Substrate and Dilution Effects on the Inhibition of Acetylcholinesterase by Carbamates

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1. The kinetics of acetylcholinesterase (EC 3.1.1.7) activity and its inhibition by eserine or by Sevin (1-naphthyl *N*-methylcarbamate) have been studied over the substrate concentration range 5×10^{-8} to 2.5×10^{-2} M. 2. Equations are given for inhibition as a function of time, substrate and inhibitor concentrations, and the relevant parameters determined at 25° and 37°. 3. The observed and calculated effects of time, dilution, substrate addition and enzyme concentration were in good agreement and consistent with a steady-state carbamylation by eserine or by Sevin in the presence of excess of inhibitor. 4. The quantitative destruction of either inhibitor at high enzyme concentrations implied by the carbamylation hypothesis has been confirmed experimentally. 5. The importance and possibility of allowing quantitatively for dilution and substrate effects when estimating carbamate inhibition are demonstrated.

In a radiometric study of the reversible inhibition of acetylcholinesterase by an insecticidal carbamate (Winteringham & Disney, 1964a) it was shown that addition of acetylcholine or dilution of the inhibited enzyme reversed the inhibition by the carbamate, but not that by organophosphorus compounds. That such effects could lead to serious underestimates of carbamate inhibition with conventional methods of assay has been confirmed (Disney, 1966). Winteringham & Disney (1964a) recognized that the reversibility of the carbamate inhibition could be explained either by the formation of an enzyme-carbamate complex or by a steady-state carbamylation as indicated by Wilson, Hatch & Ginsburg (1960) and Wilson, Harrison & Ginsburg (1961). In the latter case the molar concentration of inhibitor was so much larger than that of the enzyme that it could be regarded as constant during the experiment. In either case, under conditions of equilibrium between enzyme, substrate and inhibitor, the effects on the measured inhibition of diluting the enzyme-inhibitor mixture or of adding acetylcholine would be expected to follow the same equation. However, the significance of two constants in the equation would differ (Winteringham & Disney, 1964a). The effects of dilution and of acetylcholine addition on the measured inhibition of cholinesterase preparations by the insecticidal carbamate, *o*-isopropoxyphenyl *N*-methylcarbamate, approached, though were usually less than, the calculated values. This suggested that condi-

tions of equilibrium were not achieved during the experiment and therefore that the effects depended on time. To take time into account it would be necessary to evaluate separately the constants for the rate of formation of enzyme-carbamate complex or of carbamyl enzyme and for the rate of regeneration of free enzyme.

This paper describes the evaluation of the relevant parameters for a purified bovine erythrocyte acetylcholinesterase preparation and two *N*-monomethylcarbamates of widely differing properties: eserine and the water-insoluble insecticide Sevin (1-naphthyl *N*-methylcarbamate). The radiometric technique was used throughout, since this permitted the use of such short times and low substrate concentrations that carbamate inhibition was not significantly changed during the measurement.

MATERIALS AND METHODS

Bovine erythrocyte acetylcholinesterase. A purified, dried preparation (1–1.5 mg. of protein with 20–30 mg. of gelatin carrier; Sigma Chemical Co., St Louis, Mo., U.S.A.) was dissolved in 1.0 ml. of buffer (see below) at pH 7.4, and this stock solution was stored at –15°.

Units of enzyme activity. At 25° near-maximal activity was observed with an initial substrate concentration 1.0 mM-acetylcholine. Under these conditions 1.0 ml. of stock solution would hydrolyse acetylcholine at an initial rate 31.4 μ moles/min. and was therefore equivalent to 31.4 units of enzyme (EC 3.1.1.7) as defined by the Commission on Enzymes of the International Union of Biochemistry (1961).

In this paper all enzyme concentrations are expressed in units/ml. and activities as μ moles of acetylcholine hydrolysed, min^{-1} units $^{-1}$.

Buffer solution. Sodium barbital (2.062 g.) and KH_2PO_4 (0.272 g.) were dissolved in water to give final vol. 1 l. The pH was brought to 7.4 with HCl.

