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A COMPARISON OF TWO METHODS FOR THE MEASUREMENT OF CHOLINESTERASE INHIBITION IN HUMAN BLOOD

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Abstract—A comparison has been made between the radiometric method and an electrometric method for the measurement of cholinesterase activity in human blood. Blood was inhibited *in vitro* with Paraoxon, Baygon and Sevin and the percentage inhibition measured by the two methods. Similar results were obtained by both methods for inhibition by Paraoxon but the radiometric method indicated considerably greater inhibition by the carbamates Baygon and Sevin. The results emphasize the need to obviate substrate and dilution effects when measuring carbamate inhibition.

ANTICHOLINESTERASE insecticides such as organophosphates and carbamates are being used on an increasing scale to control agricultural pests and vectors of disease. Measurement of blood cholinesterase activity provides a useful indication of human exposure to these poisons, particularly as "it is possible to have depression of activity in the complete absence of symptoms".[1]

Conventional methods of measuring cholinesterase activity are likely to underestimate inhibition caused by carbamates which *behave* as reversible inhibitors.[2] The high substrate concentrations and considerable dilution of the enzyme, involved in most of these methods tends to reverse the inhibition it is desired to detect.

A radiometric method which largely overcomes these difficulties has been developed by Winteringham and the author.[3] A modified form of this method[4] has been used in a comparison with the electrometric method of Michel[5] as described for use with whole blood by Aldridge and Davies.[6] In the present experiments human blood was inhibited *in vitro* with Baygon*, Sevin† or Paraoxon‡.

MATERIALS AND METHODS

Samples of the author's blood (0·2 ml) were drawn from finger pricks in a heparinized pipette in the usual way. This blood was diluted for assay by the radiometric method by addition to 2·0 ml of haemolysing solution (1·0 g KCl and 50 mg saponin in 100 ml water). The haemolysed blood solution was divided into two equal parts (1·1 ml) and placed in 2-ml vials. To the first of these was added 1·0 μl of acetone as a control and to the second 1·0 μl of a solution of inhibitor in acetone. These additions were made from a Drummond Microcap pipette. The blood solutions were left to stand for 60 min. Two 0·22-ml aliquots were then transferred from the "control" and

* *o*-isopropoxyphenyl N-methylcarbamate.
† 1-naphthyl N-methylcarbamate.
‡ *O,O*-diethyl *p*-nitrophenyl phosphate

"inhibited" vials to tubes containing 0·78 ml distilled water and 1·0 ml Michel II buffer (0·1237 g Sodium barbital, 0·0136 g KH_2PO_4 and 1·7535 g NaCl in 100 ml water and the pH adjusted to 8·0). Exactly 20 min later cholinesterase activities were measured by the electrometric technique as follows: substrate solution (0·2 ml of 0·165 M acetylcholine chloride) was added to the first tube and exactly 1 min later the pH was read to the nearest 0·01 pH. One minute later the substrate was added to the second tube and so on until all four tubes had been dealt with. The pH for each tube was measured again exactly 60 min after the initial reading. The mean change of pH for each pair of tubes was used to calculate the percentage inhibition of the cholinesterase as described.[5, 6] Cholinesterase activities of a second series of four 0·22-ml samples were determined in exactly the same manner except that the substrate was added 4 hr after the final dilution instead of 20 min.

The cholinesterase activity in the original two vials of haemolysed blood was measured by the radiometric method[4] about 15 min after the initial pH readings had been taken for the electrometric assay. A 10·0 μl sample was pipetted on to the centre of a plain microscope slide and a 5·0 μl drop of ^{14}C-acetylcholine chloride ($1{\cdot}5 \times 10^{-4}$ M) was touched off the needle of an Agla micrometer syringe and mixed rapidly with the blood solution. Exactly 20 sec later a 5 μl drop of 1·0 N hydrochloric acid was similarly added and mixed. The slide was dried in a draught of warm air and counted by a thin-end window G-M counter. Three slides were prepared for each tube and four reference slides were prepared by adding the acid before the substrate. The cholinesterase activity was calculated from the mean counts for each group of slides as already described.[4] All sample preparations, treatments and measurements were conducted at 25°. The final blood dilutions and substrate concentrations were 110 fold and $1{\cdot}5 \times 10^{-2}$ M respectively for the electrometric method and 16·5 fold and 5×10^{-5} M respectively for the radiometric method.

