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SHORT COMMUNICATIONS

Radiometric assay of cholinesterases in intact tissues in the nanomolar concentration range of acetylcholine*

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KNOWLEDGE of the properties *in situ* of cholinesterases (ChE's) localized at cholinergic sites is a primary requirement in defining the function of these enzymes at such sites. Investigations of these enzymes usually involves the use of tissue homogenates or purified ChE's from various sources. Recently, several questions have been raised concerning the validity of extrapolating results obtained with such preparations to physiological and pharmacological situations.[1–3] First, tissue ChE's are known to be membrane-bound[4,5] and drastic methods are required for their solubilization.[6–11] These two factors might explain the differences which have been demonstrated in those few studies where membrane-bound enzymes have been compared with soluble preparations.[12–14] Second, there is evidence for species and tissue differences among ChE's.[1,15] Thus, comparing ChE from a specialized tissue, e.g. electric organ, with that present at mammalian junctions may not be justifiable. Third, the concentrations of substrate used in studying ChE's is generally about 10^{-3}M, a concentration one or more orders of magnitude greater than that which the enzyme encounters in most pharmacological experiments or under normal physiological conditions.

In an attempt to eliminate some of these problems, we have developed a radiometric assay procedure to determine the hydrolytic activity *in situ* of those ChE molecules which are accessible to acetylcholine (ACh) applied externally to a whole tissue *in vitro*.[16–18] Published radiometric methods for assay of ChE's have all used substrates labeled in the acyl moiety and involve the separation of radioactive acid from unhydrolyzed substrate by volatilization,[19] extraction,[20] ion exchange[21] or precipitation of the cholinester substrate.[22] These methods have been utilized primarily to assay ChE's in very small amounts of homogenized tissue at substrate concentrations greater than 10^{-4}M. The intent of the present radiometric procedure has been to measure enzymatic activity at substrate concentrations in the pharmacological range, i.e. 10^{-5} to 10^{-9}M, and on intact preparations used for pharmacological studies. In the present study, which represents a refinement of that previously described,[16–18] data are presented for activity *in situ* of ChE's of intact pieces of guinea pig ileum-jejunum. Procedures have been developed to eliminate some of the problems inherent in assaying enzymes of a complex heterogeneous system, i.e. a viable organ.

Starting at 5 cm from the ileocecal junction, ileum from male guinea pigs (250–500 g) killed by cervical fracture was flushed thoroughly in gassed Krebs' or Tyrode's-acetate solution (containing 1-g/l. sodium acetate trihydrate) and cleaned by stripping off the mesenteric membrane. A single piece of cleaned tissue, 2–6 cm in length, was used for the assay with the lumen either open or ligated; alternatively, the section was cut into pieces about 0·5 cm in length. The weight of tissue used depended on the total assay time and the volume of bathing solution, being about 800 mg wet weight for assays of 20 min and 200 mg for assays over 1 hr with bath volume of 50 ml. The volume of bathing solution and the tissue weight should be adjusted so that 10–30 per cent of the substrate is hydrolyzed. The tissue was put in a jacketed vessel (37° ± 0·1°) containing 20–50 ml Krebs' or Tyrode's-acetate solution appropriately gassed; a small magnetic bar provided stirring. After 10 min of equilibration, ACh labeled in the acyl moiety was added to the bath at a final concentration of 5×10^{-8} to 4×10^{-6} M and, after 3 min, aliquots of the bath solution were removed at suitable time intervals for subsequent processing (see below). Before starting the assay and again after the last aliquot was removed, 1 ml of the bath fluid was added directly to a scintillation vial containing 1·5 ml of 0·1 M sodium phosphate buffer (pH 7·2) and 10 ml of Bray's solution to determine total radioactivity.

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When the lumen was open, enzyme was progressively extracted into solution and separately assayed to determine tissue-bound activity. To do this, two samples of 2 ml were removed from the bath at each time interval. The first sample was added to a test tube containing 0·1 ml of a solution of neostigmine methyl sulfate (1 mg/ml) to stop further hydrolysis. The second sample was maintained at the assay temperature for 10 min before adding 0·1 ml neostigmine. A 1-ml aliquot of both test tubes was added to a short column (15 × 6 mm in diameter) of a cation-exchange resin (BioRad AG, 50W-X8, 200–400 mesh) equilibrated in 0·1 M sodium phosphate buffer (pH 7·2) and the effluent was collected in a scintillation vial. The column was then washed successively with 1 ml and 0·5 ml of phosphate buffer, and the washings were collected in the same vial. To each vial, 10 ml of Bray's solution was added and the vials, together with the vials for total radioactivity taken at the beginning and the end of the assay, were counted in a liquid scintillation counter. The first sample of each set gave the amount of acetate produced by tissue-bound enzyme plus that due to enzyme in the bath up to the time the sample was taken. The sample incubated for a further 10 min gave the amount of acetate in the first sample together with acetate produced by the solubilized enzyme. Thus, by difference, the enzyme activity in the bath solution was obtained and the correction made so as to obtain net tissue activity. After completing the assay (at least 4 points), the tissue was blotted between two pieces of filter paper and weighed.