[carboxy- ^{14}C]Acetylcholine. The anhydrous chloride (0.71 mg., at specific radioactivity $6.4 \mu\text{C}/\mu\text{mole}$) purchased from The Radiochemical Centre, Amersham, Bucks., was dissolved in 7.81 ml. of water to give a 0.50 mM stock solution at pH 4.5. This was stored at -15° .

Liquid phosphor. A 7.5% solution in scintillation grade dioxan of Scinstant NE 572 [Nuclear Enterprises (G.B.) Ltd., Edinburgh]. This was stored in the dark.

Acetone-hydrochloric acid. Aqueous 2.75 N-HCl (5.0 ml.) was diluted to 100 ml. with acetone.

Inhibitor solutions. A stock solution of 2.5 mM-Sevin was prepared by dissolving 50.3 mg. of recrystallized Sevin (Union Carbide Co., Clayton, N.C., U.S.A.) in 100 ml. of acetone and was stored at -15° . A stock solution of 5.0 mM-eserine was prepared by dissolving 162 mg. of dried eserine sulphate (British Drug Houses Ltd.) in 100 ml. of water and was stored at -15° . Water-diluted solutions were freshly prepared. The equivalent volume of acetone was added to the controls of experiments with Sevin.

Procedure. Enzyme activities were assayed radio-metrically as described by Winteringham & Disney (1964a) with slight modification to achieve greater simplicity and accuracy. Buffer solution (0.98 ml.) containing small proportions by volume (< 5%) of enzyme, and of inhibitor or water, was preincubated in a stoppered tube for the times and temperatures indicated. Diluted or undiluted labelled substrate, to which had been added when necessary the calculated weight of non-radioactive acetylcholine chloride, was measured (20 μl .) from an Agla micrometer syringe (Winteringham & Disney, 1964b). This substrate on the tip of a small glass or plastic rod was added to the buffered enzyme sample with immediate stirring and the time noted to the nearest second. At suitable intervals 0.10 ml. samples were pipetted into 10 ml. counting-phials, 0.2 ml. of acetone-HCl was added rapidly and the time of mixing noted. All solvent was evaporated from the phial by directing inside for 5 min. a jet of air at approx. 75° . The residue containing unhydrolysed ^{14}C -labelled substrate was redissolved in 0.5 ml. of 0.01 N-HCl and 5 ml. of the liquid phosphor added. ^{14}C activities were assayed in a scintillation counter with a sensitivity of approx. 50%.

With an enzyme concentration 4×10^{-3} unit/ml. and initial substrate concentration 1.0 mM it was found convenient to sample at 5–10 min. intervals over a period of 1 hr. or more, when not more than 50% of the substrate was removed. With initial substrate concentration 1 μM samples were taken at 1–2 min. intervals over a period of about 10 min., when 50% or more of the substrate had been removed. At the higher substrate concentrations enzymic activities were estimated by drawing tangents to the slight curves obtained when substrate concentration was plotted against time. At the lower concentrations activities were calculated from the straight lines obtained by plotting the logarithm of substrate concentration against time (cf. Winteringham & Disney, 1964a). Straight lines were invariably obtained at the low substrate concentrations, provided that sufficient time had been allowed for effective equilibration between enzyme and inhibitor before adding substrate.

An alternative procedure was employed when it was important that the time of incubation with ^{14}C -labelled substrate should be small in relation to the effective half-time of reaction between enzyme and inhibitor or of regeneration of the free enzyme (see below). In this procedure a 0.20 ml. sample was transferred from the buffered enzyme mixture to a counting-phial suspended in the water bath. A calculated volume of ^{14}C -labelled substrate solution (< 10.0 μl .) was added. After an exactly timed period of 15–60 sec. acetone-HCl (0.4 ml.) was blown into the phial. The drying period was increased to 8 min. to allow for evaporating the greater volume of solvent. Initial enzyme concentrations were chosen so that the labelled substrate was reduced by 20–80% during the incubation period. Initial reaction rates were deduced from their known relationship to the fraction of substrate removed over the observed period (see below).

