Analysis of cholinesterases of intact cat cornea, ciliary body, lens, and retina

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A radiometric method has been used on preparations of intact ocular tissue to dissociate the effects of surface cholinesterase from intracellular enzymes. This method provides a technique to determine the effects of surface enzyme potentiation or inhibition, and may be helpful in evaluating the degree of surface enzyme inhibition with pharmacologic response. Surface cholinesterase activity was found to be highest in ciliary body, lower in retina, and least in cornea and crystalline lens. Butyrylcholinesterase was found to contribute some 50 per cent of surface enzyme activity in the ciliary body, retina, and lens, and 80 per cent in the cornea.

Key words: acetylcholine, acetylcholinesterase, cholinergic, butyrylcholine, butyrylcholinesterase, mecholyl, ciliary body, cholinesterase, lens cholinesterase, retinal cholinesterase.

Efforts to elucidate the dynamics of the ocular cholinergic systems have been limited to histochemical methods or to analysis of enzymes obtained from tissue homogenates or of purified enzymes.1-3 Though histochemical studies delineate the precise anatomic localization of enzyme sites, the lack of quantitation limits their use in understanding the physiologic mechanisms by which these enzymes function in ocular structures. Similarly, it has yet to be demonstrated that the properties of cholinesterases (ChE) determined by analysis of enzyme extracts or tissue homogenates can be extrapolated to explain the physiologic and pharmacologic dynamics of these compounds at cholinergic sites in intact tissues.4

The current investigation is a study of surface ChE of cat ocular tissue by a radiometric technique which permits detailed kinetic analysis of these enzymes in intact structures. The assay method employs radioactive esters of choline as substrates in the pharmacologic concentration range of 10^-6 M.5 This concentration is the threshold for contractile responses of the iris and ciliary body to these drugs in vitro.

This method has several advantages over the conventional histochemical and biochemical methods of ChE assay: (1) The enzyme is assayed at substrate concentrations which are probably nearer to the physiologic range than those used for...
either the histochemical or the biochemical method. (2) The enzymes remain in their native state bound to membrane sites, and are not subject to artifacts introduced by freezing, fixing, homogenization, or purification procedures. (3) The assay is a nondestructive method and pharmacologic responses of iris or ciliary body to various drugs can be correlated with the drug's effect on the enzymes in the identical tissue. The most serious disadvantage is the problem of slow diffusion of the substrates to enzyme sites within the tissue.

While the in vitro assay of intact ocular tissues does not duplicate the in vivo situation, it does represent a more physiologic method. Results obtained by this method must be regarded as complementary to the histochemical and conventional biochemical methods for ChE determination.

Materials and methods

Ocular tissues were removed from adult cats weighing between 3 and 6 kilograms, which were anesthetized with intravenous sodium pentobarbital. The cornea and iris were excised and the intact lens was extracted with the aid of an eviscerator and scissors. The eye was then enucleated to facilitate removal of the retina and ciliary body. Tissue specimens were thoroughly cleansed of pigment and blood by gentle agitation and washing and immediately placed in an oxygenated (95 per cent O₂, 5 per cent CO₂) Krebs-Ringer solution at a constant temperature (37°C). Within ten minutes, tissues were transferred to a 25 c.c. jacketed glass bath containing the gassed buffer at 37°C. at a pH of 7.40. The intact tissues were allowed to equilibrate for 15 minutes and were then assayed for ChE activity.

The assay procedure involves the immersion of intact tissues in a solution of 10⁻⁶ to 10⁻⁴M, ¹⁴C-labeled acetylcholine (ACH), butyrylcholine (BuCh), or acetyl β-methylcholine (MeCh), labeled in the acyl moiety. Aliquots (1 c.c.) of the incubation mixture are removed for analysis at predetermined intervals. The unhydrolyzed acetylcholinesterase (ACHE) substrate is trapped in a short column of cation exchange resin (Bio-Rex 40, 200 to 400 mesh) already equilibrated in 0.1M sodium phosphate buffer, pH 7.40, and the radioactive acid is eluted for liquid scintillation counting. Unlabeled sodium acetate, 1 mg. per milliliter, is incorporated in the assay medium to eliminate uptake of radioactive acetate by the tissue preparation.

