-22.2	6.5	10
Ciliary body	1.9	0.3-6.9	2.1	10
Lens	0.0*	---	0.1	2
Vitreous	0.9	0.5-1.3	0.6	2
Retina	13.0	4.0-31.4	9.8	10
Pigment epithelium-choroid	4.6	0.0-10.9	4.5	10
Optic Nerve	0.3	---	---	1
Sclera	1.8	---	---	1

*-0.15 nanomoles ACh formed/hr/mg protein

Table 5. Distribution of choline acetyltransferase activity in the ocular tissues of humans (per mg protein).

Tissue	nanomoles ACh formed/hr/mg protein			
	Average	Range	Std Dev.	n
Cornea-epithelium	11.2	---	---	*
stroma-endothelium	0.4	---	---	*
Iris	2.5	0.1-5.3	1.8	10
Ciliary body	16.8	0.1-38.0	14.3	11
Lens	0.0**	---	0.01	6
Vitreous	2.2	---	---	*
Retina	6.1	0.9-23.1	6.7	10
Pigment epithelium-choroid	5.1	0.6-11.0	4.5	10
Optic nerve	0.7	---	---	*
Sclera	1.2	---	---	*

* pooled sample of 12 eyes

** $\leq$0.027 nanomoles ACh formed/hr/mg protein

Table 6. Distribution of choline acetyltransferase activity in Dutch Belt rabbit ocular tissues (per whole tissue).

Tissue	nanomoles ACh formed/hr/whole tissue			
	Average	Range	Std Dev	n
Iris-ciliary body	24.8	10.9-52.0	7.8	45
Retina	108.3	65.6-157.6	22.6	47
Pigment epithelium-choroid	8.8	1.8-35.9	6.0	44

Table 7. Distribution of choline acetyltransferase activity in feline ocular tissues (per whole tissue).

Tissue	nanomoles ACh formed/hr/whole tissue			
	Average	Range	Std. Dev.	n
Iris	33.3	26.7-36.6	3.8	6
Ciliary body	39.2	26.1-47.5	7.4	6
Retina	17.4	9.7-25.4	5.5	8
Pigment epithelium-choroid	35.9	26.3-49.8	9.9	6

Table 8. Distribution of choline acetyltransferase activity in bovine ocular tissues (per whole tissue).

Tissue	nanomoles ACh formed/hr/whole tissue			
	Average	Range	Std. Dev.	n
Iris	256.7	29-538	183.5	10
Ciliary body	77.5	10-239	73.0	10
Retina	469.0	157-943	282.1	10
Pigment epithelium-choroid	220.3	3-532	212.8	10

Table 9. Distribution of choline acetyltransferase activity in human ocular tissues (per whole tissue).

Tissues	nanomoles ACh formed/hr/whole tissue			
	Average	Range	Std. Dev.	n
Iris	11.6	1.3-34.9	13.5	8
Ciliary body	105.7	0.3-220.7	89.7	8
Retina	49.7	22.9-97.4	29.6	8
Pigment epithelium-choroid	52.3	17.0-102.9	34.7	8

Table 10. Choline acetyltransferase activity in aqueous humor.

Tissue	nanomoles ACh formed/hr/ml aqueous humor			
	Average	Range	Std. Dev.	n
Rabbit	0.0	0.0-0.1	0.0	14
Bovine	1.4	---	---	*
Human	7.8	5.4-10.1	3.3	7

* 2 pooled samples, one of 40 eyes and the other of 8 eyes

and cats). The retinal choline acetyltransferase activity of rabbits and cattle was, on the average, similar while human and, especially, feline enzyme activity was far less. The average value of rabbit, cattle, and human pigment epithelium-choroid choline acetyltransferase activity was, at 4-5 nanomoles ACh formed/hr/mg protein, approximately ten times that of cat. Underlying all these averaged values is a wide range in choline acetyltransferase activity that indicates considerable intraspecies, as well as interspecies, variation.

6. Intraspecies variation in ocular choline acetyltransferase activity of Dutch Belt rabbits:

Figures 7-10 show the distribution pattern of choline acetyltransferase activity in the different ocular tissues of Dutch Belt rabbit using histograms. The values tend to be grouped around a peak central value.

7. Concordance of choline acetyltransferase activity in the two eyes of the same animal:

Assuming that the enzyme activity is approximately the same in the two eyes of the same animal, then the technique of tissue dissection that gives the highest degree of concordance would be preferred.

A. Corneal epithelium - This tissue was assayed after being scraped off the cornea with a scalpel blade or after being removed as part of a full thickness 9 mm central cornea trephine button. Table 11 shows the closer approximation of the two eyes when trephination was used. On the average, trephined rabbit corneal epithelia of the two eyes of the same animal agreed to within 20%, while the deviation was more than 60% when scraped samples were used. It was assumed that

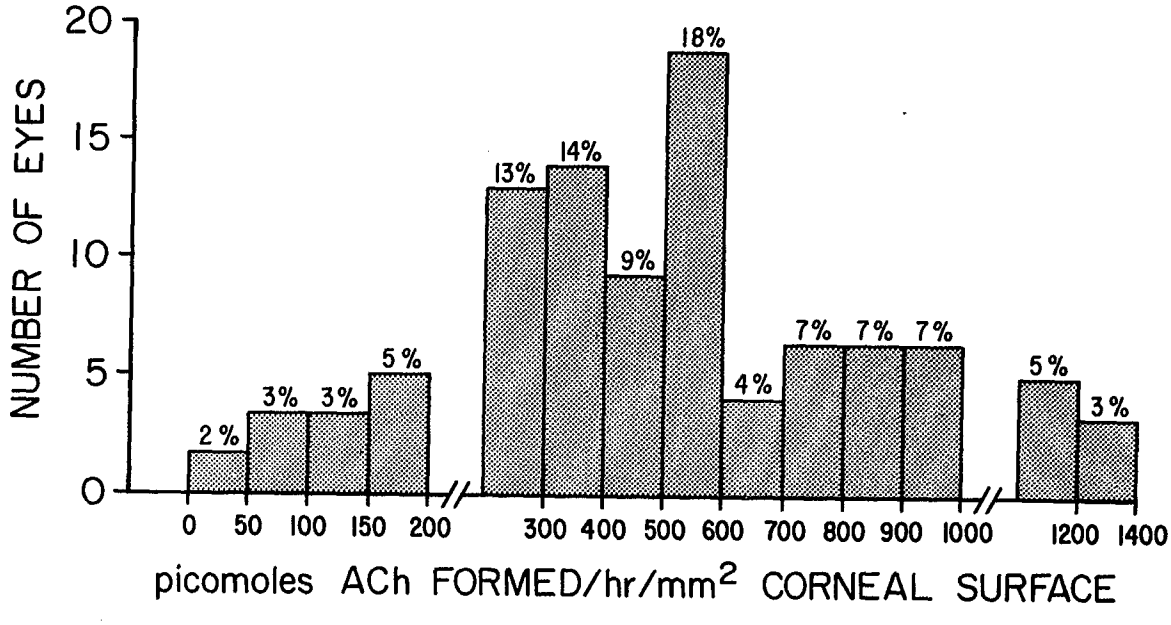


Figure 7. Distribution of choline acetyltransferase activity in the corneal epithelium of 100 eyes from 100 Dutch Belt rabbits.

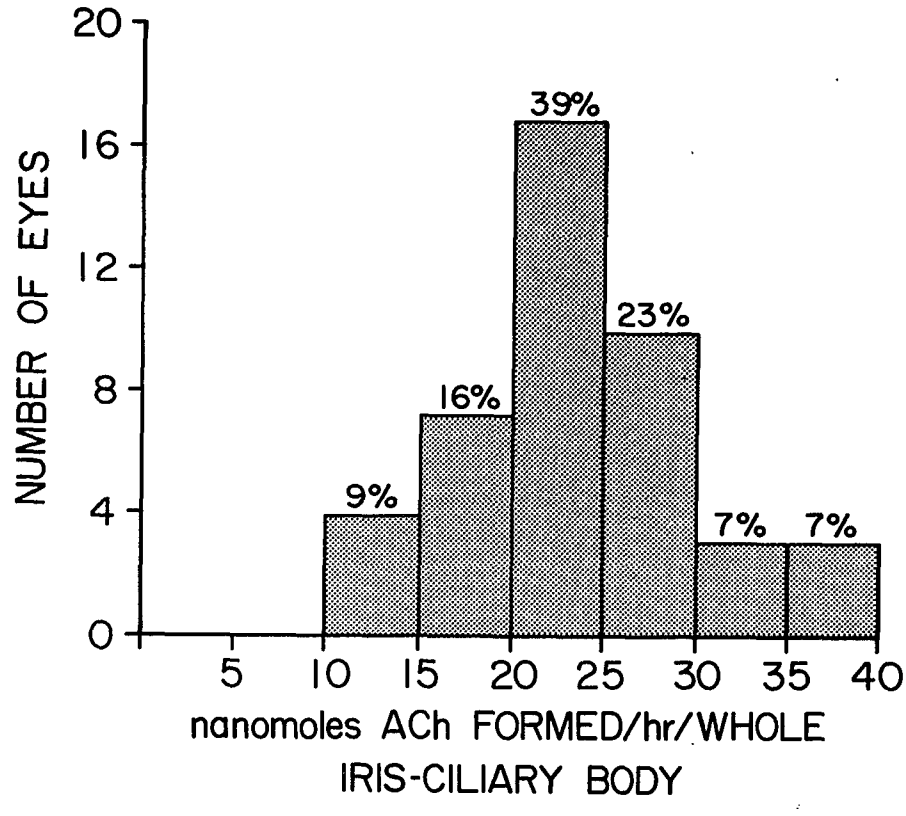


Figure 8. Distribution of choline acetyltransferase activity in iris-ciliary body of 44 eyes from 44 Dutch Belt rabbits.

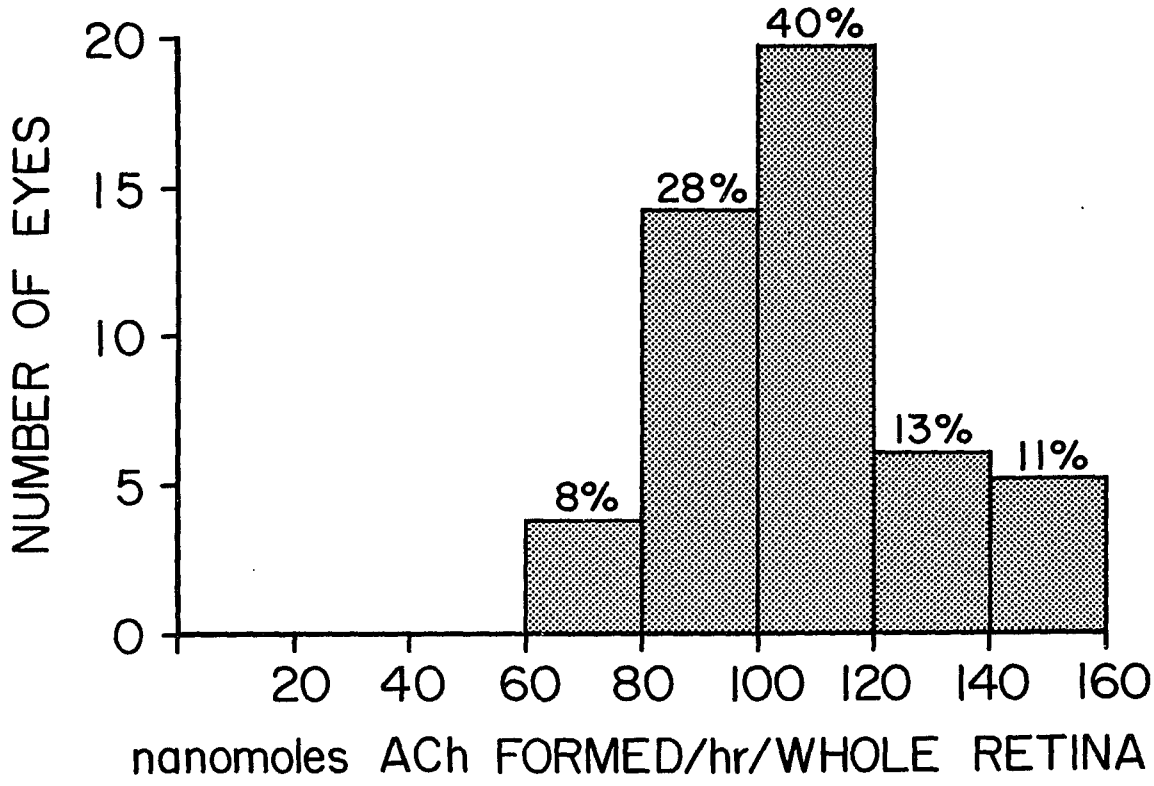


Figure 9. Distribution of choline acetyltransferase activity in retinas of 47 eyes from 47 Dutch Belt rabbits.

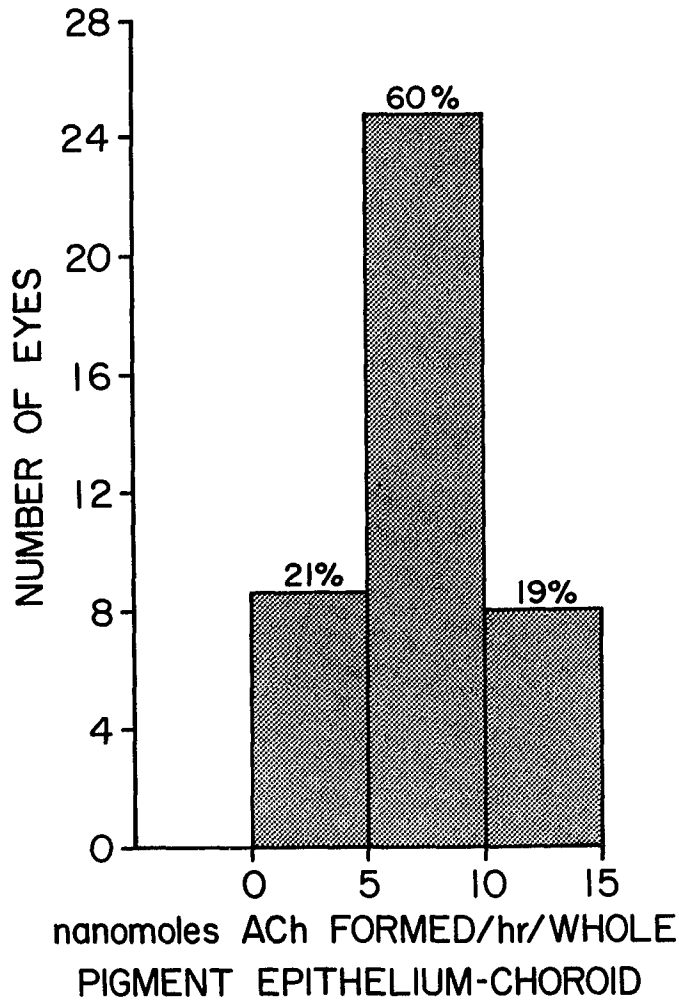


Figure 10. Distribution of choline acetyltransferase activity in pigment epithelium - choroid of 42 eyes from 42 Dutch Belt rabbits.

Table 11. Concordance of choline acetyltransferase activity in the two corneal epithelia of the same animal: scraping vs trephination.

	Number Eyes	nanomoles ACh formed/hr/mg protein		
		A.Ave/Eye	B.Ave.Variation/Animal	$\frac{B}{A}$ as%
1. Rabbit Corneal Epithelium				
a) Scraped	10	14.6	8.9	61%
b) Trephined	12	15.6	2.8	18%
2. Feline Corneal Epithelium				
a) Scraped	8	0.1	0.0	0%
b) Trephined	12	0.0	0.0	0%
3. Bovine Corneal Epithelium				
a) Scraped	8	46.5	17.6	38%
b) Trephined	10	25.5	6.3	25%

incomplete removal of epithelia and loss of tissue when transferring it from scalpel blade to test tube resulted in the errors produced by the scraping technique.

A further refinement was to calculate values for corneal choline acetyltransferase activity on a per mm^2 surface basis. Table 12 shows the same data calculated both as per mg corneal protein and as per mm^2 corneal surface. It was assumed that inability to completely and uniformly break up the tough corneal stroma by homogenization led to the errors produced by calculating data on a per mg cornea basis.

B. Iris, ciliary body, retina, pigment epithelium choroid.

These tissues had their choline acetyltransferase activities calculated on a per mg protein basis and on a per whole tissue basis (Tables 13 and 14). A statistical analysis, using a t-test to evaluate the percent deviation of each pair of eyes from the mean value, failed to show a clear-cut superiority of either method except for the rabbit retinas ($p<0.01$) and feline pigment epithelium-choroid ($p<0.05$) where calculated on a per whole tissue basis was superior. It had been anticipated that per tissue calculations would show better interocular agreement for retinas because vitreous is adherent to sensory neuroepithelium and its incomplete removal contaminates retinal samples.

8. Relationships of choline acetyltransferase activities of ocular tissues:

The cornea, iris-ciliary body, retina, and pigment epithelium-choroid of 26 rabbits were analyzed statistically to see if there was a relationship between any two different tissues. For example, as corneal choline acetyltransferase increased in a given eye, did the

Table 12. Concordance of choline acetyltransferase activity in the two corneal epithelia of the same animal: per mg protein vs per mm^2 surface.

	Number Eyes	nanomoles ACh formed/hr/mg corneal protein			picomoles ACh formed/hr/mm^2 corneal surf		
		A. Ave / Eye	B. Ave Variation / Animal	$\frac{B}{A}$ as%	C. Ave / Eye	D. Ave. Variation / Animal	$\frac{D}{C}$ as%
1. Rabbit	12	15.6	2.8	18%	354	38	11%
2. Feline	12	0.0(1)	0.0(2)	0%	0(3)	0(4)	0%
3. Bovine	10	25.5	6.3	25%	1628	110	7%
4. Human	18	1.0	0.3	30%	31	11	35%

(1) $\leq$ 0.023 nanomoles ACh formed/hr/mg corneal protein

(2) $\leq$ 0.045 nanomoles ACh formed/hr/mg corneal protein

(3) $\leq$ 0.085 picomoles ACh formed/hr/mm^2 corneal sufface

(4) $\leq$ 0.016 picomoles ACh formed/hr/mm^2 corneal surface

Table 13. Concordance of choline acetyltransferase activity in the tissues of the two eyes of the same rabbit and cat: per mg protein vs. per whole tissue.