RESULTS

Effects of substrate concentration in the absence of inhibitor. Logarithms of initial rates at 25° are plotted against negative logarithms of initial substrate concentration (pS) in Fig. 1. The mean corresponding rates over a limited range of substrate concentrations at 37° are also shown. The average temperature coefficient was 1.28 and is similar to the value of 1.32 indicated by the observations of Winteringham & Disney (1964b). Although there was an indication that the temperature coefficient varied with substrate concentration, being minimal at the optimum concentration, it was

Fig. 1. Bovine erythrocyte acetylcholinesterase: substrate effects on initial reaction rates. ○, 25° ; △, 37° . The theoretical curve at 25° is shown as the broken line (see the Discussion section).

Fig. 2. Inhibition of acetylcholinesterase in the presence of $1\ \mu\text{M}$ -Sevin. $\circ$, 25° ; $\triangle$, 37° .

Fig. 3. Inhibition of acetylcholinesterase in the presence of $0.02\ \mu\text{M}$ -eserine. $\circ$, 25° ; $\triangle$, 37° .

assumed for the purpose of the calculations below that the entire rate- pS curve at 37° would have the same shape and optimum pS value as that at 25° , with a constant temperature coefficient of 1.28.

Rates of inhibition. Rates of inhibition in the presence of $1\ \mu\text{M}$ -Sevin or $0.02\ \mu\text{M}$ -eserine were determined at 25° and at 37° with the alternative short-time incubation procedure (see above) at an initial substrate concentration $1\ \mu\text{M}$ -acetylcholine. The results are shown in Figs. 2 and 3. It will be seen that conditions of steady state or of equilibrium were achieved in all cases within 2 hr. In all these experiments inhibition is expressed as substrate-hydrolysis rate in the presence of inhibitor relative to that in the absence of inhibitor (v'/v).

Reactivation of inhibited enzyme in the presence of approx. $1\ \mu\text{M}$ -acetylcholine. Buffered enzyme at a nominal concentration 4×10^{-3} unit/ml. was incubated for not less than 60 min. at 37° with either $1\ \mu\text{M}$ -Sevin or $0.02\ \mu\text{M}$ -eserine, so that a steady state of inhibition could be assumed. Enzyme activity at $1\ \mu\text{M}$ -substrate was then assayed. At intervals of approx. 5–15 min. further additions of substrate were made so that activity of the inhibited or uninhibited enzyme could be followed for 1 hr. in the presence of substrate at a concentration of the order $1\ \mu\text{M}$. After each substrate addition straight lines were obtained by plotting logarithm of substrate concentration against time. Inhibition was estimated as the ratio (v'/v) of the slopes of the curves for the inhibited and control preparations. The results are given in Table 1. The exponential nature of the substrate-time curves enabled effective mean substrate concentrations to be evaluated by the expression

$$\frac{a}{t} \int_0^t e^{-Vt/K_m} dt$$

where t is the time in min., a the substrate concentration at $t = 0$, and the constants V and K_m have the

usual meanings (see below). These concentrations are also given in Table 1. The results show that there was little or no reversal of the inhibition by Sevin at a mean concentration $1.65\ \mu\text{M}$ -acetylcholine for 1 hr. There was similarly no detectable reversal of eserine inhibition at a mean concentration $2.22\ \mu\text{M}$ -acetylcholine.

Reactivation of inhibited enzyme in the presence of approx. $1\ \text{mM}$ -acetylcholine. Enzyme activities were determined at initial substrate concentration $1\ \text{mM}$ after the preincubation period of not less than 1 hr. The results are summarized in Table 2.

In these experiments when substrate concentration was plotted against time straight lines were not obtained. The curves for the controls were slightly convex to the time axis, and those for the inhibited preparations were slightly concave, owing to reactivation of the inhibited enzyme in the presence of substrate. The curvature was more noticeable when a large proportion of the substrate was removed as in Fig. 4. Therefore in estimating v'/v the control rate v was measured at the substrate concentration obtaining in the inhibited preparation at the time chosen for measuring v' .

