Radiometric Estimation of Blood Cholinesterase Levels in Domestic Animals

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A radiometric method has been adapted for measuring domestic animal blood cholinesterase activity and its inhibition as an index of possible exposure to organophosphorus or carbamate insecticides under field conditions. Small samples of blood are diluted (not more than 11-fold), haemolysed, and incubated on a glass slide with 0.5 × 10⁻⁴ M (¹⁴C) acetylcholine as substrate. After ca. 40 sec the reaction is stopped and the volatile (¹⁴C) acetate formed enzymically removed by drying. Enzymatic hydrolysis rate is calculated from the fraction of radioactive substrate remaining under standardised conditions.

INTRODUCTION

The estimation of blood cholinesterase provides a valuable indication of the degree of exposure of an animal to cholinesterase-inhibiting chemicals because it is possible to have depression of activity in the complete absence of external symptoms. A simple radiometric method of estimating blood cholinesterase has been described by WINTERINGHAM and his colleagues which enables cholinesterase inhibition by both organophosphate and carbamate insecticides to be readily measured. The method described by WINTERINGHAM and DISNEY is specially designed for man and some modifications to their method are described so as to adapt it for the easy measurement of the lower cholinesterase activity levels found in domestic animals.

PRINCIPLES OF THE METHOD

Known quantities of haemolysed blood and ¹⁴C-acetyl labelled acetylcholine are mixed together and incubated on a microscope slide for a known time. The radioactive substrate is hydrolysed enzymically and after incubation is complete the reaction is stopped by acidifying the mixture. After the slide has been forcibly dried the ¹⁴C is counted using a thin end-window Geiger–Müller tube with scaler. The radioactive acetic acid produced from the hydrolysed acetylcholine evaporates on drying the slide and the amount may be determined by comparison with a reference slide on which the reaction is not allowed to take place. The rate of hydrolysis is expressed as the number of moles of acetylcholine, at constant concentration, which would be hydrolysed in one hour by 1 ml of whole blood at 25°C. Thus if the net counting rate for the reference slide is a counts per minute and that for the test slide is x after t hr incubation with b ml blood, then the apparent average rate of hydrolysis \( V_t \) would be given by:

\[
V_t = \frac{A(a - x)}{a \cdot b \cdot t} \mu M \text{ hr}^{-1} \text{ ml}^{-1}
\]  

where \( A \) represents the initial quantity of acetylcholine in micromoles (µM).

At the very low concentrations of substrate used in this technique the rate of hydrolysis at any instant is proportional to the concentration of substrate remaining and this falls off exponentially with time so that the initial rate of hydrolysis \( (V_o) \) is given by:

\[
V_o = \frac{V_t \cdot \ln x/a}{(a - x)/a}
\]

and by substitution of \( V_t \) from equation (1) this becomes:

\[
V_o = \frac{A \cdot (a - x)}{a \cdot b \cdot t} \cdot F \mu M/\text{hr}^{-1} \text{ ml}^{-1}
\]
Values of the factor $F$ are given by Winteringham and Disney (2) for ratios of $x/a$ from (0–1). A simplified version is given in Table 1. The factor $F$ represents the invariable ratio of initial enzymatic hydrolysis rate at the known initial substrate concentration to the apparent rate observed for different fractions of substrate remaining. (2)

### REAGENTS AND METHODS

The (1-14C) acetylcholine chloride solution was prepared at a concentration of $1.5 \times 10^{-4}$ M and the pH adjusted so as to lie between 4 and 5. With a specific activity in the range 5–25 Ci/mol, 5 $\mu$l solution provides (8–42) $10^3$ disintegrations per minute. The solution was divided into a number of small bottles which were kept in a domestic deep freezer (−15°C). An all-glass “Agla” micrometer syringe with glass needle (27 S.W.G) was also used and, after drawing air into the barrel, this was likewise kept in the deep freezer when not in use.

Haemolysing solution contained 50 mg saponin, 1g potassium chloride and 0.05 ml toluene (added as a preservative) made up to 100 ml in glass-distilled water, whilst the acid inhibitor was 1.0-N hydrochloric acid.