	Number Eyes	nanomoles ACh formed/hr					
		per mg protein			per whole tissue		
		A. Ave / Eye	B. Ave. Variation / Animal	B/A as%	C. Ave / Eye	D. Ave. Variation / Animal	D/C as%
1. Rabbit							
Iris-ciliary body	12	5.0	0.6	12%	26.7	4.0	15%
Retina	16	26.3	13.0	49%	114.9	20.0	17%
Pigment epithelium-choroid	14	2.5	1.4	56%	7.9	3.1	39%
2. Cat							
Iris	6	0.8	0.1	12%	33.3	3.9	12%
Ciliary body	6	0.8	0.2	25%	39.2	9.5	24%
Retina	8	0.6	0.2	33%	17.4	7.6	44%
Pigment epithelium-choroid	6	0.5	0.2	40%	35.9	6.4	18%

Table 14. Concordance of choline acetyltransferase activity in the tissues of the two eyes of the same cow and human: per mg protein vs per whole tissue.

		Number Eyes	nanomoles ACh formed/hr					
			per mg protein			per whole tissue		
			A. Ave / Eye	B. Ave. Variation / Animal	B/A as%	C. Ave / Eye	D. Ave. Variation / Animal	D/C as%
1.	Bovine							
	Iris	10	10.8	1.5	14%	256.7	9.8	4%
	Ciliary body	10	1.8	1.0	56%	77.5	38.2	49%
	Retina	10	13.0	3.2	25%	469.0	133.2	28%
	Pigment epithelium-choroid	10	4.6	0.5	11%	220.3	25.8	12%
2.	Human							
	Iris	8	2.2	0.4	18%	11.6	1.4	12%
	Ciliary body	8	15.6	2.3	15%	106.0	11.5	11%
	Retina	8	4.0	1.2	30%	49.7	5.1	10%
	Pigment epithelium-choroid	8	4.8	0.6	12%	52.3	7.6	15%

retinal enzyme activity decrease? Or could any reciprocal relationship be found for the parasympathetically innervated structures of the eye, i.e. iris and ciliary body, with those tissues that are cholinergic but not parasympathetic, i.e. retina and cornea? A continous simple linear regression failed to show any significant correlations (all correlation coefficients $\leq$ 0.52).

9. Regional distribution of corneal choline acetyltransferase activity:

Five corneal buttons, 3 mm in diameter, were trephined from each of eleven rabbit eyes. The buttons were removed from central, temporal, superior, nasal, and inferior corneal areas. The choline acetyltransferase activities were assayed and the values calculated on a per mm^2 corneal surface basis (Table 15). An analysis of variance was performed by making the total activity of the five buttons of each eye equal to 100% and then giving the individual buttons the appropriate proportion of this value. The analysis of variance indicated that buttons from different locales represented different populations. A paired t-test ($p =< 0.05$) indicated that three different populations existed: (1) superior-temporal cornea, (2) inferior-nasal cornea, and (3) central cornea. Figure 11 demonstrates this distribution of choline acetyltransferase activity. The central cornea contains the most enzyme activity and the superiotemporal area contains the least.

10. Factors determining choline acetyltransferase activity in ocular tissues:

The intraspecies variation of ocular choline acetyltransferase activity has been shown to be great, while the two eyes of the same animal agree well. For example, the corneas of 45 Dutch Belt rabbit

Table 15. Regional distribution of corneal choline acetyltransferase activity in Dutch Belt rabbits.

Eye #	picomoles ACh formed/hr/mm^2 corneal surface				
	Central	Temporal	Superior	Nasal	Inferror
1	644	545	288	510	950
2	1117	286	386	1136	945
3	1769	1076	813	800	1726
4	9.6	493	338	722	607
5	822	473	308	388	1123
6	1283	397	309	1219	1205
7	1623	308	426	995	636
8	505	118	70	95	93
9	314	62	58	96	82
10	330	103	153	144	149
11	415	90	144	157	160

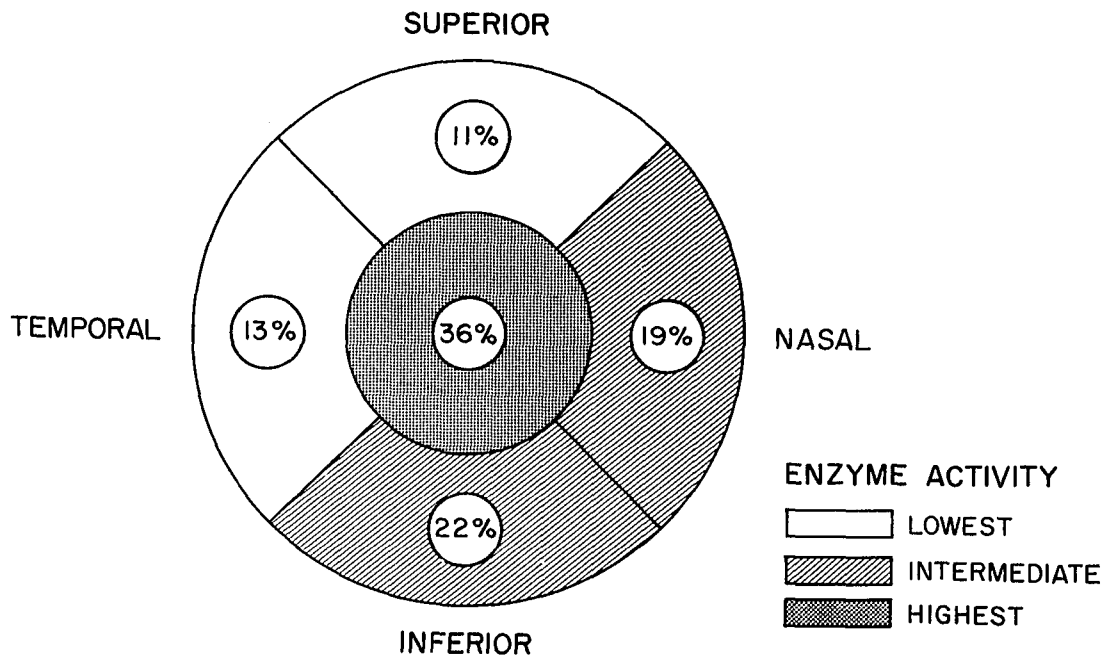


Figure 11. Regional distribution of choline acetyltransferase activity in 11 Dutch Belt rabbit corneas. Central cornea had significantly ($p < 0.05$) higher enzyme activity than nasal or inferior cornea which, in turn, had higher enzyme activity ($p < 0.05$) than superior or temporal cornea. Percentages designate each segment's portion of total corneal enzyme activity.

eyes had an average value of 545 picomoles ACh formed/hr/mm^2 corneal surface; the range was 136-1048 picomoles ACh formed and the standard deviation was 261 picomoles ACh formed. However, the two eyes of the same animal varied, on the average, only 11% from the mean value. Various factors that might explain the inter-animal variation were examined:

A. Genetic - Enzyme activity was determined in two groups of Dutch Belt rabbits with increased genetic homogeneity. A low degree of inbreeding was present in two groups of littermates whose parents had been chosen at random (Table 16). A high degree of inbreeding (F = 82-85%) was present in a specially purchased group of four Dutch Belt rabbits (Table 17). These values were compared to 33 eyes of Dutch Belt rabbits that were not inbred (Table 18). A statistical analysis was performed by expressing the deviation of each eye from the mean value divided by the mean value of the appropriate group as a per cent; these values were subjected to a t-test. It was assumed that if a genetic component influenced the tissue levels of choline acetyltransferase, then the individual values would deviate less and less from the mean value as the degree of inbreeding increased. In littermates, only the iris-ciliary body reached a degree of significance ($p \leq 0.05$) that indicated there was less variation in sibling choline acetyltransferase activity than in random eyes. However, in the highly inbred Dutch Belt rabbits the decline in interanimal variation was highly significant ($p < 0.01$) for all four ocular tissues: corneal epithelium, iris-ciliary body, retina, and pigment epithelium-choroid.

B. Tarsorrhaphy - Unilateral closure of the eyelids produced a marked fall in corneal choline acetyltransferase activity. The

Table 16. Choline acetyltransferase activity in ocular tissues of littermates (Dutch Belt rabbits).

Litter	Rabbit No.	picomoles ACh formed/hr/mm^2 surface	nanomoles ACh formed/hr/whole tissue		
		Cornea	Iris-ciliary body	Retina	Pigment Epithelium-choroid
A	1 OD	459	14.5	94.4	8.1
	OS	554	17.2	123.9	7.6
	2 OD	199	18.8	53.9	8.7
	OS	257	----	55.3	7.9
B	1 OD	172	24.8	90.8	4.2
	OS	275	23.5	100.5	4.5
	2 OD	255	27.9	114.8	5.7
	OS	331	22.7	140.5	8.7
	3 OD	136	18.3	110.8	4.4
	OS	137	18.0	76.6	10.2

Table 17. Choline acetyltransferase activity in ocular tissues of highly inbred (F=82-85%) Dutch Belt rabbits.

Rabbit No.	picomoles ACh formed/hr/mm^2 surface	nanomoles ACh formed/hr/whole tissue		
	Cornea	Iris-ciliary body	Retina	Pigment epithelium-choroid
1 OD	1703	29.5	125.9	14.1
OS	1403	21.1	85.4	9.0
2 OD	1825	24.7	113.0	9.3
OS	2057	25.0	116.4	9.3
3 OD	2082	22.6	118.9	10.2
OS	1393	22.5	134.0	14.0
4 OD	1953	21.4	110.5	15.0
OS	1751	20.7	112.3	14.0

Table 18. Effect of inbreeding on interanimal variation of ocular choline acetyltransferase activity.

A. Random breeding

	picomoles ACh formed/hr/mm^2 surface	nanomoles ACh formed/hr/whole tissue		
	Cornea	Iris-ciliary body	Retina	Choroid-pigment epithelium
Average	545	24.8	108.3	8.8
Std Deviation	261	7.8	22.6	6.0
Std Dev/Ave	48%	31%	21%	68%

B. Siblings

	Cornea	Iris-ciliary body	Retina	Choroid-pigment epithelium
Average	218	22.5	105.7	6.3
Std Deviation	81	3.8	22.0	2.6
Std Dev/Ave	37%	17%	21%	41%

C. Highly Inbred (F=82-85%)

	Cornea	Iris-ciliary body	Retina	Choroid-pigment epithelium
Ave	1771	23.4	114.6	11.9
Std Deviation	267	2.9	14.2	2.6
Std Dev/Ave	15%	12%	12%	22%

lids were united by various methods: (1) excising lid margins and running 6-0 silk or 5-0 chromic sutures; (2) leaving the lid margins intact and running 6-0 silk or 5-0 chromic sutures; and, (3) applying collodion to intact lid margins. In 14 control rabbits where no tarsorrhaphy was performed, the choline acetyltransferase activity of the right eye's cornea was, on the average, 91% that of the left eye; the standard deviation of the difference in enzyme activity for the two eyes of the same animal was 24%. In 12 rabbits whose right eyes were tarsorrhaphied 8 to 56 days, the choline acetyltransferase activity of the right cornea fell, on the average, to 28% that of the left eye; the standard deviation for the difference in enzyme activity of two eyes of the same animal increased to 72% (Table 19). Using a paired t-test, the decline in choline acetyltransferase activity in these 12 rabbits was found to be highly significant ($p<0.001$). The fall was maximum by 8 days for there was no statistical difference in those animals assayed at day 8 or day 56. There were an additional 9 rabbits assayed after 24-48 hours of unilateral tarsorrhaphy. Here, too, the tarsorrhaphied eye had a significantly lower ($p<0.002$) corneal enzyme activity (Table 20). The closed right eye's corneal choline acetyltransferase activity fell, on the average, to 65% that of the left eye; the standard deviation for the difference in the enzyme activity of the two eyes of the same animal was 39%. However, this decline was not maximal since there was a statistically significant ($p<0.01$) larger fall in choline acetyltransferase activity at 8-56 days than at 24-48 hours. The recovery of enzyme activity on re-opening the eyelids required more than 24 hours. Thus, a significant ($p<0.01$) difference in choline acetyltransferase activity of the two eyes of

Table 19. Effect of unilateral tarsorrhaphy on corneal choline acetyltransferase activity of Dutch Belt rabbits.

Rabbit Number	Length Tarsorrhaphy (days)	picomoles ACh formed/hr/mm^2 corneal surface	
		Tarsorrhaphied Eye	Open Eye
1	8	154	374
2	8	285	380
3	8	168	714
4	8	22	236
5	8	138	336
6	8	175	538
7	9	77	357
8	9	75	537
9	21	77	261
10	21	61	306
11	56	41	371
12	56	92	801

Table 20. Effect of unilateral tarsorrhaphy on corneal choline acetyltransferase activity of Dutch Belt rabbits.

Rabbit Number	Length Tarsorrhaphy (days)	picomoles ACh formed/hr/mm^2 corneal surface	
		Tarsorrhaphied Eye	Open Eye
1	2	739	850
2	2	607	810
3	2	825	1081
4	1	231	827
5	1	339	566
6	1	34	151
7	1	80	270
8	1	234	334
9	1	678	910

the same animal remained one day following the re-opening of the right lids of three rabbits tarsorrhaphied 8 days. The right eye's corneal enzyme activity was only 48% that of the left 24 hours after lid separation.

The choline acetyltransferase activities of rabbit iris-ciliary body and retina were also affected by prolonged tarsorrhaphy. There were 10 rabbits with unilateral tarsorrhaphy of the right lids for 1 to 28 days duration. Those eyes whose lids were closed 9 or more days had a small but significant decrease in enzyme activity in the iris-ciliary body ($p \leq 0.05$) and retina ($p<0.02$) when compared to the corresponding open eyes of the same animals. The closed right eye's iris-ciliary body choline acetyltransferase activity fell, on the average, to 74% of that of the left eye; the standard deviation for the difference in the enzyme activity of the two eyes of the same animal was 31%. The closed right eye's retinal choline acetyltransferase activity fell, on the average, to 80% of that of the left eye; the standard deviation for the difference in the enzyme activity of the two eyes of the same animal was 24%. Of the four tissues assayed, only the pigment epithelium-choroid was unaffected by tarsorrhaphy: the choline acetyltransferase activity was elevated in the tarsorrhaphied eye of 5 of 8 rabbits but statistical analysis gave little significance ($p<0.4>0.2$) to this finding.

An attempt was made to determine if the fall in ocular choline acetyltransferase activity produced by tarsorrhaphy would also result in an increase in intra-ocular pressure. Such a correlation was investigated because of the known ability of cholinergic drugs, as pilocarpine and diisopropylfluorophosphate, to lower intra-ocular pressure. This suggested

the possibility that some cholinergic defect was corrected by the medications and perhaps the tarsorrhaphy-induced decline in ocular choline acetyltransferase activity might mimic this defect. Three weeks of unilateral tarsorrhaphy failed to cause a significant rise ($p < 0.4 > 0.2$) in rabbit intra-ocular pressure. In addition, 6 human volunteers maintained a temporary tarsorrhaphy for 102 hours by keeping one pair of eyelids taped shut. Intra-ocular pressures were measured every 3 hours during the last 54 hours of the test period. Two volunteers were brothers whose two sisters had chronic simple glaucoma; in both, the intra-ocular pressure of the closed eye failed to increase above that of the open eye. The differences in pressure between the two eyes of the same individual did not show a tendency to increase or decrease with time in any of the 6 subjects. The intra-ocular pressure of a given eye tended to be maximum at noon and minimum at 9 PM ($p<0.01$). The changes in intra-ocular pressure that occurred in the open eye were paralleled by changes in the closed one.

C. Light stimulation - Three Dutch Belts rabbits were placed in continuous light for six weeks. One eye was removed and the animals were then subjected to 8 weeks of continuous dark. The second eye was removed and the animals killed. There was no statistically significant effect of prolonged light or dark on corneal, iris - ciliary body or retinal choline acetyltransferase activity.

D. Temperature alteration - Twelve Dutch Belt rabbits were maintained at 15° C for 17 days. One eye was removed and the animals placed in a 30° C room for 7 days. The second eye was removed and the animals killed. The corneal choline acetyltransferase activity of the eyes kept in the 30° C room (Table 21) was significantly lower than that of the eyes kept in the 15° C room ($p<0.001$). If rabbits were maintained at 15° C for only 10 days,

Table 21. Effect of temperature alteration on corneal choline acetyltransferase activity.

Rabbit #	picomoles ACh formed/hr/mm^2 corneal surface OD-17 days at 15^oC followed by OS-7 days at 30^oC	
1	1117	561
2	1248	437
3	948	383
4	950	445
5	348	204
6	379	263
7	495	335
8	511	377
9	177	122
10	603	351
11	318	283
12	311	161

instead of 17, the difference in corneal choline acetyltransferase activity produced by temperature alteration was not significant ($p<0.1>0.05$). This indicated that lowering the corneal temperature for a prolonged period, i.e. 17 days, caused an elevation in corneal choline acetyltransferase activity. This inverse relationship between corneal temperature and corneal choline acetyltransferase activity might explain the effect of tarsorrhaphy in lowering enzyme activity. The tarsorrhaphied rabbits had been kept at a 21° C room temperature so that the effect of the lid closure had been to elevate the corneal temperature of the right eye so that it more closely approximated body temperature, 39.5° C. The prolonged elevation of corneal temperature in the tarsorrhaphied eye could then result in a lowered corneal choline acetyltransferase activity. To test this theory, 9 rabbits had a unilateral tarsorrhaphy of the right eye and were placed immediately in a room kept at their body temperature, 39.5° C. Since the temperature of both corneas would be approximately the same, the fall in corneal choline acetyltransferase activity of the two eyes should be about equal. Table 22 gives the data from these nine rabbits. After 48 hours in the 39.5° C environment the enzyme activity was lower in the tarsorrhaphied eye ($p < 0.05$). When compared to the nine rabbits in Table 20 that had a 24-48 hour tarsorrhaphy but were kept at room temperature 21° C, the two groups were not significantly different ($p<0.5 >0.9$). The average decline in corneal choline acetyltransferase activity in the tarsorrhaphied animals kept in a 39.5° C room was only 10% less, on the average, than those animals kept at 21° C.

E. Humidity alteration - Four rabbits were placed in a 22° C, 69% relative humidity environment for 8 days. One eye was removed and the animals then were placed in a 22° C, 28% relative humidity environment for

Table 22. Comparison of corneal choline acetyltransferase activity in the two eyes of a unilaterally tarsorrhaphied Dutch Belt rabbit placed in a 39.5°C environment for 48 hours.

Rabbit No.	picomoles ACh formed/hr/mm^2 corneal surface	
	Tarsorrhaphied Eye	Open Eye
1	447	724
2	245	213
3	717	711
4	324	765
5	531	746
6	774	1123
7	760	1973
8	1195	1215
9	407	503

13 days. The second eye was removed and the animals killed. There was no detectable effect of humidity alteration on corneal choline acetyltransferase activity ($p>0.9$).