The total number of counts (unreacted substrate + acid in the bath) remains virtually constant over the entire assay period, which may last up to 50 minutes. Phosphate buffer (1.5 c.c.) is used to wash the column and is collected in the same counting vials; following this procedure, Bray's solution (10 c.c.) is added to each vial. The total radioactivity of the bath medium is determined by removing 1 c.c. from the bath (by-passing the exchange column) and adding it directly to a counting vial, which contains 1.5 c.c. of phosphate buffer and 10 c.c. of Bray's solution. The counting vials are then placed in a liquid scintillation counter (Nuclear-Chicago, Mark I) at 5°C. at the balance point for the isotope being used. Channel-ratio method is used to check sample uniformity and counting efficiency. After completion of the assay (at least four points), the tissue is carefully blotted with absorbent tissue paper and weighed. The calculation of the rate constant (k) is made by substitution of numerical values in the equation:

\[ k = \frac{R \times V_t}{W} \times 10^{-3} \]

where \( R = \) rate in counts per minute per milliliter of bath corrected for background autohydrolysis, \( V_t = \) bath volume in milliliter at zero time, \( W = \) tissue weight, \( T = \) total counts per minute per milliliter in the bath, and \( A_t = \) acetate concentration at zero time in counts per minute per milliliter of bath solution. The rate constant is expressed in units of liters per minute per gram of tissue (wet weight).

Results

The rate of autohydrolysis for ACh was determined from assays performed without tissue in the bath (Figs. 1 and 2). This introduced a correction factor of 0.03 to 0.01 per cent of the total substrate in 1 ml. per minute, which was subtracted from the rate (R) when calculating the value of the pseudo first-order rate constant k. In the case of MeCh and BuCh, the correction factor was 0.01 to 0.003 per cent of the total per minute. Previous work has demonstrated that tissue uptake of substrate and loss of enzyme from the tissues into the bath are negligible factors.

When radioactivity is plotted against time, a straight line is obtained with the slope representing the rate of hydrolysis.
for ciliary body (Fig. 1) and for lens (Fig. 2). Since substrate concentrations are below the \( K_m \) for these enzymes, pseudo first-order kinetics may be expected when the substrate is in excess over the enzyme.

Table I shows the corrected mean pseudo first-order rate constants \( (k) \) for each tissue with ACh as the substrate.

A comparison of the right and left eyes for each set of tissues assayed shows the \( k \) value to be approximately equal in each case (Table II).

Between assays, ocular tissues were maintained in oxygenated Krebs-Ringer solution. Reassay of tissues after several hours of storage under these conditions and several washes showed no significant change in rate constants. The ChE appear to be firmly attached to the tissues, since no enzyme is washed out into the bathing medium and the hydrolytic activity toward exogenously applied substrates remains constant. These results show that contralateral tissues can be used as controls in drug studies and that in vitro drug studies can be done by sequential assay on a single tissue.

Intact ciliary body, retina, cornea, and lens preparations were assayed with ACh, and then reassayed with BuCh or MeCh to determine the relative amounts of butyrylcholinesterase (BuChE) and AChE. The corrected \( k \) values are shown in Table III.

Discussion

An understanding of the hydrolytic properties of ChE at cholinergic sites is necessary in order to define the function of these enzymes at effector loci. An ap-
Table III. Mean rate constants ± S.E.M. in liters per minute per gram of tissue with MeCh and BuCh substrates at 10^-6M

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of assays</th>
<th>Substrate (10^-6M)</th>
<th>Mean rate constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciliary body</td>
<td>13</td>
<td>MeCh</td>
<td>3.400 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>BuCh</td>
<td>13.500 ± 0.90</td>
</tr>
<tr>
<td>Retina</td>
<td>7</td>
<td>MeCh</td>
<td>2.100 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>BuCh</td>
<td>8.600 ± 0.10</td>
</tr>
<tr>
<td>Lens</td>
<td>5</td>
<td>MeCh</td>
<td>0.044 ± 0.0011</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>BuCh</td>
<td>0.150 ± 0.012</td>
</tr>
<tr>
<td>Cornea</td>
<td>5</td>
<td>MeCh</td>
<td>0.050 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>BuCh</td>
<td>0.880 ± 0.06</td>
</tr>
</tbody>
</table>