RESULTS AND DISCUSSION

Table 1 shows the results obtained by the two methods for blood inhibited by various concentrations of Paraoxon. In the case of this irreversible phosphorylating inhibitor both methods would be expected to give the same results and this is born out by the figures in the Table. Aldridge[7] has shown that the cholinesterase of intact

TABLE 1. COMPARISON OF THE INHIBITION OF WHOLE BLOOD CHOLINESTERASE BY PARAOXON AS MEASURED BY THE RADIOMETRIC AND ELECTROMETRIC METHODS

Inhibitor concentration	Percentage inhibition			
	Radiometric method (after 35 min)	Radiometric method (after 4 hr)	Electrometric method (after 20 min)	Electrometric method (after 4 hr)
$2{\cdot}7 \times 10^{-9}$ M	5	23	8	10 (34)
	7	9	12	9
$9{\cdot}1 \times 10^{-9}$ M	31	39	32	36 (74)
	27	35	33	35
$2{\cdot}7 \times 10^{-8}$ M	73	71	69	75 (99)
	73	81	71	76
$9{\cdot}1 \times 10^{-8}$ M	95	95	94	93 (100)
	98	96	95	95

red cells of goat blood is inhibited irreversibly by Paraoxon. He also showed that the inhibition followed the characteristics of a bimolecular reaction—where one of the reactants is in excess. The equation of the reaction becomes:

$$k = \frac{1}{I.t} \ln \frac{100}{b} \tag{1}$$

where k = second order reaction constant, I = inhibitor concentration, t = time, and b = percentage residual activity. When I can be assumed to be constant a plot of ln b vs. t should yield a straight line. The results given in Table 1 do not follow this equation. The figures in parenthesis in the 4-hr column under the heading electrometric method are calculated from equation (1) using $k = 5{\cdot}0 \times 10^5$ l. mol^{-1} . min^{-1}. This mean value of k was calculated at $I = 9{\cdot}1 \times 10^{-9}$ M and $2{\cdot}7 \times 10^{-8}$ M taking the mean inhibition obtained for the first observations by both methods. The discrepancy between the observations at 4 hr and the calculated inhibition indicates that the whole blood enzyme preparation is not inhibited in the expected manner.

These observations were confirmed by time course experiments using the radiometric method. A whole blood sample was haemolyzed for the radiometric method and inhibited by $1{\cdot}8 \times 10^{-8}$ M Paraoxon. Samples were taken at various times from both the control and inhibited haemolysate and their cholinesterase activity determined. The same procedure was also carried out using a preparation of bovine erythrocyte cholinesterase. Figure 1 shows the results obtained. The inhibition of the bovine erythrocyte cholinesterase follows the expected course but the whole blood preparation reaches an equilibrium after about two hours. These curves show that the unexpected results of Table 1 are due either to some property of whole blood or to the

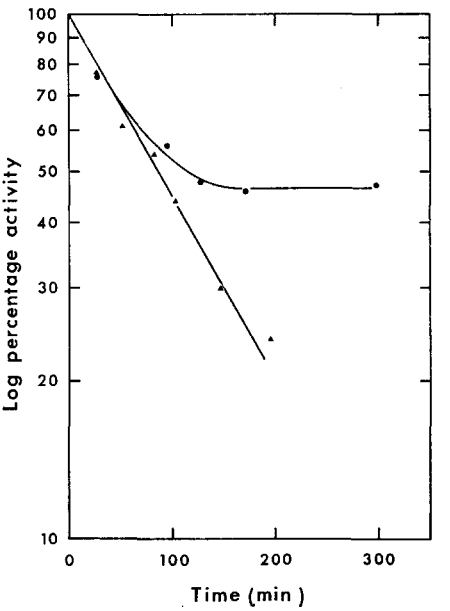

FIG. 1. Rate of inhibition of whole blood and bovine erythrocyte cholinesterase by approximately $1{\cdot}8 \times 10^{-8}$ M paraoxon.
●, whole blood. ▲, bovine erythrocytes.

decomposition of Paraoxon in the whole blood preparations. A further investigation of this phenomenon is in progress.

Figures 2 and 3 show the results obtained by the two methods for Baygon and Sevin respectively. In the case of these carbamates the two methods give different results. The radiometric method gives consistently higher percentage inhibition for

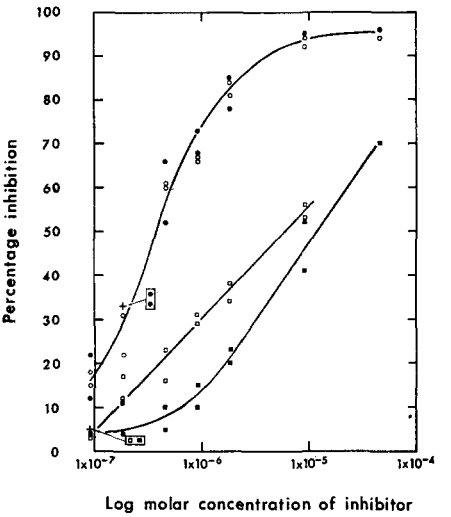

FIG. 2. Inhibition of whole blood cholinesterase plotted against the log concentration of Baygon.
○ and ●, first and second radiometric determinations.
□ and ■, first and second electrometric determinations.

