Inhibition of Housefly Acetylcholinesterase by Carbamates

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A kinetic study was undertaken of housefly cholinesterase inhibition by two methyl- and two dimethylcarbamates. The constant determining the relative concentration of the reversible enzyme-inhibitor complex ($\bar{K}_i$) and the rate constants for carbamylation and decarbamylation were determined. Contrary to the situation described in the literature for electric eel cholinesterase, the decarbamylation constant is larger for the methylcarbamyl than for the dimethylcarbamyl enzyme. The fly-head cholinesterase appears to be more sensitive to the carbamates studied than bovine erythrocyte cholinesterase. The importance for the in vivo action of the constants determining inhibition and reactivation is discussed.

Carbamate intoxication is generally believed to be due to inhibition of acetylcholinesterase (AChE). Wilson *et al.* (*13*, *14*) provided strong evidence that carbamates are, in fact, poor substrates for this enzyme. The inhibition of the hydrolysis of the natural substrate acetylcholine depends on a relatively high affinity of the carbamates combined with a low decarbamylation rate. The decarbamylation rate is, however, not to be neglected and has been suggested to be responsible for the recovery after carbamate intoxication (*5*). For an understanding of the in vivo intoxication and recovery, a knowledge of the kinetics of carbamate hydrolysis by the AChE of the insect studied is a first requirement. Carbamate hydrolysis follows a scheme identical to that for the normal substrate

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} E' \xrightarrow{k_3} E$$

E = free enzyme, I = free inhibitor, EI = reversible enzyme-inhibitor complex, E' = carbamylated enzyme.

Wilson *et al.* derived equations for the reaction obtained on preincubation of the enzyme with the inhibitor, assuming that the reversible enzyme-inhibitor complex (EI) will dissociate upon dilution before the assay. Under these conditions, the AChE activity assayed is due to the sum (e) of free enzyme (E) and reversible enzyme-inhibitor complex (EI) and the inhibition measured will depend only on the fraction of the carbamylated enzyme. Steady state inhibition, reached when the rate of carbamylation is equal to the rate of decarbamylation, follows the equation

$$\left(\frac{e}{E'}\right)_{ss} = \frac{k_3}{k_2}\left(1 + \frac{\bar{K}_i}{I}\right) \qquad (1)$$

where $(e/E')_{ss}$ is the ratio of active enzyme to carbamylated enzyme under steady state conditions and $\bar{K}_i = (k_{-1} + k_2)/k_1$. The time course of the inhibition is described by

$$-\ln\left[\frac{e}{E_0} - \frac{E'}{E_0}\left(\frac{e}{E'}\right)_{ss}\right]/t = k_2/\left(1 + \frac{\bar{K}_i}{I}\right) + k_3 = a \qquad (2)$$

where E_0 is the total enzyme concentration and t the duration of the preincubation period before addition of the substrate. Equation 2 presumes that $k_{-1} \gg k_2$. So $\bar{K}_i$ will be practically identical to $K_i = k_{-1}/k_1$, the classical competitive inhibitor constant. Wilson *et al.* (*14*) studied carbamate inhibition of the electric eel AChE where $\bar{K}_i$ values appeared to be much larger than the inhibitor concentrations applied. In this case, Equation 1 reduces to $(e/E')_{ss} = k_3\bar{K}_i/k_2I$, and the constants $\bar{K}_i$ and k_2 can, of course, not be separated. Instead of this, a bimolecular velocity constant $k_2/\bar{K}_i = k_2'$ has been determined by these and other authors (*7*, *12*, *13*, *14*, *16*).

In the present experiments with housefly AChE, the $\bar{K}_i/I$ ratios were not too high for the determination of the constants $\bar{K}_i$ and k_2. The values for $1/\bar{K}_i$ and k_3/k_2 were actually found from the intercept with the x-axis and the y-axis, respectively, in an $(e/E')_{ss}$ *vs.* $1/I$ plot, according to Equation 1. The rate constants for decarbamylation (k_3) have been calculated from

$$k_3 = a(e/E_0)_{ss} \qquad (3)$$

according to Equation 2. The rate of decarbamylation has also been directly determined from the time course of reactivation of the inhibited enzyme after extensive dilution, according to the first order equation

$$\ln (E'/E_0) = -k_3t \qquad (4)$$

The bimolecular velocity constant (k'_2) and decarbamylation rate constant (k_3) have been determined by Kunkee and Zweig (*7*) for the carbamate Sevin and the AChEs from housefly and bee heads. In the present paper, more observations are obtained with two methyl- and two dimethylcarbamates and fly-head AChE. An attempt is made to relate the kinetic constants to the in vivo action.