F. Effect of 5% CO_2 - Two rabbits raised in room air, whose CO_2 content is 0.03%, had one eye enucleated. The animals were then placed for 7 days in an environment containing more than 100 times the normal CO_2 concentration. This atmosphere was created by supplying a 5% CO_2 - 95% O_2 gas mixture to a closed container in which the rabbits were maintained. Raising the CO_2 concentration failed to alter the corneal choline acetyltransferase activity when the two eyes of the same animal were compared following the enucleation of the second eye.

11. Specificity of choline acetyltransferase activity decline in tarsorrhaphied eyes:

To determine whether the decrease in corneal choline acetyltransferase represented a specific, or relatively specific, response of the corneal epithelium to conditions of tarsorrhaphy, or whether the decrease only represented a generalized alteration in the biochemistry and physiology of the cornea, the following experiments were performed:

A. Lactic dehydrogenase activity - Three rabbits were unilaterally tarsorrhaphied for four weeks. At the end of this period the animals were killed and the corneal epithelium assayed in both eyes for lactic dehydrogenase activity. Table 23 shows the results. There was no significant difference between tarsorrhaphied eyes and open eyes ($p<0.4>0.2$).

B. Amino acid incorporation - Five rabbits had a unilateral tarsorrhaphy 9 days or 5 weeks. At the end of these periods, the animals were killed and the corneas removed. These corneas were used to measure the rate of incorporation of the amino acids leucine and alanine. Table 24

Table 23. Effect of unilateral tarsorrhaphy on corneal epithelial lactic dehydrogenase activity of Dutch Belt rabbits.

Rabbit No.	LDH Activity: I.U./ml corneal epithelial homogenate	
	Tarsorrhaphied Eye	Open Eye
1	13.1	16.0
2	13.8	14.0
3	6.4	7.7

shows the results. The tendency of the tarsorrhaphied eye to incorporate more amino acid was not statistically significant. When a paired t test was applied to the data, the probability that leucine incorporation differed in the two eyes was $p = <0.9 > 0.5$ and for alanine, $p = <0.2 > 0.1$.

C. Histology - Figure 12 shows photomicrographs of the corneas of a rabbit with a unilateral tarsorrhaphy for 5 weeks. The corneal epithelium shows no evidence of atrophy in the tarsorrhaphied eye.

D. Per mg protein enzyme assays - To ensure that the fall in choline acetyltransferase activity found by calculating data on a per mm^2 surface basis was not due simply to a thinning of the tarsorrhaphied corneal epithelium, the data was calculated on a per mg protein basis as well. This confirmed the significance of the dramatic fall in choline acetyltransferase activity in tarsorrhaphied eyes ($p<0.001$).

12. Cellular location of corneal choline acetyltransferase activity:

To determine if corneal choline acetyltransferase activity resides in the sensory nerve endings of the trigeminal nerve or in the epithelial cells, the following experiments were performed:

A. Retrobulbar injection of absolute ethanol- Retrobulbar alcohol injection produced a marked fall in choline acetyltransferase activity in cat iris and ciliary body (Table 25). The decline took more than 24 hours to begin, was maximal by day 7 and had not recovered by day 14. Eleven rabbits were then injected unilaterally with 0.3 - 0.5 ml absolute ethanol given in the retrobulbar space. In three rabbits a second injection of 0.3 ml ethanol was given 48 hours after the first. From 6-21 days following the last ethanol injection, the animals were killed and the choline acetyltransferase activity assayed. Table 26 shows the fall in enzyme activity that occurred; by day 21 post-injection, the enzyme

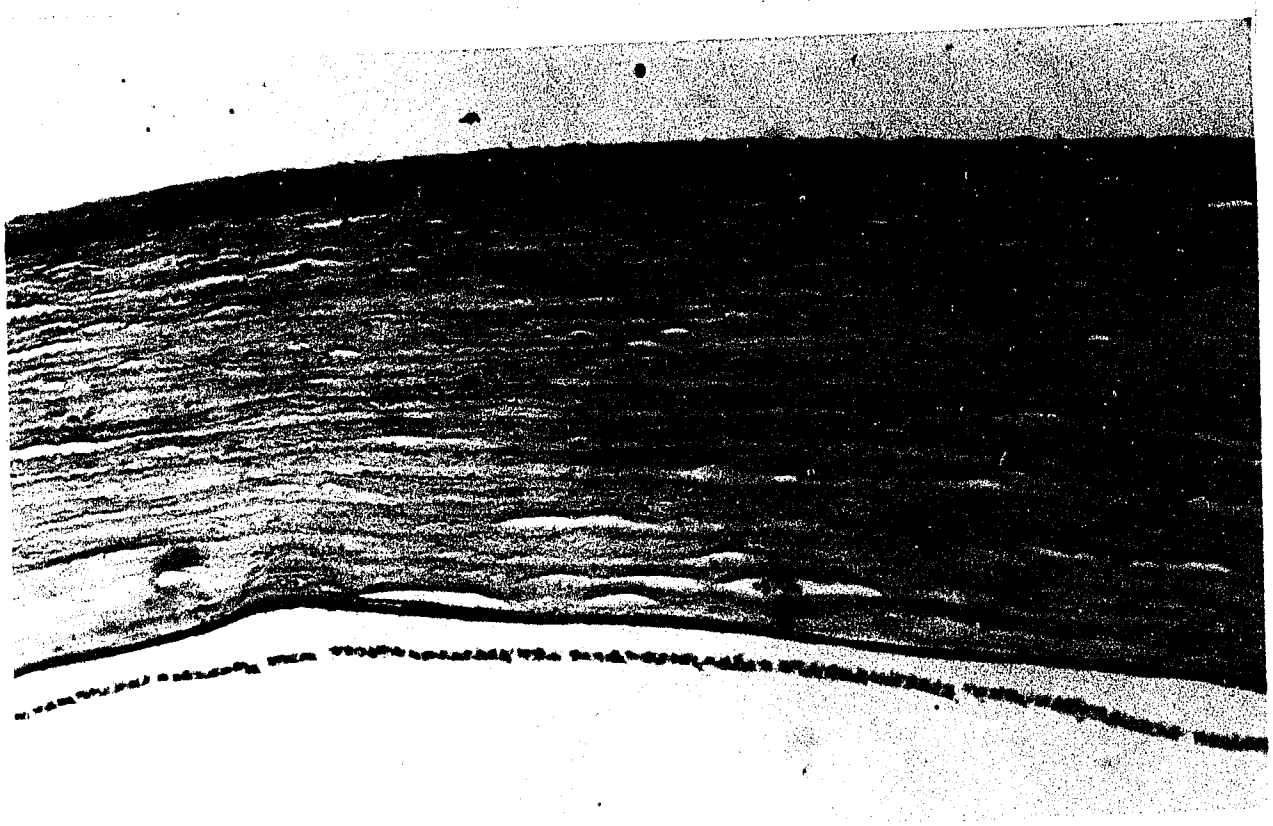


Figure 12. Rabbit cornea photomicrographs: effects of unilateral OD tarsorrhaphy. a) OD, tarsorrhaphied 5 wks, X 25

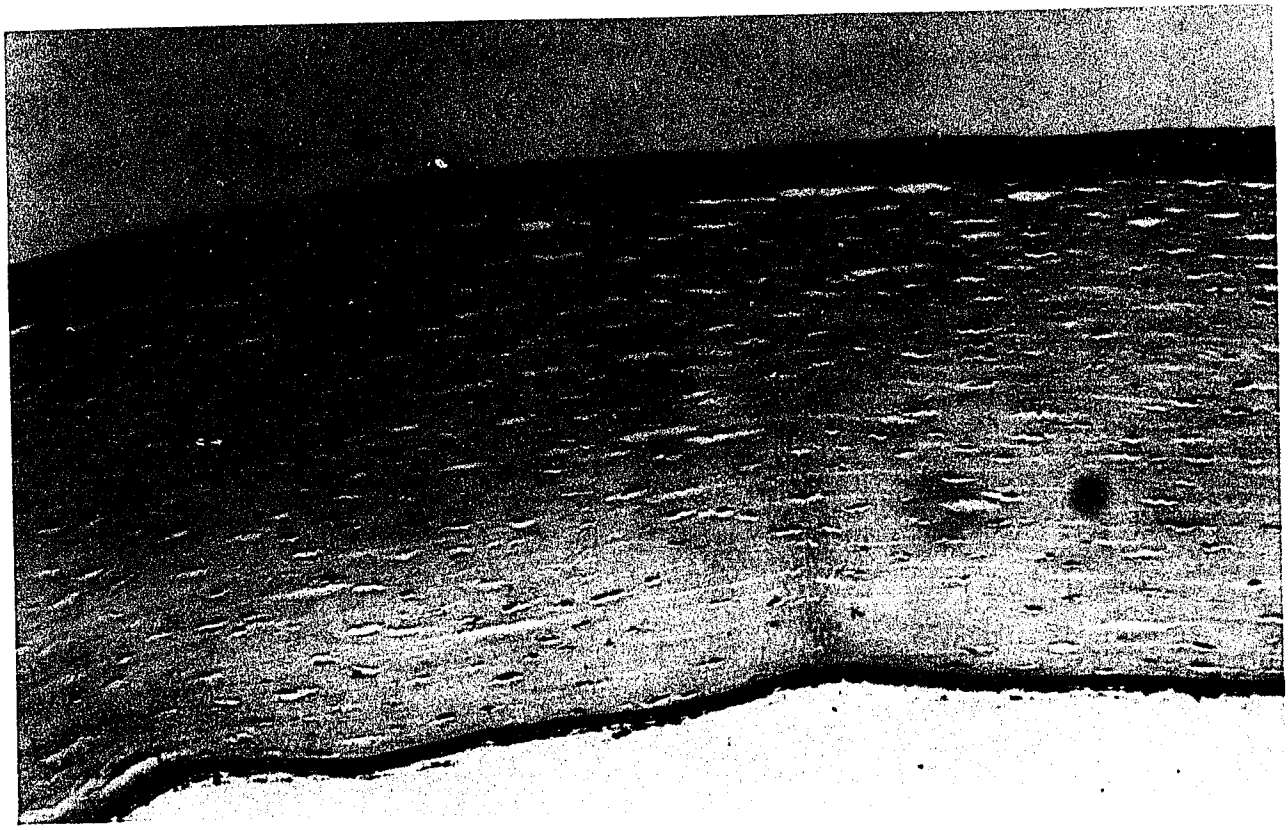


Figure 12. Rabbit cornea photomicrographs: effects of unilateral OD tarsorrhaphy. b) OS, X25

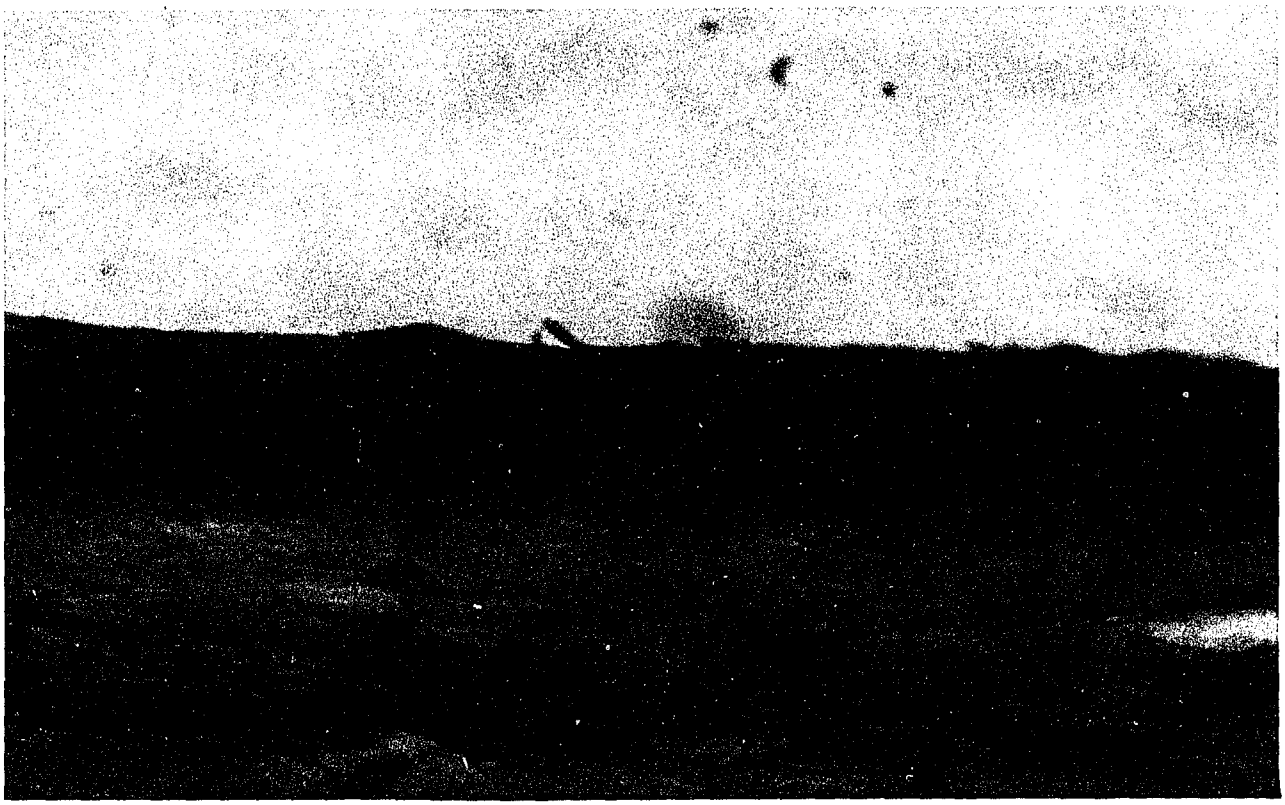


Figure 12. Rabbit cornea photomicrographs: effects of unilateral OD tarsorrhaphy. c) OD tarsorrhaphied 5 wks, X 100

Figure 12. Rabbit cornea photomicrographs: effects of unilateral OD tarsorrhaphy. d) OS, X 100

Table 24. Effect of tarsorrhaphy on amino acid incorporation of Dutch Belt rabbit corneas.

Rabbit No.	Days Tarsorrhaphied	nanomoles amino acid incorporated/6 hrs/cornea			
		Leucine		Alanine	
		Tarsorrhaphied Eye	Open Eye	Tarsorrhaphied Eye	Open Eye
1	9	3587	3330	3572	3284
2	9	3863	3304	3978	3132
3	9	6444	5609	6548	5557
4	35	5471	5360	3999	3349
5	35	4853	5995	3223	3816

Table 25. Effect of unilateral retrobulbar ethanol, 0.2 ml, on feline iris and ciliary body choline acetyltransferase activity.

Cat No.	Tissue	Days Post-ethanol	picomoles ACh formed/hr/mg tissue	
			Treated Eye	Control Eye
1	Iris	1	266	468
	Ciliary body	1	548	590
2	Iris	1	498	531
	Ciliary body	1	697	621
3	Iris	1	310	181
	Ciliary body	1	459	393
4	Iris	1	363	342
5	Iris	7	6	147
	Ciliary body	7	7	640
6	Iris	7	0	354
	Ciliary body	7	20	585
7	Iris	14	2	610
	Ciliary body	14	25	729

Table 26. Effect of retrobulbar ethanol on iris-ciliary body choline acetyltransferase activity of Dutch Belt rabbits.

Rabbit No.	Post-ethanol (Days)	Ethanol Injected (ml)	nanomoles ACh formed/hr/whole tissue		Pupil reaction to light
			Treated Eye	Control Eye	Treated Eye
2	6	0.3	2.2	18.1	Absent
3	7	0.3	1.4	16.4	Absent
4	7	0.3	8.6	23.1	Minimal
5	9	0.3	5.9	38.6	Absent
6	9	0.3	14.8	30.4	Normal
9	21	0.3x2	29.4	37.6	Almost Norma
10	21	0.3x2	26.4	29.8	Normal
11	21	0.3x2	45.4	39.5	Almost Norma

activities and pupillary reactions had returned to near normal for these parasympathetically innervated structures. However, the fall in corneal choline acetyltransferase activity that occurred through nine days post-ethanol injection was not as significant ($p < 0.1 > 0.05$). By days 12-21 (Table 27) there was no difference ($p > 0.9$) between the corneas of treated and untreated eyes. An attempt to correlate corneal choline acetyltransferase activity with corneal touch-blink reflex sensitivity,, using a Cochet and Bonnet aesthesiometer, was unsuccessful. There was too much variability in the response of the same eye of the same rabbit to the same stimulus. Retinal choline acetyltransferase activity was significantly lowered ($p < 0.001$) by retrobulbar ethanol through day 9 also (Table 28). By day 21, the differences were not significant ($p < 0.2 > 0.1$). The pigment epithelium-choroid did not have a significant reduction in choline acetyltransferase activity thorugh day nine ($p < 0.9 > 0.5$).

B. Tissue culture of corneal epithelium - Rabbit corneal epithelium was cultured for 7 days; at the end of this period, cells covered 65-80% of the Petri dish bottom. These cells were assayed for choline acetyltransferase activity. Table 29 shows the results. Choline acetyltransferase activity for these eyes averaged 19.1 nanomoles ACh formed/hr/mg corneal epithelial protein. This agrees well with the value of 20.6 nanomoles ACh formed/hr/mg corneal protein found in noncultured epithelium (Table 2).

13. Effect of drugs on choline acetyltransferase activity: The decrease in ocular choline acetyltransferase activity produced by tarsorrhaphy did not correlate with an alteration in intra-ocular pressure. However, since cholinergic drugs are used to alter intra-ocular pressure in glaucoma treatment, an attempt was made to determine if these medications

Table 27. Effect of retrobulbar ethanol on corneal choline acetyltransferase activity of Dutch Belt rabbits.

Rabbit No.	Days Post-ethanol	Ethanol Injected(ml)	picomoles ACh formed/hr/mm^2 corneal surface	
			Treated Eye	Control Eye
1	6	0.5	294	378
2	6	0.3	554	1047
3	7	0.3	232	712
4	7	0.3	416	556
5	9	0.3	259	528
6	9	0.3	416	231
7	12	0.3	451	298
8	12	0.3	403	739
9	21	0.3x2	525	578
10	21	0.3x2	1239	1262
11	21	0.3x2	754	574

Table 28. Effect of retrobulbar ethanol on retinal choline acetyltransferase activity of Dutch Belt rabbits.