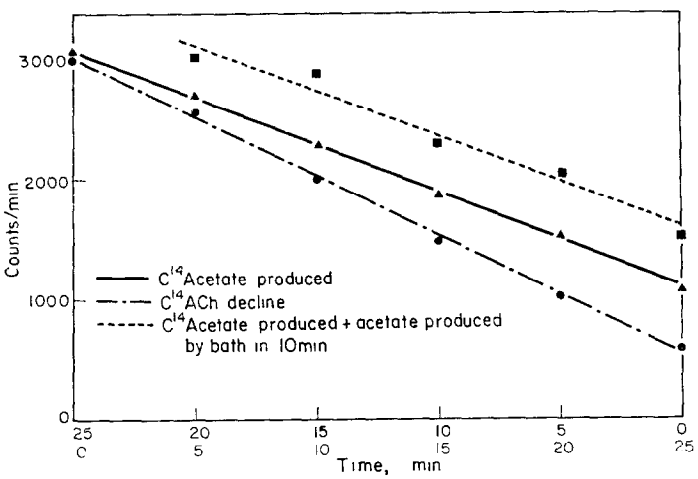

FIG. 1. Decline of ^{14}C-ACh in incubation medium in the presence of guinea pig ileum at 37° (bottom curve) and accumulation of ^{14}C-acetate (middle) plus ^{14}C-acetate produced by additional 10-min incubation in the absence of tissue (top). Time scale reads from right to left for middle and top curves

When radioactivity was plotted against time, a straight line was obtained (Fig. 1) and, after the necessary corrections for bath enzyme, the slope of this line represented tissue hydrolysis together with spontaneous background hydrolysis which was found to be 0·03 per cent per min for ACh under these conditions. Substitution of numerical values into the following equation gives a rate constant

$$k = \frac{R \times V_{t0} \times 10^{-3}}{W(T - A_{t0})}$$

for hydrolysis of substrate by the tissue enzyme; the dimensions are liter min^{-1} gram wet weight^{-1}. R = rate in cpm per ml of sample corrected for soluble enzyme and background hydrolysis; V_{t0} = bath volume in ml at zero time; W = tissue weight; T = total cpm per ml of bath; A_{t0} = cpm acetate per ml bath at zero time.

When a single piece of tissue with a ligated lumen was used, no enzymatic activity appeared in the bath solution, and it was unnecessary to do the second assay for solubilized enzymes. Rather, 1 ml aliquots were placed directly on the column, the effluent was collected, and the column washed with buffer to further elute the radioactivity.

In addition to, or as an alternative, to measuring the rate of acetate production, the rate of disappearance of radioactive ACh may be determined. After applying an aliquot, the ion-exchange column was washed to remove all traces of radioactive acetate. The adsorbed ACh was completely hydrolyzed on the column by adding 0·5 ml of 0·05 M NaOH and collecting the effluent. After 10 min, the resulting acetate was eluted by two 1-ml portions of phosphate buffer. Total radioactivity of the bath solution was determined by adding a 1-ml aliquot to a vial along with 1 ml of phosphate buffer, 0·5 ml of the NaOH, and 10 ml of Bray's solution. The assay was carried out in this way when the concentration of ACh or its specific activity was too low to measure the rate of actate production in an 1-ml aliquot. By increasing the length of the ion-exchange resin column to 30 × 6 mm in diameter, up to 10 ml of bath fluid could be applied, thereby increasing the sensitivity of the method 10-fold.

When intact pieces of intestinal tissue are bathed in radioactive ACh in normal Krebs' solution, a loss of radioactivity occurs with time. In a typical experiment, an 18 per cent loss occurred over a period of 20 min. The bulk of the loss was undoubtedly due to tissue uptake of radioactive acetate, since it was considerably reduced by adding nonradioactive sodium acetate to the buffer. Even under these conditions, the rate of ACh disappearance was still somewhat greater than the production of acetate (Fig. 1). This was attributed to uptake of ACh and accounted for the remaining 3 per cent loss. Since the rate of uptake of ACh was so much lower than the hydrolytic rate (Table 1), no correction of the latter was necessary.

TABLE 1. RATE CONSTANTS FOR ACh HYDROLYSIS BY INTACT GUINEA PIG ILEUM IN KREBS'-ACETATE, 37°, AT VARIOUS INITIAL SUBSTRATE CONCENTRATIONS.

Initial substrate conc* (ACh × 10^8)	Rate constant for hydroysis (k) (1 $min^{-1}g^{-1}$ × 10^4)
366	4·9
183	4·9
55	5·8
18·3	5·1
5·5	11·8
13·5	12·6
27·5	10·0
27·5	8·4
41·4	8·3
3·3–25·0*	4·8 (S.E. 1·6)†

* The bracketed values are for tissues from the same animal, uncorrected for soluble enzyme or background hydrolysis.