MATERIALS AND METHODS

A susceptible strain of houseflies, *S*, has been used, which has been reared under standard conditions for many years (*11*). Rates of inhibition and steady state inhibition were measured with a slightly purified AChE from fly heads. For competitive inhibition experiments, direct estimation of the reactivation rate, and AChE activity measurements in the in vivo experiments whole fly-head homogenates were used.

The partially purified enzyme was obtained as follows: Fly heads were homogenized in distilled water and centrifuged for 30 minutes at 170,000 × G. The precipitate was incubated with toluene for about one week at 2° C. with several replacements of the toluene. Thereafter the dry material was shaken for about one hour at 31° C. in pyrophosphate buffer of pH 8.0 (0.08*M*) containing 3%

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NaCl. The supernatant of this extract (30 minutes at 170,000 × G) was brought upon a Sephadex G 200 column and eluted with 3% NaCl-containing phosphate buffer of pH 7.5. The active fractions were combined and concentrated under reduced pressure. The purification factor was not more than about 20 times; however, any material and lipoids, which may act as solvents for the carbamates (*15*), are removed by the toluene treatment.

The carbamates used were phenyl *N*-methylcarbamate (PMC), 1-naphthyl *N*-methylcarbamate (NMC, Sevin), phenyl *N,N*-dimethylcarbamate (PDC), and 1-isopropyl-3-methylpyrazolyl-5-*N,N*-dimethylcarbamate (Isolan). The synergist used was 2-(3,4-methylenedioxyphenoxy)-3,6,9-trioxaundecane (sesamex).

In most AChE estimations, the hydrolysis of acetylthiocholine was measured (*3*). The reaction mixture contained 0.1*M* pyrophosphate buffer pH 7.5; 3% NaCl; $3.6 \times 10^{-4}M$ 5,5-dithiobis-2-nitrobenzoic acid; $10^{-3}M$ acetylthiocholine; and 0.017–0.03 fly head per ml. or a corresponding amount of purified enzyme. Some experiments were done by automatic titration of a solution containing 3% NaCl, $10^{-3}M$ acetylcholine bromide and 0.5 fly head per ml. The acetic acid produced was titrated with $5 \times 10^{-3}M$ NaOH. The change in optical density in the first method and the volume of NaOH solution added in the second method were recorded automatically.

EXPERIMENTAL

For the measurements of the rate of inhibition a concentrated AChE solution containing 3% NaCl, 0.1*M* pyrophosphate buffer, and the desired inhibitor concentration was incubated at pH 7.5 and 35° C. After suitable periods, aliquots were diluted 100-fold to avoid inhibition by the enzyme-inhibitor complex and immediately assayed (spectrophotometric method). The increase in inhibition was measured until the steady state was reached. The rate of substrate hydrolysis was calculated from the initial change in optical density so that decarbamylation after dilution could be neglected.

For the reactivation experiments, five fly heads stored at −16° C. were homogenized in an ice-cooled Potter-Elvehjem all-glass tissue grinder in 0.02 ml. buffer of pH 7.5 containing 0.1*M* pyrophosphate, 3% NaCl, and an inhibitor concentration producing about 70% inhibition. After half an hour at 35° C., the enzyme inhibitor mixture was diluted 10,000 fold and the increase of activity measured for 20 to 30 minutes in the presence of $10^{-3}M$ acetylthiocholine and conditions as described for the assay. The highest dilution possible under the author's experimental conditions was used, since even a 1000-fold dilution did not cause a maximal reactivation velocity in every case. A control without inhibitor showed a constant hydrolysis rate during the time of measuring. Reactivation experiments without the substrate showed that $10^{-3}M$ acetylthiocholine does not influence the reactivation rate.

In other experiments to determine k_3, the carbamylated enzyme was obtained by inhibition in vivo. Flies were decapitated when the AChE inhibition in vivo had passed its maximum. The heads were immediately homogenized, diluted about 30,000 fold, and the increase of AChE activity measured as described before.

Titrimetric experiments were done in an analogous manner for the determination of the inhibition and reactivation of the AChE in vivo. Because of the lower sensitivity of this method, the maximal possible dilution was only about 1000-fold.

The competitive inhibition was also measured with the titrimetric method by adding the inhibitor and the substrate simultaneously to the crude homogenate and measuring the activity immediately thereafter, so that carbamylation could be neglected.