**Blood sampling**

Generally blood was obtained from a vein so that a few millilitres were available. The haemolysed dilutions never exceeded 11-fold. Haemolysis was complete in about one minute and there was enough blood present to provide its own buffer at ca. pH 7.0.

### Procedure

Exactly 10 $\mu$l of diluted blood was transferred to the centre of a numbered microscope slide. Generally 5 $\mu$l of the labelled substrate was added and mixed using the tip of a fine glass rod. After the appropriate time (usually 40 sec) a 5-$\mu$l drop of acid inhibitor was similarly added from a second “Agla” micrometer syringe. At all stages the mixture was confined to a circular area of ca. 1 cm diameter and it was important that sample and substrate were mixed as quickly as possible. It is an advantage if the slides are dipped in very dilute ethereal petroleum jelly so as to ensure that they are coated with a thin grease film, so discouraging the unnecessary spreading of the drops on the slides (WINTERINGHAM, personal communication). The air temperature was recorded and the slide dried in hot air by placing it on a level surface some 30 cm below a domestic hair dryer at an air temperature of ca. 60°C.

Reference slides were prepared in exactly the same way except that the acid was added before the labelled substrate thus inhibiting all cholinesterase activity.

**Counting**

A Geiger–Müller tube (Mullard Type MX 163/01) was used attached initially to an Ecko Scaling Unit, type 1287A. This scaling unit
had the advantage that it was completely portable and could be operated for several hours on its own battery. However, for general laboratory use a Nuclear Enterprises SR3 Scaler-Ratemeter was more convenient.

Following earlier recommendations, each slide was first read twice, but a comparison of a series of counts showed that the differences between successive counts were negligible. Using the SR3 Scaler Ratemeter it was possible to count each slide over a period of 300 sec. This time gave a convenient working rate as a whole and resulted in counts of 2000 or more.

Calculation of results
The observed counting rates represent the sum of the counts due to the radioactive substrate and that due to background radiation. The fraction of substrate lost, \((a - x)/a\) in equation (2), is given by the expression \((a' - x') (a' - B)^{-1}\) where \(a'\) and \(x'\) are the observed counting rates for the reference and sample slides respectively measured over a time of 100-1000 seconds, and \(B\) the background counting rate measured over a similar period of time.

The appropriate values were then inserted for the other variables in equation (3) as follows: \(F\) was obtained from Table 1 once the fraction of substrate lost was known; \(A\) the quantity of acetylcholine which varied with the volume used but the concentration was always \(1.5 \times 10^{-4} \mu M\) per \(\mu l\) solution, \(t\) the time of reaction expressed in hours, and \(b\) the amount of whole blood in \(10 \mu l\) of the haemolysed sample.

Two slides were always prepared from each sample and the mean count used unless they were very different in which case a third slide was prepared.

Since the hydrolysis rate was dependent on temperature the slides had to be kept away from draughts and at a constant temperature as possible. When the room temperature was significantly different from 25°C corrections were made to the observed hydrolysis rate as given in Table 2 following earlier data.

### ACCURACY OF THE METHOD

Preliminary trials showed that the accuracy was not as great as was desired. It was thought likely that this was due to variations in blood film thickness and size, which could not be strictly controlled. It was decided to compare the Geiger–Müller counting technique with a liquid scintillation counting method which should eliminate errors due to sample geometry.

For this series of experiments the technique was the same except that a small coverslip (1 cm²) was attached to the slide by a minute trace of silicone grease. 5 \(\mu l\) of haemolysed blood and 5 \(\mu l\) of normal hydrochloric acid were added to each slide and treated as usual. After reading by Geiger–Müller counting the coverslip was carefully removed and placed in a scintillation counting vial. The dried blood film was washed off with exactly 0.5 ml of 0.01 N HCl and then 5 ml of a naphthalene-dioxan based scintillator was added. The reaction on each slide was thus measured four times, twice by the Geiger–Müller counting and twice by scintillation counting. Three series of readings were done: one to check the linearity of response by adding different quantities of radioactive acetylcholine, the others to check repeatability of reference and test slides so that a comparison of the ratios obtained could be made.