Rabbit No.	Days Post-ethanol	Ethanol Injected(ml)	picomoles ACh formed/hr/whole retina	
			Treated Eye	Control Eye
3	7	0.3	67.6	86.6
4	7	0.3	62.8	83.8
5	9	0.3	79.0	80.7
6	9	0.3	62.9	94.7
9	21	0.3x2	95.5	148.9
10	21	0.3x2	86.8	82.6
11	21	0.3x2	110.0	153.5

Table 29. Choline acetyltransferase activity of tissue cultured Dutch Belt rabbit corneal epithelium.

Rabbit No.	nanomoles ACh formed/hr/mg corneal epithelial protein
	Cultured 7 days
1	23.3
2	17.7
3	16.3

influence enzyme activity. All four drugs were given topically as eye drops, three times a day unilaterally for 2-3 week periods to rabbits. The three pressure-lowering medications were:

A. pilocarpine nitrate 4%[1], a direct acting cholinergic agonist.

B. diisopropylfluorophosphate 0.1%[2], an indirect acting cholinergic drug.

C. L-epinephrine[3] bitartrate 2%, an alpha and beta adrenergic agonist.

In addition, dexamethasone 0.1%[4], a drug known to frequently elevate human intra-ocular pressure, was given to a fourth group of rabbits.

Corneal choline acetyltransferase was not significantly altered by any of the drugs (pilocarpine, $p = <0.9>0.5$; DFP, $p = <0.9>0.5$; epinerphrine, $p = <0.4>0.2$; and dexamethasone, $p = <0.5>0.4$). Iris-ciliary body enzyme activity was increased by diisopropylfluoro-phosphate, though not to a significant level ($p<0.1>0.05$). The other drugs had no effect (pilocarpine, $p = <0.4>0.2$; epinerphrine, $p = <0.9>0.5$; and dexamethasone, $p = <0.4>0.2$). Retinal choline acetyltransferase activity was unaffected by these medications (pilocarpine, $p = <0.9>0.5$; DFP, $p = >0.9$; epinephrine, $p = <0.9>0.5$; and dexamethasone, $p = <0.4>0.2$). Pigment epithelium-choroid choline acetyltransferase activity was generally lowered by pilocarpine and elevated by diisopropylfluorophosphate and epinephrine, though none of these changes was significant (pilocarpine, $p = <0.1>0.05$; DFP, $p = <0.0>0.05$; epinephrine, $p = <0.1>0.05$; and dexamethasone, $p = <0.9>0.5$).

---

1. P.V. Carpine 4%; Allergan Pharmaceuticals; Irvine, California.
2. Floropryl; Merck, Sharp and Dohme; West Point, Pennsylvania.
3. Lyophrin; Alcon Laboratories; Fort Worth, Texas
4. Decadron; Merck, Sharp and Dohme; West Point, Pennsylvania

Discussion

1. Pyrolysis - gas chromatography assay of acetylcholine

The pyrolysis - gas chromatography assay of acetylcholine described by Szilagyi, Schmidt, and Green (1968) and developed by them and their co-workers (Schmidt, Szilagyi, Alkon, and Green, 1969; Alkon, Schmidt, Green, and Szilagyi, 1970; Green, Alkon, Schmidt, and Szilagyi, 1970) was stated to be capable of measuring 2-5 nanograms of acetylcholine in tissue perfusates. The method, when applied to brain tissue, allowed measurement of 25 nanograms of acetylcholine (Szilagyi, Green, Brown, and Margolis, 1972). An attempt was made to use this assay on the various ocular tissues. However the low acetylcholine content of these tissues taxed the sensitivity of the pyrolysis-gas chromatography assay and several problems manifested themselves. The first was that when the total amount of acetylcholine and internal standard propionylcholine on the pyrolysis ribbon was less than 100 ng, the chromatograms of duplicate samples were not reproducible. The values calculated for acetylcholine varied because the ratios of dimethylaminoethylacetate to dimethylaminoethylpropionate were inconsistant. A second problem was that extraneous peaks, ultimately identified as coming from the ion exchange resin, were appearing on the chromatograms. When assaying large quantities of brain acetylcholine, this resin contaminent rarely was a problem, but when assaying the smaller amounts of ocular acetylcholine, the sensitivity of the recorder had to be increased and the extraneous peaks commonly became interfering peaks of significant size (Figure 1). The third problem was that the vitreous humor contained a material that passed through the extraction procedure and gave interfering chromatogram peaks. This prevented use of the assay to measure vitreous humor acetylcholine and often caused problems when tissues adherent to the vitreous humor, i.e. retina, ciliary body, lens, and optic nerve head, were assayed.

The reproducibility of the dimethylaminoethylacetate to dimethylaminoethylpropionate ratio was improved by adding microgram quantities of butyrylcholine to the pyrolysis ribbon. This increased the sensitivity of the method as shown by the increased size of the chromatogram peaks in Figures 2 and 3. Along with the increase in sensitivity went an increase in reproducibility. However the reproducibility of chromatograms was still not good enough to give quantitiative data when dealing with ocular tissue samples containing less than 50 nanograms of acetylcholine. This was determined by adding known quantities of acetylcholine to a solution of homogenized corneal tissue (the solution had been kept 48 hours at room temperature to permit hydrolysis of any endogenous acetylcholine). This lack of reproducibility was a major reason why the results of the pyrolysis-gas chromatography assay were interpreted in a qualitative fashion rather than quantitatively: acetylcholine was either stated to be present or not detectable. The only exception was in the case of the pooled sample of bovine corneal epithelium. Here, scraping the corneal epithelium from 40 eyes produced several grams of tissue rich in acetylcholine; the chromatograms were reproducible and easily measured (Figure 6).

The reason why the addition of microgram quantities of butyrylcholine to the pyrolysis ribbon increased the sensitivity of the assay is not known. Perhaps a certain amount of the pyrolysis product is adsorbed onto the walls of the pyrolysis chamber and chromatograph column. By increasing the bulk of the pyrolysis sample through the addition of butyrylcholine, proportionately less of the dimethylaminoethylacetate formed from acetylcholine would be adsorbed. Another possibility is that the efficiency of dimethylaminoethylacetate formation from acetylcholine may have been improved by the butyrylcholine addition. Dimethylaminoethylacetate is measured because it is the major product of pyrolysis characteristic of

acetylcholine. In an early paper describing the pyrolysis, Szilagyi, Schmidt, and Green (1968) stated that under their experimental conditions, pyrolysis of acetylcholine chloride required a ribbon temperature of at least 540°C but that dimethylaminoethylacetate was detectable at temperatures up to 900°C. The acetylcholine molecule can fracture to form products other than dimethylaminoethylacetate. For example ethylacetate and trimethylamine are possible products, as well as monomethylaminoethyl-acetate and others. The demethylation reaction would be favored because the C-N bond energy (48.6^{kcal}/mole) is less than those of the C-C (58.6^{kcal}/mole), C-O (70.0^{kcal}/mole), and C-H (87.3^{kcal}/mole) bonds. Perhaps the addition of microgram quantities of butyrylcholine to the pyrolysis ribbon increases the efficiency with which dimethylaminoethylacetate is formed and thereby increases the sensitivity of the assay.

The problem of a soluble resin contaminant producing interfering peaks was solved by altering the extraction procedure so that the ion-exchange resin was not used. Attempts to remove the contamination from the resin prior to its use in the assay were unsuccessful.

As stated before, a qualitative rather than a quantitative interpretation was given to the chromatograms: acetylcholine was stated to be present or not detectable. The sample of pooled bovine corneal epithelium was the lone exception. Here the dimethylaminoethylacetate to dimethylaminoethyl-propionate ratio was reproducible and the value obtained of 35.5 nanograms ACh/mg bovine corneal epithelium agrees very well with that obtained by Williams and Cooper (1965) using bio-assay, 38 ± 2.2 nanograms ACh/mg bovine corneal epithelium. However, as these cattle eyes were delivered by the abattoir hours after death, the level of acetylcholine in the living eye is probably considerably higher. This may explain why Brucke, Hellauer, and Umrath (1949) found 100-200 nanograms ACh/mg bovine corneal epithelium

by bio-assay, assuming their tissue was more fresh than ours.

Acetylcholine was detectable not only in corneal epithelium, but in iris, ciliary body, retina, and pigment epithelium-choroid as well. Finding acetylcholine in these tissues is consistent with our knowledge of these structures: the iris and ciliary body are innervated by the parasympathetic ciliary ganglion. The retina and pigment epithelium-choroid have been shown to contain either choline acetyltransferase activity (Hebb, 1955; de Roetth, 1950A; Lam, 1972; Ross and McDougal, 1974; Alphen, 1957; Williams and Cooper, 1965; Howard and Wilson, 1972; and Howard, Wilson, and Dunn, 1973) or acetylcholine (Brucke, 1938; Brucke, Hellauer, and Umrath, 1949; Hellauer, 1950; Williams and Cooper, 1965; Fitzgerald and Cooper, 1971; Howard and Wilson, 1972; Howard, Wilson and Dunn, 1973; Velhagen, 1930, 1936; Ehinger, Falck, Persson, Rosengren, and Sporrong, 1970; Kovacik, Jezek, and Mrazova, 1972, 1973). Similarly, the inability to detect acetylcholine in the lens is consistent with an absence of indicators of cholinergic activity in this structure save for the presence of small amounts of acetylcholinesterase (de Roetth, 1966; Michon and Kinoshita, 1967).

The uniform inability to detect even the presence of acetylcholine in feline corneas using the pyrolysis-gas chromatography assay appears significant in light of the consistent ability to detect it in the corneas of cattle, rabbits, and humans. This correlates well with the relative choline acetyltransferase activities found in these species. While the feline corneal epithelium contained enzyme activity sufficient to form only 0.1 nanomoles ACh/hr/mg protein, those of rabbit, bovine, and human contained sufficient enzyme to form, respectively, 20.6, 46.5, and 11.2 nanomoles ACh/hr/mg protein (Tables 2,4,5). Hellauer (1950) also found a much lower level of corneal acetylcholine in cats compared to rabbit,

bovine, and human eyes. The cat contained 0.16 nanograms ACh/mg tissue while the respective values found for rabbit, bovine, and human corneas were 13.3, 21.9, and 3.0 nanograms ACh/mg tissue.

A species difference was also found in aqueous humor acetylcholine. Rabbit fluid contained detectable acetylcholine in half of the samples while human samples, even those pooled from 10-15 patients pretreated with cholinesterase-inhibitor eye drops, were uniformly without acetylcholine. Both Velhagen (1930) and Engelhart (1931) were unable, using a bioassay, to detect acetylcholine in all rabbit aqueous humor samples. However, pretreatment of rabbits with the cholinesterase-inhibitor physostigmine permitted detection of acetylcholine levels consistently. Similar pre-treatment of cats was unsuccessful in producing detectable levels of acetylcholine. This inability to find acetylcholine in human aqueous humor, as was Engelhart's inability in feline eyes, is somewhat surprising if one assumes that the source of aqueous humor acetylcholine is either the structures that secrete this fluid or those that are in direct contact with it. These tissues include the cornea, iris, ciliary body, and lens, only the first three of which contain detectable acetylcholine and significant amounts of choline acetyltransferase activity. Dutch Belt rabbit iris and ciliary body choline acetyltransferase activities are, on the average, similar to or less than those of cat and human eyes. And while the rabbit corneal epithelium may have a somewhat higher choline acetyltransferase activity than man (20.6 vs 11.2 nanomoles ACh formed/hr/mg protein) and a much higher choline acetyltransferase activity than that found in cats (20.6 vs 0.1 nanomoles ACh formed/hr/mg protein), there is no detectable enzyme activity in the corneal stroma and endothelium of any of the three species except for a small amount in man (0.4 nanomoles ACh formed/hr/mg protein). Perhaps acetylcholine is transported across the cornea, from epithelium to aqueous humor, to a greater degree in rabbit than in feline and human eyes. Or perhaps rabbits, unlike humans and cats, have parasympathetic nerve fibers that are capable of releasing

acetylcholine into the aqueous humor. The possibility that feline and human eyes have higher cholinesterase activities than rabbit eyes does not seem a likely explanation because pre-treatment of human eyes for days with cholinesterase inhibitors did not result in detectable acetylcholine levels. Bloomfield (1947) and Schumacher and Ehl (1974) reported findings that are different from those reported here. Bloomfield was able to detect acetylcholine-like activity in the aqueous humor of 25% of glaucoma patients and 85% of non-glaucoma patients. However the frog heart bioassay used in this study lacked specificity since any agent capable of stimulating cardiac vagal ganglion cells would elicit depressor activity. Schumacher and Ehl found pooled samples of feline aqueous humor contained 360 nanograms ACh/ml using a photometric assay that was much less sensitivie than pyrolysis-gas chromatography: they stated that their lower limit of detection was 500 nanograms acetylcholine. A more serious problem with their assay is that its specificity for acetylcholine detection, as opposed to other esters, has not been demonstrated.

The pyrolysis-gas chromatography assay for acetylcholine was also used in an experiment to determine if acetylcholine played a role in the miosis of intra-ocular inflammation. Miosis, along with dilatation of the peri-limbal blood vessels, increase in aqueous humor protein content (Krause and Raunio, 1970) and ocular pain, is a cardinal sign of iridocyclitis. Some workers have attempted to attribute all these signs to the effects of endogenously formed prostaglandins. Waitzman and King (1967) reported that PGE_1 and PGE_2 produced miosis in rabbits. However, Starr (1971) was unable to confirm the miotic activity of these compounds when he placed them on the cornea. Beitch and Eakins (1969) reported an occasional miosis produced by intracameral injection of PGE_1, PGE_2, and $PGF_{2\alpha}$. An alternative explanation for the miosis of intra-ocular inflammation would be an increased release of parasympathetic acetylcholine by the

nerve endings of the iris sphincter. Uusitalo, Stjernschantz, and Palkama (1974) have attempted to implicate the parasympathetic nervous system in the control of the blood-aqueous barrier. If the miosis of inflammation were due to increased parasympathetic tonus, then perhaps greater amounts of acetylcholine would be consistently detected in the aqueous humors of inflammed rabbit eyes. For this reason, PGE_1, PGE_2, and $PGF_{2\alpha}$ were individually placed on 20 different rabbit corneas in sufficient quantity to produce intra-ocular inflammation. No miosis resulted and only 50% of the rabbit eyes had detectable acetylcholine in the aqueous humor. These findings seemed to indicate that prostaglandins did not, in themselves, produce miosis but left the question of the role of acetylcholine in the miosis of inflammation, unanswered. Another attempt was made to create inflammatory miosis by trauma in 8 rabbit eyes. The trauma technique used was to aspirate the aqueous humor through a needle placed in the anterior chamber. The primary aqueous humor of 4 of these 8 eyes contained acetylcholine and the same 4 eyes contained acetylcholine in the secondary aqueous humor aspirated 1-3 days later. Only 3 pupils became miotic, all of which had acetylcholine detectable in the aqueous humor. The fourth eye with detectable acetylcholine did not exhibit miosis. If increased acetylcholine release is the explanation for inflammatory miosis, perhaps a threshold level is required that was not reached in this fourth eye or in those eyes where prostaglandins were applied topically.

2. Interspecies variation in ocular choline acetyltransferase activity:

Most of the early studies of ocular choline acetyltransferase activity dealt with the optic nerve (Feldberg and Mann, 1946; Nachmansohn and Berman, 1946; Loewi and Hellauer, 1938) not so much because of an interest, per se, in this tissue but because it was an easily accessible, large nerve conducting sensory impulses. The presence of acetylcholine

Table 30. Summary of ocular acetylcholine content and average choline acetyltransferase activity of four mammalian species

		Corneal epithelium	Iris	Ciliary body	Retina	Pigment epithelium-choroid
Rabbit-	acetylcholine	detectable	detectable		detectable	detectable
	choline acetyltransferase (nm ACh formed/hr/mg protein)	20.6	1.9		15.9	4.0
Cat -	acetylcholine	not detectable	-	-	-	-
	choline acetyltransferase (nm ACh formed/hr/mg protein)	0.1	2.0	2.9	0.7	0.5
Bovine-	acetylcholine	detectable	-	-	-	-
	choline acetyltransferase (nm ACh formed/hr/mg protein)	46.5	10.8	1.9	13.0	4.6
Human-	acetylcholine	detectable	detectable	detectable	detectable	detectable
	choline acetyltransferase (nm ACh formed/hr/mg protein)	11.2	2.5	16.8	6.1	5.1

and choline acetyltransferase in such nerves was considered important supportive evidence for the theory that acetylcholine was responsible for the intra-neuronal propagation of impulses. Feldberg and Mann (1946) reported choline acetyltransferase activities of 0-15 nanograms ACh formed/hr/mg acetone dried powder for canine optic nerve; Nachmansohn and Berman (1946) reported 13-21 nanograms ACh formed/hr/mg acetone dried powder for rabbit optic nerve. de Roetth (1951) reported much higher levels of enzyme activity in both horse and rabbit optic nerve: 100-300 nanograms ACh formed/hr/mg powder. If one assumes (Hebb, 1955) that acetone dried powders have specific activities 10-15 times that of whole tissues, then Cohen's (1955) finding of 15-20 nanograms ACh formed/hr/mg whole rabbit optic nerve agrees well with that of de Roetth. The reason for the 10-15 fold greater activity of de Roetth's optic nerve acetone powder may lie in the nonspecificity of bioassay. This was strongly suggested by the work of Hebb (1955) who found optic nerve formed at least two substances that constricted the ileum used in her bio-assay. One caused reproducible contractions and was probably acetylcholine. The other, by virtue of the rapid tachyphylaxis that developed to its potent ability to constrict guinea pig ileum, may have been a prostaglandin. While one can speculate as to whether it was a prostaglandin, histamine, or some other active agent that was present, Hebb's work clearly indicates a major pitfall of bioassay: lack of specificity. The low values for dog, rabbit, and chicken optic nerve choline acetyltransferase activities she obtained using only reproducible guinea pig ileum responses, would seem more accurate than the higher values of other workers. Hebb's bioassay value of 0.00-0.09 nanomoles ACh formed/hr/mg rabbit optic nerve powder agrees well with that determined by the radioisotope assay used in these experiments (Table 2): 0.0-0.1 nanomoles ACh formed/hr/mg rabbit optic nerve protein. Human optic nerve, with 0.7 nanomoles ACh formed/hr/mg protein (Table 5), contained a higher level of choline

acetyltransferase activity than that of the rabbit. Supportive evidence for the existence of choline acetyltransferase in optic nerve would be its identification in the ganglion cell layer of the retina since this is the location of the cell bodies of the optic nerve axons. The implied assumption in this statement is that choline acetyltransferase is synthesized in the cell body and transported by axonal flow, an assumption supported by the work of Hebb and Waites (1956), Hebb and Silver (1961), Frizell, Hasselgran, and Sjostrand (1970), and Kasa, Mann, Karscu, Toth, and Jordan (1973). While Ross and McDougal (1974) have confirmed the finding of choline acetyltransferase in the ganglion cell layer of mouse, rat, and monkey retinas, it is not clear whether the enzyme resides in the amacrine cell axons terminating on ganglion cells or in the ganglion cells themselves.