RESULTS

In Vitro Experiments. Table I lists the values of k_2 and $\bar{K}_i$ derived from Equation 1 (Figure 1) and the values of k_3 calculated from Equation 3 (Figure 2) and Equation 4 (Figure 3). The decarbamylation constants (k_3) calculated with Equation 3 and those obtained by direct measurement Equation 4 agree well. The data are based on at least three determinations each, and the standard deviation of the values for k_3 and the steady state inhibition does not exceed 10.8%. The results of the experiments, in which the inhibitor was added simultaneously with the substrate, are shown in Figure 4. The value of K_i for Isolan calculated from these results is $2.85 \times 10^{-7}M$.

In Vivo Experiments. Of the carbamates used, only Isolan and PMC have a reasonable effect on houseflies after topical application. Sevin and PDC are only effective in combination with a synergist. Isolan and PMC were applied to female flies in dosages which cause a temporary knock down (KD) of all treated flies. After different periods, random samples of five heads were taken, homogenized, and the AChE activities determined immediately. In Figure 5, the resulting curves are plotted together with the time course of KD in the flies showing a good correlation between the increase of the AChE inhibition and KD in both cases. Reactivation of the AChE and recovery of the flies takes longer after application of PMC than after application of Isolan.

The synergist sesamex influences the effects of the two carbamates to a different degree. When 10 μg. per ♀ sesamex is applied 60 minutes before the carbamate the LD_{50} of Isolan is lowered from 1 μg. per ♀ to 0.02 μg. per ♀ and the LD_{50} of PMC from 5 μg. per ♀ to 0.25 μg. per ♀.

DISCUSSION

In Vitro Experiments. The partially purified enzyme preparation was used, because some of the constants could not be determined satisfactorily with a crude fly-head homogenate. A comparison of the two enzyme preparations showed that with the same inhibitor concentration, the inhibition was somewhat slower and the steady state inhibition lower for the enzyme in a crude homogenate. In the $(e/E')_{ss}$ vs. $1/I$ plot, the point of intersection with the y-axis (k_3/k_2) was the same, but the slope of the line was steeper with the crude homogenate, so that the resulting values of $\bar{K}_i$ were too high. This is probably caused by the fact that, in a similar manner as described for human serum (*15*), the carbamates are partly dissolved in the lipoidal fraction of the rather concentrated homogenate, so that the effective concentration is lowered. In accordance with this explanation, the values of k_3 obtained from

Table I. Kinetic Data for Inhibition and Recovery of Fly-Head AChE with Methyl- and Dimethylcarbamates

	PDC	Isolan	PMC	NMC
k_3(min.$^{-1}$)[a] from Equation 4	0.028		0.048	
k_3(min.$^{-1}$)[b] from Equation 3	0.027		0.048	
k_2(min.$^{-1}$)[b] from Equation 1	0.50	0.60	0.28	0.80
$\bar{K}_i(M)$[b] from Equation 1	3.3×10^{-6}	2.5×10^{-7}	6.7×10^{-6}	5.0×10^{-7}
$k_2/\bar{K}_i = k_2'$[b] (M^{-1} min.$^{-1}$)	1.5×10^{5}	2.4×10^{6}	4.2×10^{4}	1.6×10^{6}
$(I_{50})_{ss}(M)$[b]	1.9×10^{-7}	1.2×10^{-8}	1.4×10^{-6}	3.2×10^{-8}

[a] Whole fly-head homogenate.
[b] Partially purified fly-head AChE.

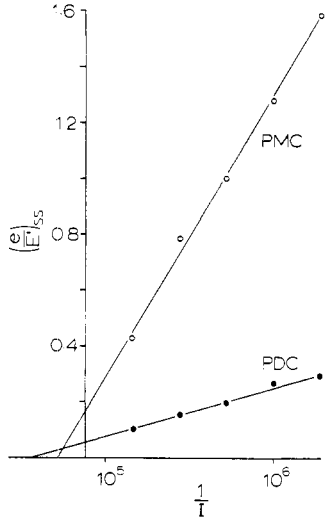

Figure 1. The relation between steady state inhibition of a purified enzyme preparation and inhibitor concentration