The approach to this goal has been achieved by utilizing intact ocular tissues for the determination of enzymatic activities. Previous studies of ocular tissues have involved either homogenized preparations, histochemical staining techniques, or assay methods utilizing purified enzymes. The technique used in the current investigation affords an advantage over other methods in maintaining relative tissue integrity and assaying only extracellular enzyme. Histochemical studies on ocular tissues have demonstrated ChE to be located on cell membranes, between cells, as well as having an intracellular location. In the iris and ciliary body, endogenous ACh is released from nerves into the extracellular space. Thus, physiologically released ACh first encounters ChE located between cells and on their outer membrane surface. The physiologic ACh sensitivity of cells is also located on the outer membrane surface and, therefore, it is likely that the extracellular pool of ChE in such tissues regulates ACh responses and can be termed the functional pool of enzyme.

Studies on several cholinergic tissues, such as nerves, ganglia, brain, and striated muscle, have shown that two major pools of ChE are present. In nervous tissues, one pool is the functional enzyme, and the second and larger fraction is nonfunctional ChE. On the basis of 2-pyridine aldoxime methiodide reactivation of organophosphate-inhibited enzyme in the brain and by selective inhibition in the ganglia, the functional enzyme was found to be located on the cell surfaces. Reserve, or nonfunctional, enzyme is found intracellularly in three- or fourfold greater amounts than the functional pool. Thus, when a tissue homogenate is used to study drug effects on AChE, more than 80 per cent of the enzyme assayed has no connection with cholinergic function in these tissues.

Our object in utilizing this assay method on intact tissues is to study the properties of ChE at extracellular locations only. Pharmacologic studies with di-isopropylfluorophosphate (DFP) on the iris and on the ciliary body, correlated with the effects of the drug on the enzyme pools, indicate that extracellular ChE is the functional pool in these tissues. The substrates used are positively charged ions and their penetration into cells through the cell membrane is very slow. Thus, the intracellular pool of ChE is inaccessible to the substrate and would not be assayed by this method.

The most serious problem with the assay procedure is that the rate of hydrolysis may be limited by diffusion of the substrate into the extracellular space. This limits the method to thin, muscular tissues and the intact cornea and lens, where the enzyme is located almost exclusively in the surface cell layers. The role of diffusion has been evaluated by studies of the temperature dependence, Km and inhibitor constants of the enzymes in the intact ciliary body and in the rat diaphragm. Results indicate that, in both of these tissues, diffusion factors are not the rate-limiting step, but partly determine the rate of hydrolysis. The problem of substrate diffusion is analogous to the limited access problem when different drugs are applied to an intact pharmacologic preparation in vitro. The absolute concentration of drugs at their cellular site of action cannot be determined and only relative activities are obtained. The ciliary body and iris give dose-dependent contractile responses to ACh and MeCh in vitro. This indicates that the epithelial layers do not
Table IV. Mean ratios $k_{\text{ACh}}:k_{\text{MeCh}}$ with percentages of true and pseudo ChE for each tissue calculated from the ratios of ACh:MeCh and ACh:BuCh data

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$k_{\text{ACh}}:k_{\text{MeCh}}$</th>
<th>True ChE (%)</th>
<th>Pseudo ChE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ACh:MeCh</td>
<td>ACh:BuCh</td>
</tr>
<tr>
<td>Ciliary body</td>
<td>8:1</td>
<td>50</td>
<td>52</td>
</tr>
<tr>
<td>Retina</td>
<td>8:1</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Lens</td>
<td>7.5:1</td>
<td>54</td>
<td>56</td>
</tr>
<tr>
<td>Cornea</td>
<td>20:1</td>
<td>20</td>
<td>10</td>
</tr>
</tbody>
</table>

Acetylcholine is hydrolyzed in vivo by both AChE and BuChE and, therefore, the $k$ value obtained with this substrate reflects the combined activity of both enzyme types. BuCh is the specific substrate for BuChE, whereas MeCh is hydrolyzed entirely by AChE. It is, therefore, possible to study the kinetics of either of these enzymes independently with the use of the specific substrate and also to estimate the relative proportions of the two enzyme types present.

The $K_m$ of ACh for AChE and of ACh and BuCh for BuChE, in many tissues, are closely similar. Assuming that this is the case for ocular tissues, the percentage of the two enzymes can be estimated from the relative rate of hydrolysis of ACh and BuCh (Table IV).