Reactivation of inhibited enzyme on dilution. Enzyme at a concentration approx. 2×10^{-2} unit/ml. was preincubated at 37° in the presence of $5\ \mu\text{M}$ -Sevin (see Table 2) for 2 hr. The mixture was then diluted 20-fold and enzyme activity at various times after dilution was assayed at an initial substrate concentration $1\ \mu\text{M}$. Control mixtures containing no inhibitor were treated identically. Activities of the inhibited preparations were determined over intervals not exceeding a few minutes after addition of the substrate, since the log concentration-time curves did not become linear until about 1 hr. after dilution. This was the time required to establish the new equilibrium between enzyme and inhibitor on dilution. The results are given in Table 3.

Table 1. *Effects of maintaining low concentrations of acetylcholine on the inhibition of acetylcholinesterase by Sevin or by eserine at 37°*

The enzyme was incubated with inhibitor for not less than 1 hr. before first addition of substrate.

	Concn. of inhibitor (μM)	Acetylcholine addition	Period (min.)	Calc. mean substrate concn. in inhibited preps. at each stage (μM)	v'/v (observed)	v'/v (calc.)
Sevin	(1.0)	None	—	—	—	0.38
		1st	0-15	0.74	0.31	—
		2nd	15-30	1.26	0.29	—
		3rd	30-45	1.91	0.28	—
		4th	45-60	2.68	0.33	—
		—	0-60	1.65	—	0.39*
Eserine	(0.02)	None	—	—	—	0.15
		1st	0-15	0.81	0.15	—
		2nd	15-30	1.63	0.15	—
		3rd	30-45	2.62	0.13	—
		4th	45-60	3.83	0.17	—
		—	0-60	2.22	—	0.16*

* Steady-state value.

Table 2. *Reversal of inhibition by adding 1 mM-acetylcholine*

Temp.		Concn. of inhibitor (μM)	Concn. of enzyme (unit/ml.)	Time after adding substrate to inhibited prepn. (min.)	Actual substrate concn. (mM)	v'/v (observed)	v'/v^* (calc.)
25°	Sevin	1.0	4×10^{-3}	0	1	0.48	0.46
				60	0.86	0.80	0.78
				120	0.62	1.00	0.89
				∞	—	—	0.95
				0	1	0.39	0.25
	Eserine	0.02	4×10^{-3}	60	0.88	0.60	0.62
				120	0.76	1.00	0.77
				∞	—	—	0.87
	37	5.0	2×10^{-2}	0	1	0.19	0.11
				20	0.87	0.48	0.44
				40	0.65	0.71	0.58
				60	0.43	0.74	0.65
				∞	—	—	0.72
	Eserine	0.02	4×10^{-3}	0	1	0.18	0.15
				30	0.97	0.31	0.56
				60	0.90	0.86	0.70
				90	0.81	1.00	0.75
				∞	—	—	0.81

* In these calculations the substrate concentration has been assumed to remain constant at 1 mM.

Dilution experiments were not undertaken with eserine.

Inhibitor as substrate. It was calculated (see the Discussion section) that if the apparent equilibrium

between enzyme and inhibitor implied by the curves of Figs. 2 and 3 were due to a steady state of carbamylation and regeneration of free enzyme, then at high enzyme concentrations the inhibitor would

Fig. 4. Substrate-time curves showing reversal of Sevin-inhibition at 37° by the addition of 1mM-acetylcholine. Enzyme concentration, 2×10^{-2} unit/ml. O, Control; Δ , 1 μ M-Sevin.

Table 3. Reactivation of Sevin-inhibited enzyme on 20-fold dilution at 37°

The enzyme (0.02 unit/ml.) and inhibitor (5 μ M) were incubated for 2 hr. before dilution.