$(e/E')_{ss} = k_3/k_2 + (k_3\bar{K}_i/k_2) \times 1/I$, Equation 1

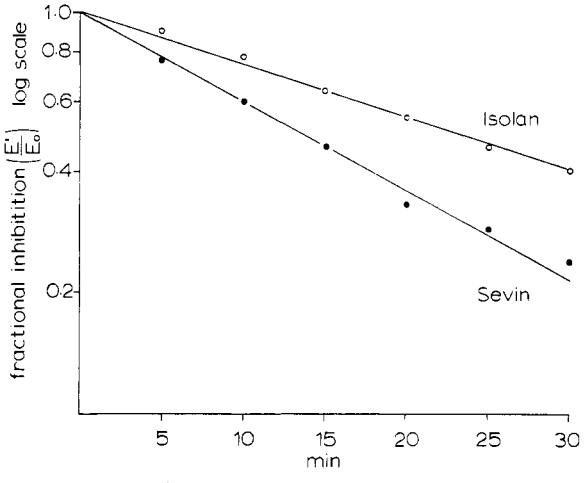

Figure 3. Rate of decarbamylation after extensive dilution of Isolan and NMC-inhibited AChE (crude fly-head homogenate)

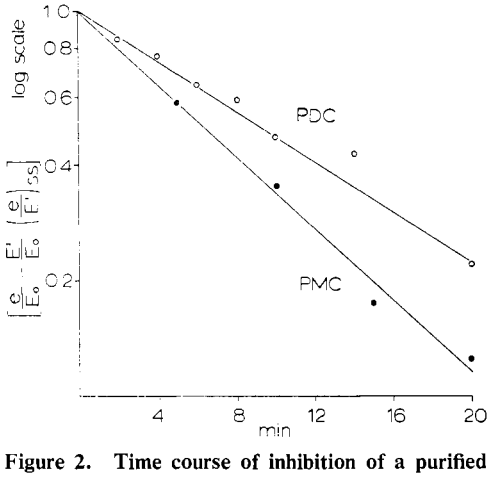

Figure 2. Time course of inhibition of a purified enzyme preparation by PDC $10^{-6}M$ and PMC $4 \times 10^{-6}M$, Equation 2

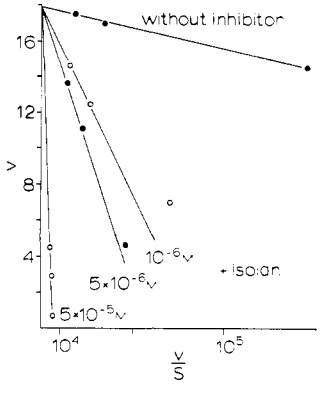

Figure 4. Influence of substrate concentration on the rate of enzymic hydrolysis under conditions of competitive inhibition according to the linear equation $v = V_m - K_m(1 + I/K_i) \cdot v/s$ (2)

Equation 3, in which the inhibitor concentration is not involved, were similar with both enzyme preparations.

In spite of the different origin of the AChEs and differences in the experimental conditions, it is worthwhile to compare the present results with those of previous authors. Kunkee and Zweig (7) obtained very similar results with housefly head AChE and Sevin at 30° C. and pH 8.0. They did not determine $\bar{K}_i$ and k_2 because they applied doses of the inhibitor, which were probably well below $\bar{K}_i$. Their bimolecular velocity constant (k'_2) is of the same order as our value for $k_2/\bar{K}_i$. Their value for k_3 is somewhat lower, probably due to the lower tempera-

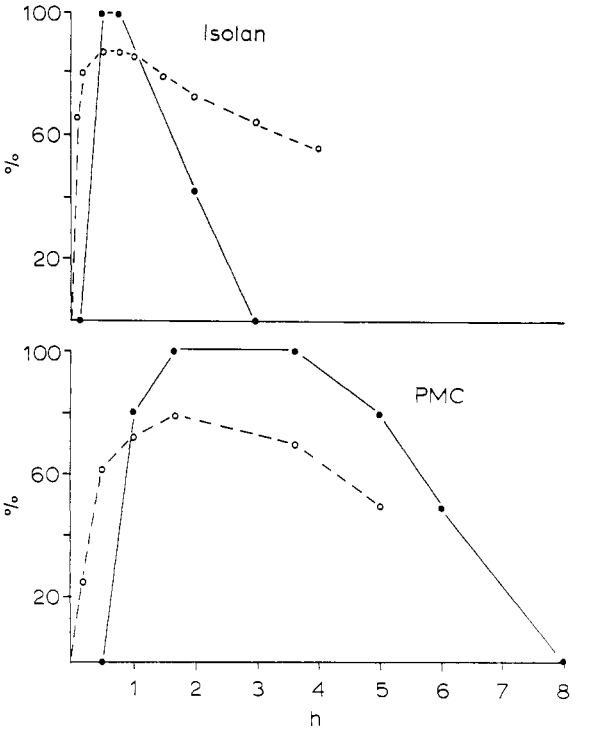

Figure 5. Percentage of flies knocked down (solid line) and of the in vivo inhibition of the fly-head AChE (broken line) after application of Isolan and PMC, respectively