MeCh, unlike ACh or BuCh, is a racemic mixture in which only the "T" form is a substrate for AChE. Although the "d" form is not hydrolyzed, it competitively inhibits the hydrolysis of the "T" form. In the presence of only true ChE or AChE, therefore, one would predict the rate of hydrolysis of racemic MeCh to be approximately one quarter of that of ACh, as has been found experimentally. In this case, the expected ratio $k_{\text{ACh}}:k_{\text{MeCh}}$ would be 4:1 in the presence of 100 per cent true ChE and 8:1 in the presence of 50 per cent true and 50 per cent pseudo ChE (BuChE). This provides a second way for estimating the proportion of the enzymes present in a tissue (Table IV). Both calculations are in good agreement but are based entirely on assumptions discussed above, which have, however, been found valid in other tissues. It is possible, however, that, in ocular tissues, there may be several pools of AChE or isoenzymes with different properties, which cannot be differentiated by the substrates used.

In comparing the percentages of the two enzymes with literature values obtained by other methods, there are points of agreement, as well as some striking differences. For the ciliary body, our results agree quite well with those of DeRoetth. The homogenate of this tissue hydrolyzed BuCh at 38 per cent of the rate for ACh, compared to 48 per cent found in this study. Both of these results do not agree with the histochemical data of Koelle and Friedenwald, who found no BuChE present in cat ciliary body. The presence of BuChE in the cat iris shows a similar discrepancy between the histochemical and the biochemical method.

Differences between the histochemical and biochemical methods were directly demonstrated by Petersen and co-work-
ers. These authors did biochemical ChE assays of rabbit cornea with BuCh in parallel with the histochemical method which uses the thiocholine ester. Butyrylcholine hydrolysis was 35 per cent that of ACh but, in the histochemical method, no BuChE was detected. The present results show cat cornea enzyme to be predominantly BuChE (80 to 90 per cent), a much greater percentage than that found in the rabbit. This may be due to a species difference, similar to differences found for the lens enzyme of various species (see below).

Intact cat lens had approximately equal amounts of the two enzyme types (Table IV). Michon and Kinoshita, by biochemical methods with the use of thiocholine esters, found the bovine lens enzyme to be entirely AChE. By contrast, Latties, with the use of the histochemical thiocholine method, found the lens enzyme in rabbit, cat, and monkey to be predominantly of the BuChE type, in agreement with the present results.

Our data for the retina show a very substantial amount of pseudo enzyme, whereas none is found in this tissue with the histochemical method.

The above results, demonstrating the presence of substantial proportions of BuChE in ocular tissues of the cat, have been confirmed with the use of the selective inhibition of BuChE by DFP. After the intact cat lens, retina, and ciliary body had been treated in vitro with DFP (10^-4 M for 15 minutes), the hydrolysis of BuChE was inhibited by 80 to 90 per cent, whereas MeCh hydrolysis was inhibited by less than 10 per cent.

It is apparent from the above discussion that there can be remarkable species differences in the composition of ChE in ocular tissues. Furthermore, biochemical and histochemical methods for detecting BuChE are not directly comparable in some tissues. The use of BuCh as substrate indicates substantial amounts of the BuChE-type enzyme, whereas they are not detected histochemically in the same tissues. This discrepancy could arise if certain ocular structures contained a BuChE isoenzyme for which BuCh was a good substrate but the thio-analogue was not. The assumption that BuCh hydrolysis is synonymous with butyrylthiocholine hydrolysis, and vice versa, may not be valid in all tissues. Another possible explanation is that the BuChE of certain tissues, such as the retina, may be readily soluble and lost during the histochemical procedure, whereas in other tissues, such as the rabbit lens, it may be fixed more firmly.

These results are somewhat at variance with those reported in the literature, but the differences seem to be minor in comparison to species differences and the non-concordance of the histochemical and biochemical results. Tissue ChE are known to be associated with, or an integral part of, membranes and, therefore, their properties can be expected to differ from enzyme preparations in solution. A comparison of membrane-bound ChE from several species and tissues has shown differences from purified enzyme studies, suggesting that the enzyme properties are altered by purification. This has also been found in the ciliary body, where the activating effect of pilocarpine on AChE is destroyed upon homogenization.

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