Time after dilution (min.)	v'/v (observed)	v'/v (calc.)
0	—	0.11
6	0.27	—
26	0.39	—
45	0.50	—
65	0.68	—
90	0.70	—
110	0.70	—
∞	—	0.71

disappear at a measurable rate. The ratio v'/v therefore would increase with time once carbamylation had reached an initial maximum. This was confirmed experimentally. Buffered enzyme (approx. 1 unit/ml.) was incubated at 37° in the presence of 0.02 μ M-eserine. After not less than 1 hr., when inhibition was assumed to have reached its maximum, enzyme activities of inhibited and control preparations were determined in 0.2 ml. samples by incubating them for 15–60 sec. with 14 C-labelled substrate at an initial concentration 1 mM. In a similar experiment with enzyme at a concentration 30 units/ml. and 1 μ M-Sevin enzyme activities were determined with 10 mM substrate.

Fig. 5. Inhibitors as substrates for acetylcholinesterase as shown by changes in inhibition (v'/v) at 37°. O, 0.02 μ M-Eserine + 1 enzyme unit/ml. Δ , 1 μ M-Sevin + 30 enzyme units/ml. Theoretical curves are shown as broken lines (see the Discussion section).

The results are shown in Fig. 5. Similar experiments with eserine and Sevin in the presence of the heat-inactivated enzyme indicated that, although there was detectable chemical decomposition of Sevin and possibly of eserine after 48 hr., in neither case did this contribute significantly to the observed changes in v'/v shown in Fig. 5.

DISCUSSION

Winteringham & Disney (1964a) suggested that when inhibition is due to a steady state of carbamylation and regeneration of free enzyme in the presence of inhibitor (concn. i) and substrate (concn. x) the velocity (v') of the enzyme-substrate reaction will be given by the equation:

$$v' = Vx/[K_m(1 + ik_1/k_2) + x + x^2/K_m] \quad (1)$$

The constants K_m and K_{ss} are respectively the Michaelis constant and the dissociation constant for the reversible reaction between substrate and normal enzyme-substrate complex to give an inactive complex. The parameter V is the maximal theoretical rate in presence of excess of substrate but in the absence of inhibitor and of inhibition by excess of substrate (see Winteringham & Disney, 1964a). The constants k_1 and k_2 respectively represent the bimolecular rate constant for the formation of carbamylated enzyme and the unimolecular rate constant for regeneration of free enzyme and carbamate decomposition products in the absence of normal substrate as follows:

Table 4. *Parameters of equations defining the behaviour of bovine erythrocyte acetylcholinesterase in the presence of acetylcholine and eserine or Sevin in sodium barbital buffer at pH 7.4*

See the Discussion section for details of column 1.

Parameter or constant	Units	Value	
		25°	37°
K_m	mole l. ⁻¹	5.63×10^{-5}	5.63×10^{-5}
K_{ss}	mole l. ⁻¹	1.12×10^{-2}	1.12×10^{-2}
V	mole l. ⁻¹ min. ⁻¹ enzyme units ⁻¹	4.59×10^{-5}	5.87×10^{-5}
x optimum	mole l. ⁻¹	7.94×10^{-4}	7.94×10^{-4}
k_1 for Sevin	l.mole ⁻¹ min. ⁻¹	2.06×10^4	4.67×10^4
k_2 for Sevin	min. ⁻¹	1.76×10^{-2}	2.87×10^{-2}
k_1 for eserine	l.mole ⁻¹ min. ⁻¹	2.03×10^6	7.95×10^6
k_2 for eserine	min. ⁻¹	1.35×10^{-2}	2.81×10^{-2}

In this equation $e - e_i$, e_i , $i - p$ and p respectively represent the concentrations of free enzyme, carbamylated enzyme, free inhibitor and inhibitor decomposition products where $e_i \ll i$. In deriving equation (1) both e and p of equation (2) are assumed to be very much less than i . In the absence of inhibitor ($i = 0$) it follows from equation (1) that:

$$v' = v = Vx/(K_m + x + x^2/K_{ss}) \quad (3)$$

hence,

$$v'/v = \frac{(K_m + x + x^2/K_{ss})}{[K_m(1 + i \cdot k_1/k_2) + x + x^2/K_{ss}]} \quad (4)$$

Evaluation of parameters. The parameters V , K_m and K_{ss} for the enzyme used in the present study were evaluated as described earlier (Winteringham & Disney, 1964a) and are given in Table 4. The observed $\log v$ - pS curve and the theoretical one based on the evaluated parameters of equation (3) are shown in Fig. 1.