ture applied. Other authors also determined k_2 at pH 7.4 for the inhibition of bovine erythrocyte AChE by Sevin at 25° C. (*12*, *16*) and at 37° C. (*16*) and by PMC at 25° C. (*12*). Their value of k'_2 were considerably lower than those for housefly AChE, indicating a greater sensitivity of the latter enzyme. O'Brien *et al.* (*10*) determined the affinity constants and the carbamylation constants for Isolan, PDC, Sevin, PMC, and some other methyl- and dimethylcarbamates with bovine-erythrocyte AChE at 38° C. and pH 7.0. Compared with their results, the housefly AChE has a much higher affinity to these carbamates and in most cases a somewhat lower carbamylation rate, but the bimolecular velocity constant of the inhibition (k'_2) is also in this case much lower for the erythrocyte AChE.

As the affinity of the carbamates to the housefly AChE appears to be rather high compared with that to the erythrocyte AChE determined by other authors (*8*, *10*), we also determined the dissociation constant for competitive inhibition (K_i) for Isolan and got a very similar value as for $\bar{K}_i$. This is an independent indication that the values for $\bar{K}_i$ calculated from the steady state experiments are in the right order. The fact that the values of k_i and $\bar{K}_i$ do not differ significantly is in accordance with the presumption of Equation 2 that $k_{-1} \gg k_2$.

Another interesting result was that the reactivation constant for methylcarbamyl-AChE was higher than for dimethylcarbamyl-AChE of the housefly. The only authors who also compared these two types of carbamylated enzyme were Wilson *et al.* (*13*), who found the reverse for the AChE of the electric eel. Therefore, decarbamylation in these two cholinesterases appears to differ in a qualitative way.

In Vivo Experiments. Recovery of insects after intoxication by carbamates has often been described (*1*, *4*, *9*). By 1947, the reversible nature of the inhibition had been suggested to be the cause of the recovery of roaches after carbamate intoxication (*1*). When the inhibition of the AChE by carbamates was found to follow the same scheme as the inhibition by phosphorus esters, the spontaneous decarbamylation was suggested to be responsible for the recovery after carbamate intoxication (*5*). A reactivation of the carbamylated AChE in vivo is only possible if the concentration of the carbamate is lowered, for instance by enzymatic degradation. Since the concentration of the carbamates is reduced only gradually by such detoxification mechanisms, the relaxation velocity is lower than found in vitro on extensive dilution.

Kolbezen *et al.* (*6*) found good correlation with several substituted phenylcarbamates between the anticholinesteratic potency and the insecticidal action. Generally, a close relation between AChE inhibition in vitro and the action in vivo is not to be expected, since there are many other factors affecting the latter such as ability to penetrate and stability. The results with the synergist sesamex show a considerable influence of the detoxication mechanisms on the action of the two carbamates. The stronger synergistic effect of sesamex on the action of Isolan indicates a faster detoxification of Isolan than of PMC. Therefore, Isolan is only about 5 times as effective as PMC with respect to the LD_{50} in spite of its much higher bimolecular velocity constant of inhibition (k'_2).

Though the decarbamylation rate of the methylcarbamyl AChE in vitro is about 1.75 times as high as that of the dimethylcarbamyl AChE, no clear difference is visible in the reactivation rates in vivo. The reason for this may be that the carbamate concentration is lowered more slowly than after Isolan intoxication owing to the larger amount of PMC taken up and the lower detoxification rate.

In the in vivo experiments, inhibition of the fly-head AChE by Isolan and its reactivation is well correlated with the symptoms. At about 60% of AChE inhibition,

the KD of the flies begins and when the enzyme is reactivated to about the same degree of inhibition all flies are recovered again. For the intoxication with PMC, the symptoms are correlated in the same way with the increase in the AChE inhibition only, while the recovery of the flies compared with the reactivation of the enzyme takes longer. This may be due also to the lower detoxification rate and the higher concentration of PMC. As a result, the AChE will remain inhibited for a longer period, which may cause secondary damages resulting in retardation of recovery.

The fact that carbamate inhibition and reactivation in vivo depends on so many different factors—i.e., affinity, carbamylation, decarbamylation, penetration, detoxification, etc., which vary widely between different compounds and different species—accounts for the rather selective action, which characterizes this group of insecticides.

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