The corneal epithelium, as mentioned previously, showed the most marked interspecies variation in choline acetyltransferase activity (Tables 2-5). In contrast to the very high choline acetyltransferase activities of rabbit (20.6 nanomoles ACh formed/hr/mg protein), bovine (46.5 nanomoles ACh formed/hr/mg protein) and human (11.2 nanomoles ACh formed/hr/mg protein) corneal epithelia, the cat contained very little (0.1 nanomoles ACh formed/hr/mg protein) enzyme. These findings are consistent with the ability to detect acetylcholine, using pyrolysis-gas chromatography, in samples of rabbit, bovine, and human corneas and the inability to detect it in feline corneas. The value reported here for bovine corneal choline acetyltransferase activity agrees well with that of Williams and Cooper (1965) who found 33 nanomoles ACh formed/hr/mg bovine corneal epithelium. van Alphen's (1957) determination of 3-7 nanomoles ACh formed/hr/mg powder rabbit epithelium is in the lower part of my range of values, 3.9 - 41.2 nanomoles ACh formed/hr/mg protein (Table 2). van Alphen's use of a high concentration of cysteine, 3.9%, in his extraction procedure was based on Balfour and

Hebb's 1952 paper. It was not until Morris, Hebb, and Bull (1966) reported 14 years later that these high concentrations of cysteine reduced acetylcholine synthesis that this practice ceased. Morris (1967) showed that cysteine nonenzymatically thiolysed the acetyl groups from acetylcoenzyme A and Smith and Weiskopf (1967) reported that cysteine was being acetylated to S-acetylcysteine. This would explain van Alphen's lower corneal choline acetyltransferase values compared to my own and those of Howard, Wilson, and Dunn. Howard and Wilson (1972) and Howard, Wilson and Dunn (1973) found a range of values for choline acetyltransferase in adult rabbit eyes of 7.1 - 123.1 nanomoles ACh formed/hr/mg protein. Their uppermost value was considerably higher than mine. However, they used a radiometric assay for choline acetyltransferase activity that would be expected to give higher apparent values for corneal epithelium than the technique I used. The reason for this is that their method did not include the use of a specific choline acetyltransferase inhibitor. As a result, acetylated cationic molecules other than choline, such as acetylcarnitine, could pass through the ion exchange resin column and contribute to falsely elevated values for acetylcholine synthesis. The experiments reported here made use of a highly specific inhibitor, naphthylvinylpyridinium hydroxyethylbromide (Cavallito, Yun, Kaplan, Smith, and Foldes, 1970; White and Cavallito, 1970B) in the appropriate blank tube permitted correction of this error. It is also quite possible, however, in light of the marked individual variation in corneal choline acetyltransferase activity found between different rabbits (Table 2), that Howard, Wilson, and Dunn were using animals whose enzyme activities were greater than mine.

The rather startling difference between cat corneal choline acetyltransferase activity and acetylcholine content compared to those of rabbit,

bovine, and human eyes also was found for retinal and pigment epithelium - choroid enzyme activities (Tables 2-5). In the retina, the feline choline acetyltransferase activity was only 0.7 nanomoles ACh formed/hr/mg protein, compared with corresponding values of 15.9, 13.0, and 6.1 nanomoles ACh formed/hr/mg/protein in rabbit, bovine, and human retinas, respectively. The pigment epithelium-choroid choline acetyltransferase activity of cats was 0.5 nanomoles ACh formed/hr/mg protein as compared to the much higher values of 4.0, 4.6, and 5.1 nanomoles ACh formed/hr/mg protein for rabbit, bovine, and human pigment epithelium-choroids, respectively. Hebb's (1955) reliance on a regional difference in myelinization to explain why pigeon central retina had lower choline acetyltransferase activity than peripheral retina would not seem to explain the inter-species variation in retinal enzyme activities. The rabbit retina is more highly myelinated (Polyak, 1957) than the other 3 species yet this was not reflected in its having the lowest enzyme specific activity.Ross and McDougal (1974) have shown that the inner plexiform, inner nuclear, and ganglion cell layers of rats and primates have significant amounts of choline acetyltransferase activity. Whether there is a similar localization of enzyme activity in the 4 species studied here cannot be stated at this time, nor can the species difference in retinal enzyme activity be explained.

The finding of choline acetyltransferase activity in the pigment epithelium - choroid samples was somewhat surprising. While neurons pass between sclera and choroid (Duke-Elder, 1961) to the cornea, iris, and ciliary body, there is no known cholinergic innervation of the choroid per se. Evidence that confirms the presence of the enzyme in this tissue is the finding by pyrolysis-gas chromatography of detectable acetylcholine

in 6 of 7 rabbit samples and 10 of 10 human samples of pigment epithelium-choroid. In addition, Kovacik (1974) found, by bioassay, that rabbit choroid, whose pigment epithelium had been removed, contained 0.6 nanograms ACh/mg tissue. Another surprising finding was that while rabbit sclera contained no choline acetyltransferase activity, bovine and human sclera did. Perhaps this reflects the difference in the number of parasympathetic neurons penetrating the sclera of the cattle and human eyes, whose iris and ciliary body are well developed, as opposed to that of the rabbit, whose iris and ciliary body are poorly developed. There is some support for this interpretation in that the bovine iris alone (i.e. without including ciliary body), on a per whole tissue basis, formed an average of 256.7 nanomoles ACh/hr (Table 8) and the human ciliary body alone formed an average of 105.7 nanomoles ACh/hr/whole tissue (Table 9). On the other hand, rabbit iris and ciliary body together formed only 24.8 nanomoles ACh/hr/whole tissue (Table 6). If one assumes that the choline acetyltransferase in these structures is located in the parasympathetic axons, then it can be concluded that there are probably more post-ciliary ganglionic fibers penetrating the bovine and human scleras than the rabbit sclera.

The parasympathetically innervated iris and ciliary body, with two exceptions, did not show the marked interspecies variation in choline acetyltransferase activity, on a per mg protein basis, found in cornea, retina, and pigment epithelium-choroid (Tables 2-5). The feline iris and ciliary body choline acetyltransferase activities were 2.0 and 2.9 nanomoles ACh/hr/mg protein, respectively. These are similar to the value for iris-ciliary body choline acetyltransferase activity of rabbit, 1.9

nanomoles ACh formed/hr/mg protein. Bovine ciliary body and human iris enzyme activities were also in this same range, being 1.9 and 2.5 nanomoles ACh formed/hr/mg protein, respectively. Considerably higher choline acetyltransferase activities were found for bovine iris, 10.8 nanomoles ACh formed/hr/mg protein, and human ciliary body, 16.8 nanomoles ACh formed/hr/mg protein. No explanation can be offered for why these two tissues were exceptionally rich in enzyme activity relative to the others.

The finding in feline eyes that corneal, retinal, and pigment epithelium-choroid choline acetyltransferase activities were reduced while those of iris and ciliary body were not, suggested the possibility that there was some inter-relationship in the enzyme activities of different tissues. For example, did a low retinal choline acetyltransferase activity in an eye mean that the corneal or pigment epithelium-choroid enzyme activity would also be reduced? A statistical analysis of the corneal, iris-ciliary body, retinal, and pigment epithelium-choroid choline acetyl-transferase activities was performed for 26 rabbit eyes. All 6 combinations of pairs of tissues were analyzed using a continuous simple linear regression. No significant correlation could be found, indicating the choline acetyltransferase activities in these tissues varied independently of each other. This is not surprising in view of the diversity of tissues being analyzed: the corneal epithelium has sensory innervation from the trigeminal nerve, the iris and ciliary body have motor innervation from the parasympathetic ciliary ganglion, and the retina is the sensory pathway for the optic nerve.

The aqueous humors of bovine and human eyes exhibited low choline acetyltransferase activities while aqueous humor from rabbit eyes did not

exhibit any enzyme activity (Table 10). The vitreous humors of these species contained enzyme activity in the following sequence: human>bovine> rabbit>cat (Tables 2-5). The amount of choline acetyltransferase activity in these fluids correlated well with the length of time between death and the removal of the ocular fluids. Human eyes, which were received up to days after death had higher enzyme activities in their fluids than the rabbit and cat eyes, which were removed within minutes of death. Bovine eyes, which were received several hours after death, formed an intermediary group. de Roetth (1951) attributed a similar increase in cholinesterase activities of aqueous and vitreous humors postmortum to cellular autolysis of tissues bordering the ocular fluids. Such a breakdown of iris, ciliary body, and retinal neurons could release choline acetyltransferase as well into the aqueous and vitreous humors.

Another possible explanation for finding choline acetyltransferase activity in vitreous humor samples from human, bovine, and rabbit eyes is that there may have been contamination from small pieces of retinal tissue. The retinas of these three species are rich in choline acetyltransferase activity (Tables 2,4, and 5). The retina lies adjacent to the vitreous humor and frequently there are vitreo-retinal adhesions. Perhaps during the collection of samples, small pieces of retina that were adherant to vitreous were also included. In both of these explanations, the source of the vitreous choline acetyltransferase activity is assumed to be the retina. Consistent with this theory is the finding that cat retina has a much lower choline acetyltransferase activity (Table 3) than the other three species and that cat vitreous humor was the only one that had no detectable enzyme activity.

3. Intraspecies variation in ocular choline acetyltransferase activity:

The choline acetyltransferase activity of a given tissue in a given species can vary widely (Tables 2-5 and Figures 7-10). For example, rabbit corneal enzyme activity varied from 3.9 - 41.2 nanomoles ACh formed/hr/mg protein and rabbit retina varied from 5.8-30.3 nanomoles ACh formed/hr/mg protein. Yet the two eyes of the same animal had similar choline acetyltransferase activities for a given tissue. This meant that the variation in enzyme activity varied not from eye to eye but from animal to animal. Initially, corneal epithelium was scraped off the stroma and then assayed. However, unlike Fitzgerald and Cooper (1971), who found excellent agreement for the choline acetyltransferase activities of the two scraped corneal epithelia of the same rabbit, the results in the studies reported here were similar to those of Howard, Wilson, and Dunn (1973) who found scraping a poor method when working with neonatal rabbits. Table 11 shows that better interocular agreement was attained for the enzyme activity of a given animal when the corneas were removed as 9 mm diameter trephine buttons. Perhaps the failure of the scraping method was related to the use of Dutch Belt rabbits, rahter than the albino rabbits of Fitzgerald and Cooper. But more important, there was difficulty transferring quantitatively the small amount of scraped epithelium to the homogenizing tube on a scalpel blade. This loss of several milligrams of tissue would not be an important source of error if the choline acetyltransferase activity were distributed uniformly in the corneal epithelium, since data was calculated on a per mg basis. That the enzyme activity of scraped corneal epithelium from the two eyes of the same animal varied, on the average, by 69% suggested that choline acetyltransferase activity was not uniformly distributed. To test this hypothesis, five 3 mm. corneal buttons were trephined from each of 11 corneas. Buttons were taken from central, superior,

inferior, nasal, and temporal cornea (Table 15). Three distinctly different populations of enzyme activity could be identified ($p < 0.05$). The central cornea, with, on the average, 36% of the total corneal choline acetyltransferase activity, was the most active area. The nasal and inferior cornea had similar enzyme activities which, together, represented 41% of the total corneal choline acetyltransferase activity. The superior and temporal cornea also had similar enzyme activities which, together, represented only 23% of the total corneal choline acetyltransferase activity. This data confirmed that a regional variation in corneal enzyme activity exists and helped to explain the low concordance between the two eyes of the same animal when the epithelium was scraped. Leaving the corneal epithelium intact on the trephine button and transporting the button, without any tissue loss, to the homogenization tube improved the concordance of choline acetyltransferase activities of the two eyes to acceptable limits, i.e. an average difference of 13%.

A further refinement was to calculate corneal epithelial activity on a per mm^2 corneal surface basis (Table 12) rather than a per mg protein basis. Use of a 9 mm diameter trephine permitted removal of corneal buttons having identical surface areas. The average difference in choline acetyltransferase activities of the two corneas of the same animal decreased from 18% to 11% in rabbits and from 25% to 7% in cattle when data was calculated on a per mm^2 surface area basis rather than a per mg protein basis. A possible explanation is as follows: The epithelium represents only a small portion of the total corneal protein while the stroma, which accounts for most of the corneal weight, is approximately 94% protein (Maurice and Riley, 1970). The corneal stroma is difficult to homogenize and any errors in this homogenization will affect enzyme activity calculated

on a per mg. basis. In addition, differences in the amount of stromal protein in the two eyes of an animal can significantly alter the value for enzyme activity calculated on a per mg. protein basis even if the enzyme activities of the epithelia are similar. The preferred method that evolved for comparing the corneal epithelial choline acetyltransferase activities of the two eyes of an animal was to use homogenized trephined corneal buttons and to calculate the data on a per mm^2 corneal surface basis.

A similar attempt was made to devise techniques that would improve the interocular agreement of choline acetyltransferase activities of irides, ciliary bodies, retinas, and pigment epithelium-choroids of an animal. In general, calculating on a per whole tissue basis was neither worse nor better than calculating on a per mg. protein basis (Tables 13 and 14). Only rabbit retinas ($p<0.01$) and feline pigment epithelium-choroid ($p<0.05$) showed a statistically significant greater degree of concordance when calculated on a per whole tissue basis. No explanation can be offered why only these tissues in these species exhibited greater intra-animal concordance.

Despite the improvements in assay technique and calculation of choline acetyltransferase activity, there still remained a marked inter-animal variation for a given species that was difficult to explain. The two corneas of 100 Dutch Belt rabbits might agree well with each other, but there was a 25-fold variation in enzyme activity between different animals (Figure 7). What was the physiologic role played by corneal acetylcholine that required its synthesizing enzyme be present in high concentration in one rabbit while another rabbit had normal corneal function with less than 4% this amount? The approach to answering this question was to

examine a variety of factors that might influence ocular choline acetyltransferase activity.

The first consideration was to determine if the differences found in corneal choline acetyltransferase activities of different rabbits was related to differences in genotype, differences in environmental factors, or both. To study the effect of genotype on choline acetyltransferase activity, three different groups of Dutch Belt rabbits were studied. Group 1 consisted of 45 randomly chosen Dutch Belt rabbits. As these rabbits came from a single supplier, there may have been some degree of inbreeding. Group 2 was genetically more homogeneous than group 1 because it consisted of littermates (Table 16). Group 3 consisted of 4 highly inbred rabbits having 82-85% genetic homogeneity (Table 17). It was reasoned that as the degree of inbreeding increased in going from group 1 to group 3, the degree of variation of ocular choline acetyltransferase activity should decrease between members within a group. Unfortunately the environmental conditions under which each group was raised were different, so that more than one variable was being examined. Group 1 rabbits were obtained from a supplier located in New York State, group 2 rabbits were born and raised in the laboratory, and group 3 rabbits were obtained from a supplier in Maine. However all rabbits were kept a minimum of 3 days in the animal quarters under standard conditions of diet, temperature, humidity, and light-dark exposure before being used. The littermates of group 2 did not exhibit a significantly smaller spread in corneal, retinal, or pigment epithelium-choroid choline acetyltransferase activities compared to group 1. Only the iris-ciliary bodies of group 2 animals had significantly better agreement ($p<0.05$) than those of group 1. However, in the highly inbred rabbits of group 3, there was a significant ($p<0.01$) decrease in

inter-animal variation not only of corneal choline acetyltransferase activities but also iris-ciliary body, retinal, and pigment epithelium-choroid enzyme activities (Table 18). The finding that the corneal enzyme activities from individual eyes in this group of highly inbred animals deviated only 15% from their averaged value was considered consistent with the belief that genetic factors play a major role in determining the level of corneal enzyme activity.

In order to assess the role environmental factors might play in determining the level of corneal choline acetyltransferase activity, a unilateral tarsorrhaphy was performed on a group of rabbits. Tarsorrhaphy was chosen because it seemed to be the procedure altering the greatest number of corneal environmental factors. The influence of light was mitigated, the corneal temperature was elevated, the effect of humidity on corneal evaporation was altered, atmospheric oxygen was no longer in contact with the epithelium, and corneal CO_2 could no longer diffuse into the atmosphere. Initially, the rabbits which were tarsorrhaphied were examined after 3 and 8 weeks. There was a dramatic fall in corneal choline acetyltransferase activity of the tarsorrhaphied eye to a level only 11-30% that of the open eye (Table 19). Additional rabbits were tarsorrhaphied for varying periods of time. To make certain that the method of tarsorrhaphy was not responsible for the change in enzyme activity, the technique was varied. Sometimes the lid margins were excised and the raw edges sewn together with silk or chromic gut sutures. At other times, the lid margins were left intact and the lids sewn together using silk or chromic gut sutures. A third technique was the use of collodion to fasten the lids together. None of these variations altered the ability of tarsorrhaphy

to produce a fall in corneal choline acetyltransferase activity. The decline was highly significant ($p<0.001$) and was maximum by day 8 for there was no statistically significant difference between those animals assayed at days 8 and 56. Even rabbits tarsorrhaphied as little as 24 hours (Table 20) showed a significant ($p<0.002$) fall in the choline acetyltransferase activity of the closed eye compared to the open one. However, the decline was not maximal at 48 hours since there was a significantly larger ($p<0.01$) difference between open and closed eyes in those rabbits tarsorrhaphied 8 to 56 days. In a few rabbits, the tarsorrhaphy was inadvertently made incomplete. In these animals the choline acetyltransferase activity after weeks of approximately 80% lid closure was about 70% that of the open eye. Relatively small imperfections in the completeness of tarsorrhaphy were, therefore, able to prevent the dramatic fall in enzyme activity.

An attempt was made to determine the rate of recovery of corneal choline acetyltransferase activity after separating the lids. Rabbits unilaterally tarsorrhaphied for 8 days had their closed lids separated. However they continued to exhibit abnormally low corneal choline acetyltransferase activities 24 hours after the lids were opened ($p< 0.01$). The significance of this finding was compromised because lid structure was sufficiently altered from the tarsorrhaphy to impair blinking and protection of the cornea. This may have further retarded recovery of enzyme activity. The lid function was so compromised that severe corneal damage resulted if the recovery period were extended beyond 24 hours.