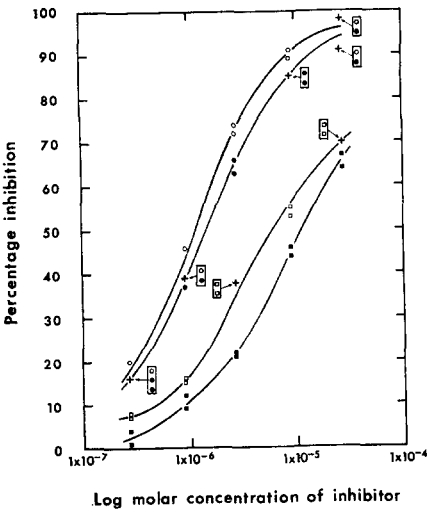

FIG. 3. Inhibition of whole blood cholinesterase plotted against the log concentration of Sevin.
○ and ●, first and second radiometric determinations.
□ and ■, first and second electrometric determinations.

any particular inhibitor concentration. There is no significant difference between the first and second radiometric determinations with Baygon but with Sevin there may be some slight reactivation over the 4-hr period. Both inhibitors show some difference between the first and second electrometric determinations, indicating that reactivation continued after 20 min as a result of the greater dilutions involved by this method.

Winteringham and the author[2] showed that the inhibition kinetics of whole blood cholinesterase by Baygon tended to follow a Michaelis–Menten,[8] Haldane-derived[9] equation for a reversible competitive inhibitor. It has been shown that under conditions of equilibrium between enzyme, substrate and inhibitor:

$$\frac{v'}{v} = \frac{K_m + S + S^2/K_s}{K_m(1 + I/K_i) + S + S^2/K_s} \tag{2}$$

Where v and v' represent enzymic reaction rates in the absence and in the presence of inhibitor at concentration I respectively, K_m is the Michaelis constant and K_i the dissociation constant for the *effectively* reversible reaction of the type $E + I \rightleftharpoons EI$. K_s is the dissociation constant for the reversible formation of the complex between the normal enzyme substrate complex and a further molecule of substrate ($ES + S \rightleftharpoons ES_2$) which is not capable of further reaction with the formation of substrate reaction products. When S is small equation (2) can be written in the form:

$$\frac{v}{v'} = 1 + \frac{K_m I}{K_i(K_m + S)} \tag{3}$$

It follows that a plot of v/v' against I should give a straight line of slope $K_m/K_i(K_m + S)$. The radiometric results for Baygon and Sevin both yielded straight lines, within the limits of experimental error, when plotted in this way. Using the previously published value of $K_m = 7{\cdot}4 \times 10^{-4}$ mol. $l.^{-1}$ for whole blood,[2] the slopes of the lines gave $K_i = 3{\cdot}8 \times 10^{-7}$ for Baygon and $9{\cdot}6 \times 10^{-7}$ mol. $l.^{-1}$ for Sevin.

The effect of differing substrate concentrations and dilution of the inhibited enzyme preparation may be calculated from equation (2). This was done for both carbamates using the values of K_m and K_i quoted above and taking K_s as $2{\cdot}13 \times 10^{-3}$ mol.$l.^{-1}$ for whole blood.[2] The predicted inhibition as measured by the electrometric method does not differ significantly from zero over the range of inhibitor concentration used. If only the dilution is considered, the effect is to move the radiometric curves to the right in Figs. 2 and 3 by a factor of ten. This then gives agreement between the two methods at lower levels of inhibition but does not account for all the difference at higher inhibitor concentrations. There are two factors concerned here. The time taken to reach equilibrium after dilution is unknown but is obviously in excess of 20 min. Previous data[2] indicate a period of about 2 hr for Baygon. The high substrate concentration used for the electrometric method should reverse the inhibition. This does not appear to occur to any great extent but the time available is only one hour and this may be insufficient for any marked reversal to occur. Also because the pH change will not be linear with time if substrate reversal is occurring, the method will underestimate both the initial inhibition and the amount of reversal.

The present results underline the conclusions of Witter[10] and of Winteringham and the author[2] that the measurement of cholinesterase inhibition by carbamates by the classical methods is subject to serious errors. Although the radiometric method partly

overcomes these difficulties, because the blood is initially diluted 11-fold for radiometric assay, even this method probably underestimates inhibition *in vivo*. The results emphasize the importance of using a method which employs a low substrate concentration and involves minimal dilution of the inhibited enzyme especially. Alternatively, a method should be employed in which the time of measurement is small compared with the time required for equilibrium between enzyme, substrate and inhibitor and measurements are made immediately after diluting samples.

It is essential that errors of this nature should be eliminated as far as possible in the measurement of blood cholinesterase activity as an index of exposure to carbamates.

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