The first series of readings to check the linearity of the response showed that for both counting methods errors due to the variability of the counting rates measured for a given quantity of radioactivity were negligible.

Then using the pairs of readings obtained from each slide by each method of measurement an analysis of variance was carried out to determine the variance due to successive readings of the same slide and that attributed

<table>
<thead>
<tr>
<th>Temperature</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>35</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor</td>
<td>1.27</td>
<td>1.12</td>
<td>1.00</td>
<td>0.89</td>
<td>0.79</td>
<td>0.72</td>
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</table>

<table>
<thead>
<tr>
<th>Counting method</th>
<th>Sample and standard deviations (c/min)</th>
<th>Estimate of radioactivity loss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test</td>
<td>Reference</td>
</tr>
<tr>
<td>Geiger-Müller Counting</td>
<td>423.7 ± 9.5</td>
<td>703.6 ± 50.00</td>
</tr>
<tr>
<td>Scintillation Counting</td>
<td>394.5 ± 15.7</td>
<td>603.4 ± 39.0</td>
</tr>
<tr>
<td>Regression Coefficients between counts</td>
<td>0.11 ± 0.12</td>
<td>0.19 ± 0.11</td>
</tr>
</tbody>
</table>

TABLE 4. Effect of varying blood dilution and reaction time on acetylcholinehydrolysis rates (with constant 7.5 × 10⁻⁴ μM acetylcholine per slide).

<table>
<thead>
<tr>
<th>Blood dilution</th>
<th>Reaction time (seconds)</th>
<th>Hydrolysis rate (μM AcOH/cm²·h) mean</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:12</td>
<td>1:6</td>
<td>1:3</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15.0</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.6</td>
<td>2.5</td>
</tr>
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<td>13.7</td>
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<td></td>
<td></td>
<td>12.9</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.4</td>
<td>2.6</td>
</tr>
</tbody>
</table>

When counts from the same slide by Geiger–Müller and scintillation counting were compared by means of a regression analysis no correlation was found (Table 3). Finally the estimates of loss of radioactivity were compared by the different methods of counting and the standard error of each calculated. Although the difference between methods was small it was nevertheless statistically significant and corresponded to hydrolysis rates of 20.6 and 17.1 μl acetylcholine hydrolysed per hour per ml whole blood for Geiger–Müller and scintillation counting respectively.

In another series of experiments the time of reaction and the concentration of blood were varied: five dilution replicates being prepared for each set of conditions from one blood sample and two slides prepared from each dilution. The different dilutions used and reaction times are shown in Table 4. All the readings were made by Geiger–Müller counting and it may be seen that the hydrolysis rates to different slides, this showed that at least 75 per cent of the variability obtained was due to differences between slides. Hence for greater accuracy it was more important to repeat the number of slides prepared for each estimate than read an individual slide more accurately by measuring over a longer time. This conclusion applied to both methods of measurement of radioactivity and was an error variability presumably due combination of variables, namely quantity of radioactivity present, reaction rate and timing. (Geiger–Müller counting; variance between slides 2268 and between readings 589; scintillation counting; variance between slides 1972 and between readings 289). The means and standard deviations of the various counting rates observed are shown in Table 3. In this series of data the standard deviation was small, the largest coefficient of variation being only 7 per cent and there was no significant difference between the variation obtained by either method of measurement.
calculated were reasonably constant for each set of conditions.

CONCLUSIONS

Both Geiger–Müller and scintillation counting gave repeatable results without too much scatter. There was no reason for choosing either counting method in preference to the other, although the methods appeared to give slightly different results. As Geiger–Müller counting was the simpler to carry out this was the method used.

Varying the blood dilution or reaction time within the narrow limits selected had no significant effect on the calculated hydrolysis rates. The scatter of these results was greater than desirable and all efforts to reduce this have so far been unavailing. However, the scatter was not sufficient to prevent blood cholinesterase measurements from being made.

REFERENCES