The results from tarsorrhaphy brought to mind the work of Howard and Wilson (1972) and Howard, Wilson, and Dunn (1973). They found that newborn

rabbits did not have detectable corneal acetylcholine or choline acetyltransferase activity until their eyelids opened at about age 10 days. Adult levels were not attained until age 50 days, approximately. It may well be that those factors preventing the development of corneal choline acetyltransferase activity when the lids are closed in a newborn rabbit also cause the decline in enzyme activity when the lids are closed in adult rabbits.

The other ocular tissues with significant amounts of choline acetyltransferase, i.e., iris-ciliary body, retina, and pigment epithelium-choroid, were also examined to see if tarsorrhaphy affected their enzyme activities. In those rabbits tarsorrhaphied 9 or more days, there was a small but significant decrease in iris-ciliary body ($p < 0.05$) and retinal ($p < 0.02$) choline acetyltransferase activities in the closed eyes. The iris-ciliary body enzyme activity fell, on the average, to 74% of that of the open eye. The retinal enzyme activity fell, on the average, to 80% of that of the open eye. It should be pointed out that the two retinas of a normal rabbit vary, on the average, by 17%. What made the retinal results of the tarsorrhaphied rabbits significant was that the variation was not random since the tarsorrhaphied eye was always the one with the lower enzyme activity. The small decrease in iris-ciliary body and retinal choline acetyltransferase activity may have resulted from a lack of light stimulation. Light not only excites retinal tissue, but also reflexly alters the tonus of the iris sphincter and ciliary muscle. The most well known example of this is light altering the size of the pupillary opening. In the adrenergic system, physiologic or pharmacologic alteration of neuron activity can result in altered tyrosine hydroxylase activity (Molinoff and Axelrod, 1971; Black and Geen, 1973). Perhaps the

light deprivation from tarsorrhaphy caused a similar decrease in choline acetyltransferase activity in cholinergic neurons. Wiesel and Hubel (1963A; 1963B; 1965) have demonstrated in the kitten that tarsorrhaphy and altered visual input can result in anatomic and physiologic alteration of the neurons of the visual pathway. The decrease in choline acetyltransferase activity in tarsorrhaphied rabbit retina and iris-ciliary body may represent part of a similar process.

Another form of physiologic tarsorrhaphy, besides that found in the newborn rabbit, is the lid closure during sleep. During sleep the intra-ocular pressure of the human eye increases. Phelps, Woolson, Kolker, and Becker (1974) wrote, "Although the peak of the diurnal variation is most frequent between 6:00 and 8:00 A.M., it may occur any time of the day or night. Furthermore, the timing and magnitude of the peak intra-ocular pressure may vary from day to day." If one assumes that the ocular choline acetyltransferase activity of man decreases during the lid closure of sleep in a manner similar to that which occurs to the tarsorrhaphied rabbit eye, then perhaps decreased cholinergic activity may result in an elevation of intra-ocular pressure. This theory is consistent with the clinical finding that increased cholinergic stimulation, as, for example, that resulting from pilocarpine eye drops, results in a lowering of intra-ocular pressure while muscarinic blocking agents, as atropine, tend to elevate the intra-ocular pressure. Duke-Elder and Gloster (1968) have written:

> "Many explanations of the basic causation of the diurnal fluctuation of intra-ocular pressure have been advanced. Some of them have involved local factors only, such as changes in the size of the pupil, the massaging of the eye by movements of the lids or the extraocular muscles during the day contrasted with the relative immobility during sleep;

> or the effects of changing bodily posture acting through concomitant adjustments in the vascular bed of the eye. Such possibilities, however, are ruled out by the persistence of the variation after the use of miotics or mydriatics or after surgical relief of the tension in cases of glaucoma, and by the fact that a sudden reversal of habits (such as if a patient remains up at night and goes to bed during the day or changes his hours of eating) does not change the pattern of the diurnal variation until a considerable period has elapsed (Raeder, 1925); only a prolonged reversal of the sleep-habits induces a reversal in the diurnal rhythm."

The theory that the decreased choline acetyltransferase activity resulting from lid closure would result in increased intra-ocular pressure was first tested in rabbits. Five rabbits were given a prolonged unilateral surgical tarsorrhaphy of 8 or more days. At the end of this period, the lids were separated and the intra-ocular pressures of the two eyes of the same rabbit were compared using a Schiotz tonometer. No significant rise in intra-ocular pressure could be found in the tarsorrhaphied eye. A second approach was to admit 6 human volunteers to the hospital where a temporary unilateral tarsorrhaphy was maintained for 2.5 days using tape to close one pair of lids of each patient. For the next 2.5 days, the closed lids were opened once every three hours, around the clock, to permit measuring the intra-ocular pressure with an applanation tonometer. The lids were open no more than 40 seconds at each pressure reading and were then resealed with tape. If persistent lid closure resulted in decreased human ocular choline acetyltransferase activity and this in turn resulted in an elevation of intra-ocular pressure, then the following would have occurred.

1. The variation in intra-ocular pressure in the closed eye, marked by rises and falls, would no longer occur while the open eye would continue to show diurnal variation.

2. The closed eye would show a slow steady increase in intra-ocular pressure with time while the open eye would continue to fluctuate within

the same limits.

3. The peak pressure difference between the closed eye and the open eye, for a given 24-hour period, would increase with each passing day.

None of these effects occurred: the intra-ocular pressures of the closed eyes showed similar diurnal patterns as the corresponding open eyes, and there was no trend towards an increased difference in peak ocular pressures with time. Two of the patients were brothers whose two sisters had chronic simple glaucoma. These patients' pressure patterns were examined separately as well as being included in the study with all 6 patients. There was no difference in these brothers' ocular pressure responses to lid closure compared to those of the patients without a family history of chronic simple glaucoma. It was concluded that lid closure is not a factor directly or indirectly in the diurnal variation in intra-ocular pressure in man. In addition, the multiple peaks and troughs that occurred for each patient's intra-ocular pressure during a 24-hour period supported the belief that man had irregular diurnal variations in his intra-ocular pressure, not a smoothly and regularly changing diurnal curve. In this group of six patients, there was a strong tendency for the intra-ocular pressure to be maximum at noon and minimum at 9 P.M. ($p<0.01$).

Another attempt to correlate intra-ocular pressure with choline acetyltransferase activity was made using medications known to affect the former. Pilocarpine, diisopropylfluorophosphate, and epinephrine are drugs used therapeutically in man to lower intra-ocular pressure (Havener, 1970). Dexamethasone, however, produces an increase in intra-ocular pressure in man (Bigger, Palmberg, and Zink, 1975). In

rabbits, topical corticosteroids are also capable of elevating the intra-ocular pressure although a longer period of chronic administration and use of a 1% dexamethasone solution are required (Levene, Rothberger, and Rosenberg, 1974). Approximately 90% of rabbits respond with an increase in intra-ocular pressure if exposed to topical corticosteroid drops for 18 weeks. To determine if any of these drugs produces its effect by altering ocular choline acetyltransferase activity, pilocarpine 4%, diisopropyl-fluorophosphate 0.1%, l-epinephrine bitartrate 2%, or dexamethasone phosphate 0.1% eye drops were administered topically to one eye of 5 rabbits three times a day for 2 and 3 week periods. No effect was noted in the choline acetyltransferase activities of the corneas, iris-ciliary bodies, retinas, and pigment epithelium-choroids of these eyes compared to the untreated eyes. It was concluded that these drugs do not affect intra-ocular pressure by influencing the choline acetyltransferase activities of the ocular tissues.

The two eyes of four patients with chronic simple glaucoma were obtained after death and compared with a group of 10 human eyes from non-glaucomatous patients. There was no obvious deficiency in choline acetyl-transferase activity in the corneas, irides, ciliary bodies, pigment epithelium-choroids, or retinas of the glaucomatous eyes. In fact, the average enzyme activity of all these tissues, save iris, was higher in the glaucomatous eyes than in the nonglaucomatous eyes. The exception was that glaucoma patients had an average iris choline acetyltransferase activity of 1.7 nanomoles ACh formed/hr/mg protein as opposed to a corresponding value of 2.5 nanomoles ACh formed/hr/mg protein in non-glaucomatous patients. The number of eyes assayed is too small to over-come the artifactual alterations in enzyme activity produced by different

storage conditions of the eyes prior to reaching me and different lengths of time between patient death and my obtaining the eyes. For example, the average length of time from death until receiving the eyes of non-glaucomatous patients was 32 hours, with a range of 9 to 72 hours; the corresponding figures for glaucomatous eyes were an average of 28 hours with a range of 16-48 hours. At present there is no evidence that choline acetyltransferase activity is deficient in patients with chronic simple glaucoma nor is there any evidence linking choline acetyltransferase activity with intra-ocular pressure.

4. Decrease in corneal choline acetyltransferase activity by tarsorrhaphy:

It has been mentioned previously that a number of environmental factors are altered by tarsorrhaphy. Which of these factors were effective in lowering corneal choline acetyltransferase activity could only be ascertained by examining one factor at a time. Before beginning these investigations, it was important to know whether the decrease in choline acetyltransferase activity produced by tarrsorrhaphy represented a specific change in this enzyme, or whether it was only part of a general metabolic alteration of the corneal epithelium. Rabbits with a unilateral tarsorrhaphy maintained more than 8 days failed to show any significant alteration in their corneal lactic dehydrogenase activity or amino acid incorporation (Tables 23 and 24). Lactic dehydrogenase activity was chosen because the corneal epithelium is extremely rich in this enzyme (Maurice and Riley, 1970). Since it was not practical to assay every known corneal enzyme individually, protein incorporation of leucine and alanine was chosen as an indicator of cellular metabolism. Histologic examination (Figure 13) of tarsorrhaphied rabbit corneas failed to reveal

any evidence of epithelial atrophy; if anything, the corneal epithelium was thicker in the tarsorrhaphied eye because the superficial layers were not being desquamated by the wiping action of the blinking lids. This impression was further confirmed by calculating choline acetyltransferase activity on a per mg. protein basis. If the decrease in corneal choline acetyltransferase activity calculated on a per mm^2 surface area were caused simply by a thinning of the corneal epithelium, then the same data calculated on a per mg protein basis would show a more normal concentration of enzyme. However, it did not matter which method of calculation was used, the corneal enzyme activity was markedly diminished by tarsorrhaphy. It was concluded that the decline in choline acetyltransferase activity produced by tarsorrhaphy was a rather specific response of the corneal epithelium to lid closure.

Which of the factors altered by tarsorrhaphy were responsible for the fall in choline acetyltransferase? Fitzgerald and Cooper (1971) had placed rabbits in the dark for periods of 2.5 to 4 hours without altering their corneal acetylcholine content. Perhaps a longer period of darkness was required. However, it was found that rabbits placed for 8 weeks in continuous darkness had unchanged corneal choline acetyltransferase activity. This indicated that light was not required for normal levels of corneal choline acetyltransferase activity. Rabbits placed for 6 weeks in continuous light also failed to have altered corneal choline acetyltransferase activity. Nor did the decrease in the rate of evaporation of water from the tarsorrhaphied cornea explain the decrease in choline acetyltransferase activity because prolonged exposure, alternately, to 28% relative humidity (for 13 days) and 69% relative humidity (for 8 days) failed to alter enzyme activity. If tarsorrhaphy lowered corneal choline acetyltransferase

activity by preventing the diffusion away of CO_2 produced by epithelial metabolism, then increasing the concentration of CO_2 bathing the cornea by more than 100-fold might be expected to mimic the effect of lid closure. However rabbits kept one week in an atmosphere of 5% CO_2 failed to have altered corneal enzyme activity.

It was not until corneal temperature was altered that a change in corneal choline acetyltransferase activity occurred. Lowering the corneal temperature by keeping rabbits in a 15°C. room for 17 days instead of a 30°C. room produced a significant ($p < 0.001$) elevation in enzyme activity (Table 21). If lowering the corneal temperature raised the choline acetyltransferase activity, then raising the corneal temperature might be expected to produce a fall in enzyme activity. This could explain how tarsorrhaphy lowered corneal choline acetyltransferase activity: it raised the corneal temperature, which was normally exposed to a 20.5°C. room temperature, toward the rabbit body temperature of 39.5°C. A second experiment was designed to test this theory. Rabbits were unilaterally tarsorrhaphied and immediately placed in a 39.5°C. room. Out of approximately 13 rabbits, 9 survived 48 hours under these conditions. It was expected that these surviving animals would have approximately equal corneal choline acetyltransferase activities since both eyes were maintained at 39.5°C. However, there was a consistently ($p < 0.05$) lower enzyme activity in the tarsorrhaphied eyes than the open eyes (Table 22). In addition the decline in choline acetyltransferase activity of tarsorrhaphied eyes compared to open eyes was not significantly less for rabbits kept at 39.5°C than for rabbits kept at room temperature. The statement was true whether the change in enzyme activity between the two eyes of the same animal were calculated as a percent difference or

as an absolute difference.

One is left, then, with the conclusion that altering corneal temperature will alter corneal choline acetyltransferase activity but this does not account for the effect of tarsorrhaphy. Other factors have yet to be elucidated. The finding that lowering corneal temperature promotes enzyme activity is unexpected since enzyme activity and metabolic rates tend in general to increase with temperature elevation. Williams and Cooper (1965) found that the corneas of freshly enucleated bovine eyes swelled at 4°C. and there was an associated decrease in corneal acetylcholine. Raising the incubation bath temperature to 37°C. caused a partial reversal of this detergesence and a return of acetylcholine content to control levels. Perhaps the increase in corneal enzyme activity detected in rabbits maintained at a lower temperature for 17 days is related to the physiologic response of the eye to prevent corneal swelling. This might explain why the enucleated bovine eyes were not able to tolerate sudden temperature changes. If one assumes that the lid closure of sleep results in a decrease in choline acetyltransferase activity, then one would expect on awakening to have a slightly thickened cornea. Mandell and Fatt (1965) have confirmed such a slight increase in corneal thickness. Maurice and Giardini (1951) have shown that removal of the rabbit corneal epithelium will result in a doubling of the corneal thickness. This suggests that the corneal epithelium may be involved in maintaining corneal dehydration and choline acetyltransferase and acetylcholine may be important in this regard.

To support the concept that the acetylcholine produced by choline acetyltransferase is involved in transport across the corneal epithelium, it would be necessary to show that the enzyme is located in the epithelial

cells rather than in the nerve endings. Two approaches were used to demonstrate that this was so. Ethanol was injected in the retrobulbar space unilaterally, first in cats (Table 25) and then in rabbits (Tables 26-28). Two to nine days after injection, iris, ciliary body and retina showed a significant ($p < 0.05$) fall in choline acetyltransferase activity. However, cornea and pigment epithelium-choroid enzyme activities did not show a significant decline. Since ethanol affected the neurons of the optic nerve and the ciliary ganglion, it is reasonable to assume that the trigeminal nerve fibers in the retrobulbar area were also affected. Kornbleuth (1949) showed that the corneal nerves of rabbits given retrobulbar injections of ethanol had normal histological staining characteristics for 4 days, but from days 6-27, the majority of nerves stained abnormally. It was not till day 48 after injection that most nerves stained normally. The mitosis rate of the corneal epithelium decreased by 30% during the first 3 days post - injection, increased to 30% above normal on the 4th day, and then decreased again to 25-30% below normal during days 6-21 post-injection. There was a slightly higher corneal water content after alcohol injection that the author did not feel was statistically significant. Maumenee (1949) has also presented evidence confirming that retrobulbar ethanol interferes with corneal sensory neuron function. He injected ethanol retrobulbarly in orbits of patients with blind painful eyes and produced an anesthesia lasting weeks. As there was no significant drop in corneal choline acetyltransferase activity in those rabbits that received retrobulbar ethanol the implication is that the enzyme is not located in the corneal sensory nerve endings. A more direct way of establishing the epithelial cell location of choline acetyltransferase was to culture rabbit corneal epithelial cells using a technique based

on that of Gnadinger, Heimann, and Markstein (1973). Their work on tissue cultures was confirmed: corneal epithelial cells cultured 7 days still retained choline acetyltransferase activity. They reported that the activity fell to about 50% that of freshly scraped tissue. However, the cultured epithelia assayed in this laboratory had an average enzyme activity of 19.1 nanomoles ACh formed/hr/mg corneal epithelial protein; this agreed well with the value of 20.6 nanomoles ACh formed/hr/mg corneal epithelial protein (Table 2) found in freshly scraped tissues.

SUMMARY

The ocular cholinergic systems were investigated using a pyrolysis - gas chromatography assay for acetylcholine and a radiometric assay for choline acetyltransferase activity. The sensitivity of the acetylcholine assay was improved by adding microgram quantities of butyrylcholine to the platinum pyrolysis ribbon. However, when tissue samples contained less than 50 nanograms acetylcholine, the pyrolysis - gas chromatography assay was not quantitatively reproducible. Therefore, ocular tissue chromatograms were interpreted as showing the qualitative presence or absence of acetylcholine. Acetylcholine was consistently detected in the corneas of rabbit, bovine, and human eyes. However, feline corneas had no detectable corneal acetylcholine. A second species variation was found in aqueous humor acetylcholine content: 50% of rabbit eyes assayed contained aqueous humor acetylcholine while none was detectable in human anterior chamber fluid even though the pooled samples were removed from eyes pre-treated with cholinesterase-inhibitors. Attempts to determine if the pupillary moisis found in intraocular inflammation were associated with an elevated aqueous humor acetylcholine level gave equivocal results: eyes with miosis had detectable acetylcholine in their aqueous humors but not all inflammed eyes with detectable aqueous humor acetylcholine had moisis.

Of the five ocular tissues found consistently to contain detectable quantities of choline acetyltransferase activity, the corneal epithelium and retina had the most enzyme activity, the iris and ciliary body had an intermediate quantity, and the pigment epithelium-choroid had the least. Several species variations were noted in choline acetyltransferase activity: the cornea, retina, and pigment epithelium-choroid of rabbit, bovine, and human eyes had far greater enzyme activities than the

corresponding tissues in feline eyes. The iris and ciliary body of these four species had similar choline acetyltransferase activities with two exceptions: bovine iris and human ciliary body had much higher enzyme activities. No explanation for these variations could be found.

While the corneal choline acetyltransferase activities of the two eyes of a given rabbit, bovine, or human agreed well, there was a marked inter-animal variation in enzyme activity, e.g., there could be a 25-fold variation in rabbit corneal choline acetyltransterase activity. In addition, a regional difference in rabbit corneal choline acetyltransferase activity was demonstrated: central cornea > inferior or nasal cornea > superior or temporal cornea. Inter-animal variation in the enzyme activities of rabbit ocular tissues decreased among highly inbreed animals indicating some genetic regulation of choline acetyltransferase activity exists.