† Composite data for ileum from 20 different animals, corrected for soluble enzyme.

The upper line in Fig. 1 was plotted from those samples of the bath fluid which were removed and incubated for an additional 10 mins in the absence of the tissue as described above. The line is almost parallel to that for total acetate production but is displaced upward due to additional radioactive acetate produced by soluble enzymatic activity. Enzymatic activity from this source constituted 10–30 per cent of the total activity measured, depending on how thoroughly the tissue was washed. The soluble ChE was probably released by the action of proteolytic enzymes present in the lumen, since activity did not appear in the bath fluid when the tissue was ligated at both ends.

Since the substrate concentrations used are well below the K_m for ChE's (5 × 10^{-4}M), first order kinetics can be expected.[23] Figure 2 shows a representative assay carried out over 90 min in which 94 per cent of the substrate was hydrolyzed. The log plot deviates from a straight line and the rate

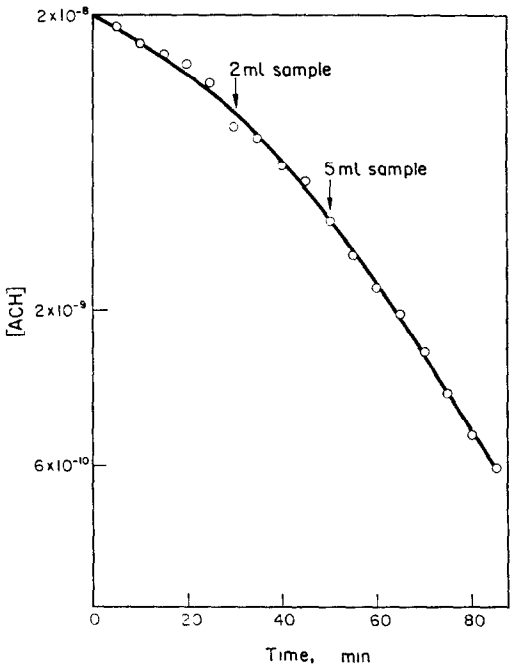

FIG. 2. Semi-log plot of rate of decline of ACh in incubation medium by 1 g guinea pig ileum (single length) in Krebs'-acetate at 37°. Sample volumes removed are indicated at the arrows.

constant appears to increase as the substrate concentration decreases. This is due to the increasing ratio of enzyme to substrate as the volume of the solution bathing the tissue-bound enzyme is reduced by removal of aliquots. This complication was negligible when the rate of hydrolysis was measured over a small fraction of the total substrate and acetate production was linear (Fig. 1).

The total activity for separate pieces of ileum from the same animal determined at varying substrate concentrations agreed quite closely, but there was considerable variation when tissues from different animals were used (Table 1). The mean value of $4{\cdot}8 \times 10^{-4}$ liter min^{-1} g^{-1} for the corrected rate constant from ileums of 20 different animals represents net tissue-bound enzyme activity. The value for the rate constant for acetylcholine uptake by the intact tissue ($1{\cdot}8 \times 10^{-5}$ liter min^{-1} g^{-1}) has been calculated from the difference between the rates for ACh disappearance and acetate production.

For the enzyme reaction,

$$\mathrm{ACh} + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ACh}.E \xrightarrow{k_3} \mathrm{AE} + \mathrm{Ch} \xrightarrow{k_4} \mathrm{A} + \mathrm{E}$$

at low substrate concentrations, the rate determining step is the acylation of the enzyme by the substrate.[24] Thus, $R = k_3(\mathrm{ACh.E}) = k_3(\mathrm{ACh})(E)/K_s$. The rate equation used in this study is $R = k(\mathrm{ACh})$, and in the absence of a diffusion barrier, k is equal to $(E)k_3/K_s$, where (E) is the number of substrate sites in 1 g tissue. However, if diffusion is rate determining, the constant, k, would include a rate constant for the diffusion of substrate into the compartment containing the tissue-bound enzyme. Results from other experiments (in preparation) suggest that such diffusional factors may be minimal. Even if a diffusion barrier were present, this should not invalidate the assay procedure, since such a barrier would be constant for a given substrate.

The hydrolysis of ACh by ChE accessible to externally applied substrate over the concentration range 10^{-9} to 10^{-5} M was found to be exponential and proportional to the concentration of the enzyme–substrate complex. Thus, the influence of cholinergic drugs, which affect either the total number of free substrate sites (E) or their turnover number, can be measured in the intact tissue by changes in the experimentally determined value of k as defined above.

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p-chlorophenylalanine depletion of gastrointestinal 5-hydroxytryptamine*

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5-HYDROXYTRYPTAMINE (serotonin, 5-HT) is widely distributed throughout the mammalian organism. The organ system which has the highest concentration of this amine is the gastrointestinal tract. The distribution of 5-HT in the gastrointestinal tract varies greatly from one portion to another. The distribution of 5-HT in the rat gastrointestinal tract has recently been carefully studied.[1] With the

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