When substrate effects can be ignored ($x \rightarrow 0$), it follows from equation (4) that:

$$v'/v = 1/(1 + ik_1/k_2) \quad (5)$$

This enables the ratio k_1/k_2 to be evaluated from the steady-state values of v'/v indicated by the data of Figs. 2 and 3. When e_i and $p \ll i$ it can be seen from equation (2) that:

$$de_i/dt = ik_1(e - e_i) - k_2e_i$$

Since $e_i = 0$ when $t = 0$, integration gives:

$$\ln \left[1 - e_i \left(\frac{ik_1 + k_2}{ik_1e} \right) \right] = -(ik_1 + k_2)t \quad (6)$$

In the absence of substrate effects $e_i = e(1 - v'/v)$ so that equation (6) may be written:

$$\ln [1 - (1 - v'/v)(1 + k_2/ik_1)] = -(ik_1 + k_2)t \quad (7)$$

Intermediate values of v'/v were obtained graphic-

ally from Figs. 2 and 3. The straight lines implied by equation (7) were indeed obtained by plotting the L.H.S. of the equation against t , so that k_1 and k_2 could be separately determined (Table 4). It should be noted that the unimolecular reaction of equation (2) is not ignored in these calculations.

Calculation of substrate and dilution effects. In the absence of substrate the ratio v'/v at an inhibitor concentration i is obtained by substituting the relevant constants of Table 4 in equation (5). This ratio indicates the steady-state inhibition achieved during the preincubation period of the reactivation and dilution experiments. Equation (4) gives the ratio in the presence of substrate. The values so calculated (Table 1) indicated that little reversal of inhibition by Sevin or eserine would be expected in the presence of 1.65–2.22 μM -acetylcholine. This was confirmed experimentally, since the times were sufficient for new steady states to be achieved.

Equation (4) indicates that at the higher substrate concentrations often used for acetylcholinesterase assay the steady-state value of v'/v would differ significantly from that achieved in the absence of substrate and would be a function of time after substrate addition. Under these conditions the proportion of substrate removed is usually small, so that the order of substrate concentration can be assumed to be constant. For the purpose of estimating v'/v as a function of time (t) under these conditions the following equation was deduced:

$$1 - v'/v = \frac{1 - \left[1 - \frac{\mu}{\theta(ik_1 + k_2)} \right] e^{-\mu}}{\mu/ik_1\theta} \quad (8)$$

where $\theta = (x/K_m + x^2/K_m \cdot K_{ss} + 1)^{-1}$

and $\mu = ik_1\theta + k_2$.

When $t = 0$ this equation reduces to the form of (5) and indicates v'/v before substrate addition. When $t = \infty$ this equation becomes equivalent to that of (4) and indicates the steady-state value of v'/v in the

presence of constant substrate. As $x \rightarrow \infty$, then $\theta \rightarrow 0$, $\mu \rightarrow k_2$, and the equation reduces to a form which describes the rate of reactivation in the absence of further carbamylation or as a result of sudden 'infinite' dilution (cf. Wilson *et al.* 1961). The values of v'/v calculated for a substrate concentration of 1 mM are compared in Table 2 with those observed. The pre-dilution and post-dilution steady-state values for v'/v in the Sevin dilution experiment can be calculated from equation (5), since v'/v was estimated experimentally at 1 μ M substrate. Therefore substrate effects could be neglected (see above), and dilution would affect only i in equation (5). Observed and calculated values are compared in Table 3.

The data of Tables 1, 2 and 3 indicate good agreement between the observed and calculated effects on v'/v of substrate addition, of dilution, and of time at 1 mM substrate.