Environmental factors also were important in determining ocular choline acetyltransferase activity. Unilateral tarsorrhaphy caused a rapid fall in the corneal enzyme activity of the closed eye. This fall was highly significant ($p < 0.002$) by 24 hours and maximal by 8 days. A smaller but still significant fall in iris-ciliary body and retinal choline acetyltransferase activity occurred by day 9. The decline in corneal choline acetyltransferase activity was a highly specific response of this tissue to tarsorrhaphy because lactic dehydrogenase activity, amino acid incorporation, and histologic appearance of the closed eye's cornea were unaffected. All attempts made to correlate ocular choline acetyltransferase activity with intra-ocular pressure were unsuccessful: prolonged tarsorrhaphy did not raise rabbit or human intra-ocular pressure nor was there a striking difference in the enzyme activities of the ocular tissues of normal and glaucomatous eyes. Three weeks of topical application of medications known to alter intra-ocular pressure, such as pilocarpine, epinephrine, diisopropylfluorophosphate, and dexamethasone failed to

alter ocular choline acetyltransferase activity.

Several of the environmental factors altered by tarsorrhaphy were investigated individually to determine the reason why lid closure resulted in decreased choline acetyltransferase activity. The prolonged presence or absence of light did not alter enzyme activity. Neither humidity alteration nor increased atmospheric CO_2 affected enzyme activity. However, an inverse relationship was found between ambient temperature and corneal choline acetyltransferase activity: as room temperature decreased, corneal choline acetyltransferase activity gradually increased. This could not explain the rapid fall in enzyme activity produced by tarsorrhaphy because more than 10 days were required for ambient temperature to produce its effect. In addition, raising the ambient temperature to that of rabbit body temperature was not as effective as tarsorrhaphy in lowering corneal choline acetyltransferase activity.

Evidence was found, using tissue cultured corneal epithelium and retrobulbar injections of ethanol, indicating that removing the influence of the trigeminal nerve did not alter corneal choline acetyltransferase activity. This gave supportive evidence to the belief that, in the cornea, this enzyme is located in the epithelial cells rather than in the sensory nerve endings.

BIBLIOGRAPHY

Alkon, D.L., D.E. Schmidt, J.P. Green, and P.I.A. Szilagyi. The release of acetylcholine from the innervated and denervated diaphragm. The Journal of Pharmacology and Experimental Therapeutics, 1970, 174:346-349.

Alpert, A., R.L. Kisliuk, and L. Shuster. A simple radioisotopic assay for choline acetyltransferase and its application in the Lactobacillus plantarum system. Biochemical Pharmacology, 1966, 15:465-473.

Baddiley, J. and E.M. Thain. Coenzyme A Part II. Evidence for its formulation as a derivative of pantothenic acid - 4'phosphate. Journal of the Chemical Society, 1951, Pt. III: 2253-2258.

Baddiley, J., E.M. Thain, G.D. Novelli, and F. Lipmann. Structure of coenzyme A. Nature, 1953, 171:76.

Balfour, W.E. and C.O. Hebb. Mechanisms of acetylcholine synthesis. Journal of Physiology, 1952, 118:94-106.

Banister, J., V.P. Whittaker, and S. Wijesundera. The occurence of homologues of acetylcholine in ox spleen. Journal of Physiology, 1953, 121:55-71.

Barker, L.A. and T.W. Mittag. Inhibition of synaptosomal choline uptake by naphthylvinylpyridiniums. FEBS Letters, 1973, 35:141-144.

Barker, L.A. and T.W. Mittag. Comparative studies of substrates and inhibitors of choline transport and choline acetyltransferase. Journal Pharmacology and Experimental Therapeutics, in press.

Barnett, R.J. and J.A. Higgins. Cytochemistry and electron microscopy: acyl hydrolase and transferase reactions. "Proceedings of the Electron Microscopy Society of America, 26th Annual Meeting". Claitor's Publishing Division. Baton Rouge, La., 1968, p. 1.

Beitch, B.R. and K.E. Eakins. The effects of prostaglandins on the intra-ocular pressure of the rabbit. British Journal of Pharmacology, 1969, 37:158-167.

Bell, K.M., A.R. Kirby, and F.C. Rodger. The source of the corneal nerve fibres in the cat. Journal of Physiology, 1952, 117:56P.

Berman, R., I.B. Wilson, and D. Nachmansohn. Choline acetylase specificity in relation to biological function. Biochemica et Biophysica Acta, 1953, 12:315-324.

Bigger, J.F., P.F. Palmberg, and H.A. Zink. In vitro corticosteroid: correlation response with primary open-angle glaucoma and ocular corticosteroid sensitivity. American Journal of Ophthalmology, 1975, 79:92-97.

Black, I.B. and S.C. Geen. Trans-synaptic regulation of adrenergic neuron development inhibition by ganglionic blockade. Brain Research, 1973, 63:291-302.

Bloomfield, S. Relative deficiency of parasympathomimetic activity in aqueous of eyes with chronic simple glaucoma. Archives of Ophthalmology, 1947, 37:608-617.

Boberg-Ans, J. Experience in clinical examinations of corneal sensitivity. British Journal of Ophthalmology, 1955, 39:705-726.

Brucke, H, v. Zur Kenntnis der Hornhautschadigung nach Trigeminusverletzung. Klinische Wochenschrift, 1938, 17:420-422.

Brucke, H.v., H.F. Hellauer, and K. Umrath. Azetylcholin-und Aneuringehalt der Hornhaut und seine Beziehungen zur Nervenversorgung. Ophthalmologica, 1949, 117:19-35.

Bull, G., A. Feinstein, and D. Morris. Sedimentation behavior and molecular weight of choline acetyltransferase. Nature, 1964, 201:1326.

Bull, G. and B. Oderfeld-Nowak. Standardization of a radiochemical assay of choline acetyltransferase and a study of the activation of the enzyme in rabbit brain. Journal of Neurochemistry, 1971, 18:935-941.

Burgin, A.S.V., G. Burke, and M. Desbarats-Schonbaum. The specificity of brain choline acetylase. British Journal of Pharmacology, 1956, 11:308-312.

Burt, A.M. The histochemical demonstration of choline acetyltransferase activity in the spinal cord of the rat. Anatomical Record, 1969, 163:162.

Burt, A.M. A histochemical procedure for the localization of choline acetyltransferase activity. The Journal of Histochemistry and Cytochemistry, 1970, 18:408-415.

Burt, A.M. and A. Silver. Non-enzymatic imidazole-catalysed acyl transfer reaction and acetylcholine synthesis. Nature New Biology, 1973, 243:157-159.

Cavallito, C.J., H.S. Yun, T. Kaplan, J.C. Smith, and F.F. Foldes. Choline acetyltransferase inhibitors. Dimensional and substituent effects among styrylpyridine analogs. Journal of Medicinal Chemistry, 1970, 13:221-224.

Cavallito, C.J., H.S. Yun, J.C. Smith, and F.F. Foldes. Choline acetyltransferase inhibitors, configurational and electronic features of styrylpyridine analogs. Journal of Medicinal Chemistry, 1969, 12:134-138.

Chang, C.H. and J.H. Gaddum. Choline esters in tissue extracts. Journal of Physiology, 1933, 79:255-285.

Chao, L.P. and F. Wolfgram. Activation, inhibition and aggregation of choline acetyltransferase. Journal of Neurochemistry, 1974, 23:697-701.

Chase, J.F.A. and P.K. Tubbs. Specific inhibitors of carnitine acetyltransferase and other acyltransferases, Biochemical Journal, 1966, 100:47-48P.

Chiou, C. and B.V. Rama Sastry. Acetylcholinesterase hydrolysis of halogenated substituted acetylcholines. Biochemical Pharmacology, 1968, 17:805-815.

Cohen, M. Concentration of choline acetylase in conducting tissue. Archives of Biochemistry and Biophysics, 1956, 50:284-296.

Csillik, E.K. and N. Halasz. Ultrastructural localization of acetylcholinesterase in autonomic postaganglionic axons. Journal of Neuro-Visceral Relations, 1968, 31:3-10.

Curtis, D.J. The fine structure of photoreceptors in Mitopus Morio (Phalangida). Journal of Cell Science, 1969, 4:327-351.

Dardenne, U., W. Leydhecker, und E. Halferich. Die cholinesterase in der Iris von Mensch und Rind. v. Graefes Archiv fur Ophthalmologie, 1957, 158:434-438.

deRobertis, E., G.R. deLores Arnaiz, L. Salganicoff, A.P. de Iraldi, and L.M. Zieher. Isolation of synaptic vesicles and structural organization of the acetylcholine system within brain nerve endings. Journal of Neurochemistry, 1963, 10:225-235.

deRoetth, A. Jr. Choline acetylase activity in ocular tissues. Archives of Ophthalmology, 1950A, 43:849-852.

de Roetth, A. Jr. Cholinesterase activity in ocular tissues and fluids. Archives of Ophthalmology, 1950B, 1004-1025.

de Roetth, A., Jr. Role of acetylcholine in nerve activity. Journal of Neurophysiology, 1951, 14:55-57.

de Roetth, A. Jr. Lens opacities in glaucoma patients on Phospholine Iodide therapy. American Journal of Ophthalmology 1966, 62:619-628.

Domino, E.F., M.E. Mohrman, A.E. Wilson, and V.B. Haarstad. Acetylseco hemicholinium-3, a new choline acetyltransferase inhibitor useful in neuropharmacological studies. Neuropharmacology, 1973, 12:549-561.

Dowling, J.E. and B.B. Boycott. Organization of the primate retina : electron microscopy. Proceedings of the Royal Society of London, 1966, 166:80-111.

Duke-Elder, S., ed. "System of Ophthalmology , Volume 2: The Anatomy of the Visual System". C.V. Mosby Company, St. Louis, 1961, p 121.

Duke-Elder, S. and J. Gloster, "System of Ophthalmology, Volume 4 : The Physiology of the Eye and of Vision". C.V. Mosby Company , St. Louis, Missouri , 1968, p. 278.

Ehinger, B. Connections between adrenergic nerves and other tissue components in the eye. Acta Physiologica, Scandinavica, 1966, 67:57-64.

Ehinger, B., B. Falck, H. Persson, A.M. Rosengren, and B. Sporrong. Acetylcholine in adrenergic terminals of the cat iris. Journal of Physiology, 1970, 209:557-565.

Emson, P.C., D. Malthe-Sørenssen, and F. Fonnum. Purification and properties of choline acetyltransferase from the nervous system of different invertebrates. Journal of Neurochemistry, 1974, 22:1089-1097.

Eng, L.F., C.T. Uyeda, L.P. Chao, and F. Wolgram. Antibody to bovine choline acetyltransferase and immunofluorescent localizaton of the enzyme in neurones. Nature, 1974, 250:243-245.

Engelhart, E. : Der humorale Wirkungmechanismus der Oculomotoriusreizung. Pflugers Archiv fur die Gesamte Physiologie, 1931, 227:220-234.

Feldberg, W. Present views on the mode of action of acetylcholine in the central nervous system. Physiological Reviews, 1945, 25:596-642.

Feldberg, Wand T. Mann. Properties and distribution of the enzyme system which synthesizes acetylcholine in nervous tissue. Journal of Physiology, 1946, 104:411-425.

Feldberg, W. and M. Vogt. Acetylcholine synthesis in different regions of the central nervous system. Journal of Physiology, 1948, 107:372-381.

Fitzgerald, G.G. and J.R. Cooper. Acetylcholine as a possible sensory mediator in rabbit corneal epithelium. Biochemical Pharmacology, 1971, 20:2741-2748.

Fonnum, F. A radiochemical method for the estimation of choline acetyltransferase. Biochemical Journal, 1966, 100:479-484.

Fonnum, F. The 'compartmentation' of choline acetyltransferase within the synaptosome. Biochemical Journal, 1967, 103:262-270.

Fonnum, F. Choline acetyltransferase binding to and release from membranes. Biochemical Journal, 1968, 109:389-398.

Fonnum, F. Radiochemical micro assays for the determination of choline acetyltransferase and acetylcholinesterase activities. Biochemical Journal, 1969, 115:465-472.

Fonnum, F. Surface charge of choline acetyltransferase from different species. Journal of Neurochemistry, 1970, 17:1095-1100.

Fonnum, F., M. Frizell, and J. Sjostrand. Transport, turnover, and distribution of choline acetyltransferase and acetylcholinesterase in the vagus and hypoglossal nerves of the rabbit. Journal of Neurochemistry, 1973, 21:1109-1120.

Fonnum, F. and D. Malthe-Sørenssen. Membrane affinities and subcellular distribution of the different molecular forms of choline acetyltransferase from rat. Journal of Neurochemistry, 1973, 20:1351-1359.

Frizell, M., P.O. Hasselgren, and J. Sjostrand. Axoplasmic transport of acetylcholinesterase and choline acetyltransferase in the vagal and hypoglossal nerve of the rabbit. Experimental Brain Research, 1970, 10:526-531.

Gardiner, J.E., and V.P. Whittaker. The identification of propionylcholine as a constituent of ox spleen. Biochemical Journal, 1954, 58:24-29.

Genovesi, E. and U. Gaspa. Cholinesterase in aqueous humor of man and open-angle glaucoma. Ophthalmologica, 1971, 162:60-64.

Giller, E., Jr. and J.S. Schwartz. Choline acetyltransferase: regional distribution in the abdominal ganglion of aplysia. Science, 1968, 161:908-911.

Glick, S.D., T.W. Mittag, and J.P. Green. Central cholinergic correlates of impaired learning. Neuropharmacology, 1973, 12:291-296.

Glover, V.A.S. and L.T. Potter. Purification and properties of choline acetyltransferase from ox brain striate nuclei. Journal of Neurochemistry, 1971, 18:571-580.

Gnadinger, M.C., R. Heimann, and R. Markstein. Choline acetyltransferase in corneal epithelium. Experimental Eye Research, 1973, 15:395-399.

Gnadinger, M.C., D. Walz., H.P. v. Hahn, and F. Grun. Acetylcholine-splitting activity of abraded and cultivated corneal epithelial cells. Experimental Eye Research, 1967, 6:239-242.

Goldberg, A.M., A.A. Kaita, and R.E. McCaman. Microdetermination of choline acetyltransferase - a comparison of reinecke vs. periodide precipitation. Journal of Neurochemistry, 1969, 16:823-824.

Goldberg, A.M. and R.E. McCaman. A quantitative microchemical study of choline acetyltransferase and acetylcholinesterase in the cerebellum of several species. Life Sciences, 1967, 6:1493-1500.

Goldberg, M.E., A.I. Salama, and S.W. Blum. Inhibition of choline acetyltransferase and hexobarbitone-metabolizing enzymes by naphthylvinyl pyridine analogues. Journal of Pharmacy and Pharmacology, 1971, 23:384-385.

Gray, E.G. and V.P. Whittaker. The isolation of nerve endings from brain: an electron-microscope study of cell fragments derived by homogenization and centrifugation. Journal of Anatomy, 1962, 96:79-87.

Green, J.P., D.L. Alkon, D.E. Schmidt, and P.I.A. Szilagyi. Confirmation of acetylcholine in perfusates of the stimulated vagus nerve by pyrolysis gas chromatography. Life Sciences, 1970, 9:741-745.

Hamprecht, B. and T. Amano. Differential assay for choline acetyltransferase, Analytical Biochemistry, 1974, 57:162-172.

Hanin, Israel, editor. "Choline and acetylcholine: Handbook of Chemical Assay Methods." Raven Press, New York, New York, 1974.

Havener, W.H. "Ocular Pharmacology". C.V. Mosby Company, St. Louis, Missouri, 1970, pp 174, 227, and 454.

Hebb, C.O. Choline acetylase in mammalian and avian sensory systems. Quarterly Journal of Experimental Physiology and Cognate Medical Sciences, 1955, 40:176-186.

Hebb, C.O. Biosynthesis of acetylcholine in nervous tissue. Physiological Reviews, 1972, 52:918-957.

Hebb, C.O. and K. Krnjevic. The physiological significance of acetylcholine. "Neurochemistry", 2nd edition. Elliott, Page, and Quastel, editors, Charles C. Thomas Publisher, Springfield, Illinois, 1962, p 460-461.

Hebb, C.O. and A. Silver. Gradient of choline acetylase activity. Nature, 1961, 189:123-125.

Hebb, C.O. and B.N. Smallman. Intracellular distribution of choline acetylase. Journal of Physiology, 1956, 134:385-392.

Hebb, C.O. and G.J. Waites. Choline acetylase in antero - and retro - grade degeneration of a cholinergic nerve. Journal of Physiology, 1956, 132:667-671.

Hebb, C.O. and V.P. Whittaker. Intracellular distributions of acetylcholine and choline acetylase. Journal of Physiology, 1958, 142:187-196.

Hellauer, H.F. Sensibilitat und Acetylcholingehalt der Hornhaut verschiedener Tiere und des Menschen. Zeitschrift fur vergleichende Physiologie, 1950, 32:303-310.

Hemsworth, B.A. and D. Morris. A comparison of the N-alkyl group specificity of choline acetyltransferase from different species. Journal of Neurochemistry, 1964, 11:793-803.

Howard, R.O. and W.S. Wilson. Development of acetylcholine, choline acetyl-transferase and acetylcholinesterase in rabbit corneal epithelium. British Journal of Pharmacology, 1972, 76:567-568P.

Howard, R.O., W.S. Wilson, and B.J. Dunn. Quantitative determination of choline acetylase, acetylcholine, and acetylcholinesterase in the developing rabbit cornea. Investigative Ophthalmology, 1973, 12:418-425.

Higgins, J.A. and R.J. Barnett. Cytochemical localization of transferase activities: carnitine acetyltransferase. Journal of Cell Sciences, 1970, 6:29-51.

Hsu, S.Y. and M.C. Gerald. Determination of femtomole levels of acetylcholine by an improved bioassay procedure. European Journal of Pharmacology, 1973, 24:269-273.

Kaita, A.A. and A.M. Goldberg. Control of acetylcholine synthesis - the inhibition of choline acetyltransferase by acetylcholine. Journal of Neurochemistry, 1969, 16:1185-1191.

Kasa, P., S.P. Mann, S. Karcsu, L. Toth, and S. Jordan. Transport of choline acetyltransferase and acetylcholinesterase in the rat sciatic nerve: a biochemical and electron histochemical study. Journal of Neurochemistry, 1973, 21:431-436.

Kasa, P. Identification of cholinergic neurones in the spinal cord: an electron histochemical study of choline acetyltransferase. Journal of Physiology, 1970, 210:89-90P.

Kasa, P., S.P. Mann, and C. Hebb. Localization of choline acetyltransferase. Nature, 1970A, 226:812-814.

Kasa, P., S.P. Mann, and C. Hebb. Ultrastructural localization in spinal neurones. Nature, 1970B, 226:814-816.

Koelle, G.B., L. Wolfand, J.S. Friedenwald, and R.A. Allen. Localizaiton of specific cholinesterase in ocular tissues of the cat. American Journal of Ophthalmology, 1952, 25:1580-1584.

Korey, S.R., B. de Braganza, and D. Nachmansohn. Choline acetylase V. Esterifications and transacetylations. Journal of Biological Chemistry, 1951, 189:705-715.

Kornblueth, W. The effect of retrobulbar alcohol injection on the eyes of experimental animals. American Journal of Ophthalmology, 1949, 32:781-792.

Kovacik, L. Acetylcholine des tissues du segment posterieur de l'oeil des bovides III. Reserve en acetylcholine de l' epithelium pigmente de la retine. Archives d 'Ophtalmologie, 1974, 34:59-64.

Kovacik, L., L. Jezek, and E. Mrazova. Acetylcholine des tissus du segment posterieur de l'oeil des bovides I-Reserve en acetylcholine de la retine de bovides. Archives d'Ophtalmologie, 1972, 32:837-842.

Kovacik, L., L. Jezek, and E. Mrazova. Acetylcholine des tissus du segment posterieur de l'oeil des bovides. II Acetylcholine sous forme de depot dana la choroide des bovins. Archives d'Ophtalmologie, 1973, 33:429-437.

Krause, U. and V. Raunio. The proteins of the pathologic human aqueous humour. Ophthalmologica, 1970, 160:280-287.

Lam, D.M.K. Biosynthesis of acetylcholine in turtle photoreceptors. Proceedings of the National Academy of Sciences, USA, 1972, 69:1987-1991.

Laties, A. and D. Jacobowitz. A histochemical study of the adrenergic and cholinergic innervation of the anterior segment of the rabbit eye. Investigative Ophthalmology, 1964, 3:592-600.

Levene, R.Z., M. Rothberger, and S. Rosenberg. Corticosteroid glaucoma in the rabbit. American Journal of Ophthalmology, 1974, 78:505-510.

Lipmann, F., N.O. Kaplan, G.D. Novelli, L.C. Tuttle, and B.M. Guirard. Coenzyme for acetylation, a pantothenic acid derivative. Journal of Biological Chemistry, 1947, 167:869-870.

Lipton, M.A. and E.S.G. Barron. On the mechanism of the anaerobic synthesis of acetylcholine. Journal of Biological Chemistry, 1946, 166:367-380.

Loewi, O. and H. Hellauer. The acetylcholine content of the nerves of warm-blooded animals. Journal of Physiology, 1938, 93:34P.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry, 1951, 193:265-275.

Luco, J.V. and K. Lissak. Chemical mediators in the aqueous humor. American Journal of Physiology, 1938, 124:271-278.

Lynen, F., E. Reichert, and L. Rueff. Biological degradation of acetic acid VI Isolation and chemical nature of activated acetic acid. Annalen der Chemie, 1951, 574:1-32.

Malthe-Sørenssen, D., R.A. Andersen, and F. Fonnum. Biochemical and pharmacological properties of acryloylcholine, an inhibitor of choline acetyltransferase. Biochemical Pharmacology, 1974, 23:577-586.

Malthe-Sørenssen, D., T. Eskeland, and F. Fonnum. Purification of rat brain choline acetyltransferase; some immunochemical properties of a highly purified preparation. Brain Research, 1973, 62:517-522.

McCamen, R.E., G.R. deLores Arnaiz, and E. deRobertis. Species differences in subcellular distribution of choline acetylase in the CNS. Journal of Neurochemistry, 1965, 12:927-937.

Malthe-Sørenssen, D., and F. Fonnum. Multiple forms of choline acetyltransferase in several species demonstrated by isolectric focusing. Biochemical Journal, 1972, 127:229-236.

Mandell, R.B. and I. Fatt. Thinning of the human cornea on awakening. Nature, 1965, 208:292-293.

Maumenee, A.E. Retrobulbar alcohol injections. American Journal of Ophthalmology, 1949, 32:1502-1508.

Maurice, D.M. and A.A. Giardini. Swelling of the cornea in vivo after destruction of its limiting layers. British Journal of Ophthalmology, 1951, 35:791-797.

Maurice, D.M. and M.V. Riley. "Biochemistry of the Eye", ed. C.N. Graymore, Academic Press, New York, N.Y., 1970, pp. 24 and 77.

McCaman, R.E. and J.M. Hunt. Microdetermination of choline acetylase in nervous tissue. Journal of Neurochemistry, 1965, 12:253-259.

Michon, J., Jr. and J. H. Kinoshita. Cholinesterase in the lens. Archives of Ophthalmology, 1967, 77:804-808.

Molinoff, P.B. and J. Axelrod, Biochemistry of catecholamines. Annual Review of Biochemistry, 1971, 40:465-500.

Morris, D. The effect of sulphydryl and other disulphide reducing agents on choline acetyltransferase activity estimated with synthetic acetyl-CoA. Journal of Neurochemistry, 1967, 14:19-27.

Morris, D. and D.S. GreWaal. Halogen substituted derivatives of acetylcholine as inhibitors of choline acetyltransferase. Life Sciences, 1969, 8:511-516.

Morris, D. and D.S. GreWaal. Human placental choline acetyltransferase. Radiometric assay, inhibition by analogues of choline and acetylcholine, and isotopic exchange between choline and acetylcholine. European Journal of Biochemistry, 1971, 22:563-572.

Morris, D., C. Hebb, and G. Bull. Inhibition of choline acetyltransferase by excess choline. Nature, 1966, 209:914-915.

Morris, D., A. Maneckjee, and C. Hebb. The kinetic properties of human placental choline acetyltransferase. Biochemical Journal, 1971, 125:857-863.

Morris, D. and S. Tucek. The influence of inorganic cations on the activities of choline acetyltransferase, phosphate acetyltransferase, and acetylphosphatase. Journal of Neurochemistry, 1966, 13:333-345.

Nachmansohn, D. and M. Berman. Studies on choline acetylase III. On the preparation of the coenzyme and its effect on the enzyme. Journal of Biological Chemistry, 1946, 165:551-563.

Nachmansohn, D., H.M. John, and H. Waelsch. Effect of glutamic acid on the formation of acetylcholine. Journal of Biological Chemistry, 1943, 150:485-486.

Nachmansohn, D. and A.L. Machado. The formation of acetylcholine. A new enzyme: "Choline Acetylase". Journal of Neurophysiology, 1943, 6:397-403.

Nachmansohn, D. and M.S. Weiss. Studies on choline acetylase IV. Effect of citric acid. Journal of Biological Chemistry, 1948, 172:677-687.

Nichols, C.W. and G.B. Koelle. Acetylcholinesterase: method for demonstration in amacrine cells of rabbit retina. Science, 1967, 155:477-478.

Nichols, C.W. and G.B. Koelle. Comparison of the localization of acetylcholinesterase and non-specific cholinesterase activities in mammalian and avian retinas. Journal of Comparative Neurology, 1968, 133:1-16.

Novelli, D., F.J. Schmetz, Jr., and N.O. Kaplan. Enzymatic degradation and resynthesis of coenzyme A. Journal of Biological Chemistry, 1954, 206:533-545.

Ochoa, S., J.R. Stern, and M.C. Schneider. Enzymatic synthesis of citric acid II Crystalline condensing enzyme. Journal of Biological Chemistry, 1951, 193:691-702.

Oer, S., v. Uber die Beziehung des Acetycholins des Hornhautepithels zur Erregungsubertragung von diesem auf die sensiblen Nervenenden. Pfluegers Archiv fur die Gesamte Physiologie des Menschen und der Tiere, 1961, 273:325-334.

Pauling, Linus. "The Nature of the Chemical Bond", 2nd edition. Cornell University Press, Ithaca, New York,1948, p. 53.

Persson, B.O., L. Larsson, J. Schuberth, and B. Sorbo. 3-Bromoacetonyltrimethylammonium bromide, a choline acetylase inhibitor. Acta Chemica Scandinavica, 1967, 21:2283-2284.

Petersen, R.A., K. Lee, and A. Donn. Acetylcholinesterase in the rabbit cornea. Archives of Ophthalmology, 1965, 73:370-377.

Phelps, C.D., R.F. Woolson, A.E. Kolker, and B. Becker. Diurnal variation in intraocular pressure. American Journal of Ophthalmology, 1974, 77:367-377.

Polyak, S.L. "The Vertebrate Visual System". The University of Chicago Press, Chicago, Illinois, 1957, pp 219 and 239.

Potter, L.T., V.A.S. Glover, and J.K. Saelens. Choline acetyltransferase from rat brain. Journal of Biological Chemistry, 1968, 243:3864-3870.

Prince, A.K. Properties of choline acetyltransferase isolated from squid ganglia. Proceedings of the National Academy of Sciences, U.S.A., 1967, 57:1117-1122.

Quastel, J.H., M. Tennenbaum, and A.H.M. Wheatley. Choline ester formation in, and choline esterase activities of, tissues in vitro. Biochemical Journal, 1936, 30:1668-1681.

Reisberg, R.B. Sulfhydryl groups of choline acetylase. Biochemica et Biophysica Acta, 1954, 14:442-443.

Reisberg, R.B. Stimulation of choline acetyltransferase activity by ethanol in in vitro preparations of rat cerebrum. Life Sciences, 1974, 14:1965-1973.

Robertson, D.M. and R.K. Winkelmann. A whole-mount cholinesterase technique for demonstrating corneal nerves: observations in the albino rabbit. Investigative Ophthalmology, 1970, 9:710-715.

Rodger, F. C. Source and nature of nerve fibers in cat cornea. Archives of Neurology and Psychiatry, 1953, 70:206-223.

Roskoski, R., Jr. Choline acetyltransferase. Evidence for an acetyl-enzyme reaction intermediate. Biochemistry, 1973, 12:3709-3714.

Roskoski, R., Jr. Choline acetyltransferase inhibition by thiol reagents. Journal of Biological Chemistry, 1974, 249:2156-2159.

Ross, C.D. and D.B. McDougal, Jr. Choline acetyltransferase in retinal layers. Federation Proceedings, 1974, 33:#1507.

Rossier, J., A. Bauman, and P. Benda. Antibodies to rat brain choline acetyltransferase: species and organ specificity. FEBS Letters, 1973, 26:43-52.

Schmidt, D.E., P.I.A. Szilagyi, D.E. Alkon, and J.P. Green. Acetylcholine: Release from neural tissue and identification by pyrolysis-gas chromatography. Science, 1969, 165:1370-1371.

Scharenberg, K. The cells and nerves of the human cornea. American Journal of Ophthalmology, 1955, 40:368-379.

Schmidt, D.E., P.I.A. Szilagyi, D.A. Alkon, and J.P. Green. A method for measuring nanogram quantities of acetylcholine by pyrolysis-gas chromatography: The demonstration of acetylcholine in effluents from the rat phrenic nerve - diaphragm preparation. Journal of Pharmacology and Experimental Therapeutics, 1970, 174:337-345.

Schrier, B.K. and L. Shuster. A simplified radiochemical assay for choline acetyltransferase. Journal of Neurochemistry, 1967, 14:977-985.

Schuberth, J. Choline acetyltransferase. Purification and effect of salts on the mechanism of the enzyme-catalyzed reaction. Biochimica et Biophysica Acta, 1966, 122:470-481.

Schuberth, J. Radiochemical determination of choline acetyltransferase. Acta Chemica Scandinavica, 1963, 17: Supplement 1:233-237.

Schuberth, J., B. Sparf and A. Sundwall. On the turnover of acetylcholine in the brain, in "Drugs and Cholinergic Mechanisms in the CNS", Holmstedt, Sorbo, Schuberth, and Sundwall, editors, Research Institute of Natinal Defense, Stockholm, Sweden, 1970, p 177-187.

Schumacher, H. and R. Ehl. Quantitative photometry detection of esters of choline. "Choline and Acetylcholine: Handbook of Chemical Assay Methods", editor I. Hanin, Raven Press, New York, 1974, p. 195-213.

Smallman, B.N. The choline acetylase activity of rabbit brain. Journal of Neurochemistry, 1958, 2:119-127.

Smith, J.C., C.J. Cavallito, and F.F. Foldes. Choline acetyltransferase inhibitors: a group of styryl-pyridine analogs. Biochemical Pharmacology, 1967, 16:2438-2441.

Smith, J.C. and R.B. Weiskopf. Effect of cysteine on acetylcholine synthesis. Nature, 1967, 215:1379-1380.

Stadtman, E.R. The net enzymatic synthesis of acetyl coenzyme A. Journal of Biological Chemistry, 1952, 196:535-546.

Starr, M. Further studies on the effect of prostoglandin on intra-ocular pressure in the rabbit. Experimental Eye Research, 1971, 11:170-177.

Stavinoha, W.B. and L.C. Ryan. Estimation of the acetylcholine content of rat brain by gas chromatography. Journal of Pharmacology and Experimental Therapeutics, 1965, 150:231-235.

Stedman, E. and E. Stedman. The mechanism of the biological synthesis of acetylcholine. 1. The isolation of acetylcholine produced by brain tissue in vitro. Biochemical Journal, 1937, 31:817-827.

Steinberg, G.M. and J.A. Cramer. (3-Bromo-2-oxoprppyl) trimethylammonium bromide, an inhibitor of acetylcholinesterase. Biochemical Pharmacology, 1970, 19:632-636.

Stephenson, M. and E. Rowatt. The production of acetylcholine by a strain of Lactobacillus plantarum. Journal of General Microbiology, 1947, 1:279-298.

Stern, J.R. and S. Ochoa. Enzymatic synthesis of citric acid I. Synthesis with soluble enzymes. Journal of Biological Chemistry, 1951, 191:161-172.

Stern, J.R., B. Shapiro, E.R. Stadtman, S. Ochoa. Enzymatic synthesis of citric acid III. Reversibility and mechanism. Journal of Biological Chemistry, 1951, 193:703-720.

Stevenson, R.W. and W.S. Wilson. Depletion of acetylcholine in the corneal epithelium. British Journal of Pharmacology, 1973, 49:167P.

Szilagyi, P.I.A., J.P. Green, O.M. Brown, and S. Margolis. The measurement of nanogram amounts of acetylcholine in tissues by pyrolysis gas chromatography. Journal of Neurochemistry, 1972, 19:2555-2566.

Szilagyi, P.I.A., J.S. Mindel, A. Goldberg, J.P. Green, and A. Blaiklock. Simplified method for measuring acetylcholine by pyrolysis gas chromatography. Submitted to Analytical Biochemistry.

Szilagyi, P.I.A., D.E. Schmidt, and J.P. Green. Microanalytical determination of acetylcholine, other choline esters, and choline by pyrolysis-gas chromatography. Analytical Chemistry, 1968, 40:2009-2013.

Tucek, S. On subcellular localization and binding of choline acetyltransferase in the cholinergic nerve endings of the brain. Journal of Neurochemistry, 1966A, 13:1317-1327.

Tucek, S. On the question of the localization of choline acetyltransferase in synaptic vesicles. Journal of Neurochemistry, 1966B, 13:1329-1332.

Umrath, K. and H. Mussbichler. Die Blockierung der Erregringsubertragung von sekundaren Sinneszellen auf die sensiblen Nerven und die Herabsetzung der Hornhautempfindlichkeit durch Atropin. Zeitschrift fur Vitamin-Hormon-und Fermentforschung, 1951, 4:182-190.

Uusitalo, R., J. Stjernschantz, and A. Palkama. Studies on parasympathetic control of the blood-aqueous barrier in the rabbit. Experimental Eye Research, 1974, 19:125-134.

van Alphen, G.W.H.M.v. Acetylcholine synthesis in corneal epithelium. Archives of Ophthalmology, 1957, 58:449-451.

Velhagen, K. Einleitende Untersuchnungen uber das Vorkommen aktwer und neurotroper Substanzen im Auge. Archiv fuer Augenheilkunde, 1930, 103:424-483.

Velhagen, K. Zur Frage der vagotropen Substanzen im Auge. Archiv fuer Augenheilkunde, 1932, 105:573-602.

Velhagen, K. Neue Untersuchungen uber aktive Stoffe im Auge. Archiv fuer Augenheilkunde, 1936, 109:195-204.

Waitzman, M.B. and C.D. King. Prostaglandin influences on intra-ocular pressure and pupil size. American Journal of Physiology, 1967, 212:329-334.

White, H.L. and C.J. Cavallito. Inhibition of bacterial and mammalian choline acetyltransferase by styrylpyridine analogues. Journal of Neurochemistry, 1970A, 17:1579-1589.

White, H.L. and C.J. Cavallito. Choline acetyltransferase. Enzyme mechanism and mode of inhibition by a styrylpyridine analogue. Biochimica et Biophysica Acta, 1970B, 206:343-358.

White, H.L. and C.J. Cavallito. Photoisomerization of styrylpyridine analogues in relation to choline acetyltransferase and cholinesterase inhibition. Biochimica et Biophysica Acta, 1970C, 206:242-251.

White, H.L. and J.C. Wu. Separation of apparent multiple forms of human brain choline acetyltransferase by isoelectric focusing. Journal of Neurochemistry, 1973, 21:939-947.

Whittaker, V.P., I.A. Michaelson, and R.J.A. Kirkland. The separation of synaptic vesicles from nerve ending particles ('synaptosomes'). Biochemical Journal, 1964, 90:293-303.

Wiesel, T.N. and D.H. Hubel. Effects of visual deprivation on morphology and physiology of cells in the cat's lateral geniculate body. Journal of Neurophysiology, 1963A, 26:978-993.

Wiesel, T.N. and D.H. Hubel. Single-cell responses in striate cortex of kittens deprived of vision in one eye. Journal of Neurophysiology, 1963B, 26:1003-1017.

Wiesel, T.N. and D.H. Hubel. Comparison of the effects of unilateral and bilateral eye closure on cortical unit responses in kittens. Journal of Neurophysiology, 1965, 28:1029-1040.

Williams, J.D. and J.R. Cooper. Acetylcholine in bovine corneal epithelium. Biochemical Pharmacology, 1965, 14:1286-1289.

Witzleb, E. Zur Frage von cholinergischen Mechanismen bei der Erregung von afferenten Systemen. Pflugers Archiv fur die Gesamte Physiologie des Menschen und der Tiere, 1959, 269:439-470.

Wolter, J.R. Double nerve supply of Krause corpuscles at the human corneal limbus. Archives of Ophthalmology, 1964, 71:409-411.

Zander, E. and G. Weddell. Observations on the innervation of the cornea. Journal of Anatomy, 1951, 85:68-99.

Zeller, E.A. Enzymology of the refractory media of the eye. IV. Direct photometric determination of cholinesterase and aliesterase in corpus vitreum. Archives of Biochemistry and Biophysics, 1956, 61:231-240.