Enzymic destruction of the inhibitor. Evidence that nearly constant inhibition is due to a steady state of enzyme carbamylation and regeneration of free enzyme derives mainly from the demonstration by Wilson *et al.* (1961) that carbamic esters which contained differently substituted tertiary or quaternary ammonium groups but the same carbamyl moieties had similar k_2 values, as now also demonstrated for the dissimilar *N*-methylcarbamates above (cf. Table 4). Conceivably, this could be due to some rate-limiting step in the release of the free enzyme which does not involve decomposition of the inhibitor itself. However, the calculations below show that, in the presence of sufficiently high concentrations of acetylcholinesterase, the inhibitors were not only progressively removed as shown in Fig. 5, but were removed at rates which quantitatively account for inhibition on the basis of steady-state carbamylation. If in equation (2) the rate of decomposition of inhibitor is slow compared with the rate of achieving a steady-state inhibition, and if i is much greater than e , it follows that:

$$dp/dt = k_2 e_i \quad (9)$$

Since $v'/v = (e - e_i)/e$ it follows from equation (5) that $e_i = e(i - p)/[(i - p) + k_2/k_1]$. Substituting e_i in equation (9), noting that $p = 0$ when $t = 0$, integration gives:

$$t = \frac{1}{e} \left[\frac{p}{k_2} + \frac{1}{k_1} \ln \left(\frac{i}{i - p} \right) \right] \quad (10)$$

This equation enables the concentration of undecomposed inhibitor, and therefore the corresponding values of v'/v (equation 5), to be calculated as a function of time. In this equation e will be the effective normality of the enzyme, since acetylcholinesterase is known to have a number of active sites per molecule. Normality was taken as the quotient: observed optimum acetylcholine hydro-

lysis rate at the enzyme concentration used/turnover number. The turnover number of 2.95×10^5 moles/min. reported by Cohen & Warringa (1953) was assumed for the present calculations. The curves so calculated are shown as broken lines in Fig. 5. Equation (10) assumes equilibration between undecomposed inhibitor and enzyme. To make some correction for this, periods of 30 and 60 min. respectively were allowed for the initial carbamylation by eserine and Sevin at 37° (cf. Figs. 2 and 3) and were not included in the time-scale of Fig. 5. The curve for eserine in Fig. 5 resembles that obtained by Ellis, Plachte & Straus (1943) with horse serum.

Estimation of acetylcholinesterase inhibition by insecticidal carbamates in vivo. For estimating inhibition *in vivo* as a result of insecticidal carbamate intoxication a sample of tissue is invariably diluted in buffer and the rate of hydrolysis of added acetylcholine or equivalent substrate is measured. This dilution and addition of substrate will tend to reverse the inhibition it is sought to measure, and should therefore be kept minimal as already stressed (Jensen-Holm, 1961; Winteringham & Disney, 1964a,b). Although substrate effects can be obviated by the use of very low substrate concentrations or by making relatively rapid measurements after adding the substrate (see above), it is difficult to avoid the effects of dilution. The present studies suggest the need to allow sufficient time after dilution, e.g. 2 hr., so that a new steady state is established. Inhibition will then be independent of time after dilution. In the absence of substrate effects it follows from equation (5) that if v'_0/v_0 represent actual inhibition *in vivo* and v'_z/v_z that observed after diluting the tissue z times

$$\frac{v'_0}{v_0} = \frac{1}{1 + z(v'_z/v_z - 1)} \quad (11)$$

The equation enables a simple but important correction to be made for the effects of dilution when estimating carbamate inhibition *in vivo*. It quantitatively represents the effects of diluting a mixture of an enzyme and reversible inhibitor under the 'zone A' conditions discussed by Straus & Goldstein (1943) in which enzyme concentration is very small compared with that of inhibitor.

Since this work was completed a paper by Reiner & Simeon-Rudolph (1966) has appeared indicating that carbamate inhibition was independent of the substrate concentration used for assay and of dilution of the inhibited enzyme. However, in their experiments no time was effectively allowed for steady-state equilibration with substrate or excess of inhibitor after substrate addition or dilution. Their findings in no way minimize the importance of taking into account substrate and dilution effects as explained above.

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