Biochemical Pharmacology, Vol. 26, pp. 757–761. Pergamon Press, 1977. Printed in Great Britain.

THE INTERACTION OF CARBAMATES WITH ACETYLCHOLINESTERASE

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(*Received* 8 *September*; *accepted* 28 *October*)

Abstract—It has been shown that the kinetic schemes proposed by earlier workers to describe the reactions of carbamates with acetylcholinesterase are incomplete. A reaction scheme has been developed and tested by the use of methyl- and dimethyl-carbamoylcholines; it adequately explains the reactions observed in the carbamate–enzyme system, including the catalysis of decarbamoylation of carbamoylated acetylcholinesterase by excess carbamate. This study has been extended to include the reaction of acetylcholinesterase with more complex carbamates, pyridostigmine, physostigmine, mobam and decarbofuran.

The inhibition of acetylcholinesterase by carbamates has been studied by many workers [1–3] and schemes to represent the inhibition have been proposed [3–9]. However, the observation [7] that the spontaneous reactivation of carbamoylated acetylcholinesterase is accelerated by free carbamate showed that these schemes did not fully account for all the facts. A reinvestigation of the carbamate–acetylcholinesterase reaction was therefore necessary.

It was decided initially to investigate the reaction of acetylcholinesterase with the structurally simple mono- and dimethyl-carbamoylcholines and then to extend the study to include the more complex carbamates, pyridostigmine (I), physostigmine (II), mobam (III) and decarbofuran (IV).

MATERIALS AND METHODS

The enzyme used was purified bovine erythrocyte acetylcholinesterase, E.C.3.1.1.7. (ex Sigma Chemical Company). Mono- and dimethyl-carbamoylcholine iodides and pyridostigmine bromide were prepared in our laboratories by published procedures [3a, b], and the identities of the compounds were confirmed by melting point and spectroscopic methods. Physostigmine sulphate, mobam, and decarbofuran were obtained from British Drug Houses (U.K.), Mobil Oil (U.S.A.) and Bayer (F.D.R.) respectively.

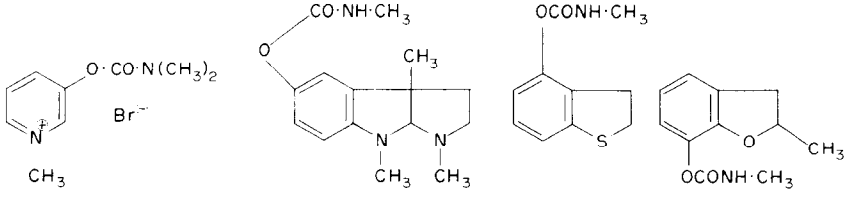
Apparatus. Potentiometric titrations were carried out using a Radiometer TTTlc Autotitrator and SBR2c Titrigraph fitted with an SBU titration assembly using a G2222B glass electrode and a K4112 reference electrode (all ex V. A. Howe Limited).

Carbamoylation. The carbamate was dissolved in water to give a 10^{-2} M stock solution (it was shown that there was no measurable hydrolysis of the compounds in this aqueous solution). 0.5 ml of enzyme solution (containing ca. 2 μmolar units of acetylcholinesterase in pH 7.4, 5×10^{-3} M phosphate buffer, made up to a total ionic strength of 0.1 M with sodium chloride) was pipetted into each of ten tubes contained in a water bath at 37°. An appropriate vol of carbamate solution was pipetted into tube 1 and after shaking a 0.1 ml sample was immediately removed and added to 10 ml of 0.1 M sodium chloride solution containing acetylcholine iodide at 5.0×10^{-4} M in a Radiometer pH-stat thermostatted cell. The solution was assayed at pH 7.4 and 37° using a twin syringe pH-stat technique so that the substrate concentration was maintained constant. The second syringe containing acetylcholine iodide (10^{-2} M) was driven synchronously with the syringe containing sodium hydroxide (10^{-2} M).

An identical amount of carbamate was added to tube 2 and a sample was removed after an appropriate time interval for assay. This procedure was repeated at varying time intervals until a constant value of enzyme activity was reached. Reactions were then carried out using different concentrations of carbamate.

At the maximum concentration of carbamate in the assay solution, further inhibition during the assay period was minimal.

Decarbamoylation. 1 ml of enzyme solution (prepared as above) was incubated at 37° for about 1 hr with the required concentration of carbamate to



(I) (II) (III) (IV)

obtain maximum inhibition of the enzyme. Of this solution, 0.5 ml was then diluted to 100 ml with pH 7.4, 5×10^{-4} M phosphate buffer (of total ionic strength 0.1 M with sodium chloride). Aliquots (10 ml) were assayed at appropriate time intervals using the method described above (with the acetylcholine iodide concentration again 5.0×10^{-4} M).

The linear rates of acid production observed indicated that negligible decarbamoylation of the inhibited enzyme occurred during the assay. The concentration of carbamate in the diluted solution was so low that no significant carbamoylation of the free enzyme would occur. The decarbamoylation rate coefficient, k_3, obtained was thus unambiguous in that it was measured in the absence of carbamate. The rate coefficient was evaluated by plotting $\ln(E_\infty - E_t)$ against time, where:

E_∞ = final measured enzyme activity
E_t = enzyme activity measured at time, t

The final enzyme activity, E_∞, was found to be in close agreement with the value obtained from a blank experiment carried out with no carbamate i.e. E_0.

RESULTS AND KINETIC ANALYSIS

It has been suggested that acetylcholinesterase (E) reacts with organophosphates and carbamates (CX) in an analogous manner to its reaction with substrates. Thus, the reaction is supposed to proceed via a complex (ECX) to give a carbamoylated enzyme (EC) which can be hydrolysed to regenerate the enzyme [3, 5, 6].

$$E + CX \overset{K_1}{\rightleftharpoons} ECX \xrightarrow{k_2} EC \xrightarrow{k_3} E + \text{product} \tag{1}$$

where

$$K_1 = \frac{[ECX]}{[E][CX]}$$

k_2 = rate coefficient for breakdown of complex to form carbamoylated enzyme

k_3 = rate coefficient for decarbamoylation of carbamoylated enzyme

The reaction reaches a steady state, where the rate of carbamoylation is equal to the rate of decarbamoylation, the extent of carbamoylation of the acetylcholinesterase depending upon the concentration of carbamate. It has been observed, however, that the carbamate itself can increase the rate of decarbamoylation of the carbamoylated acetylcholinesterase [7].

The present inhibition data were analysed using the kinetic scheme:

$$E + CX \xrightarrow{k_c} EC \xrightarrow{k_d} E \tag{2}$$

which can be considered as two opposing first order reactions provided the carbamate is in considerable molar excess, as is usually the case. The differential equation for Equation 2 is

$$\frac{d[E]}{dt} = k_d[EC] - k_c[E] \tag{3}$$

or

$$\frac{d[E]}{dt} = k_d[E_0 - E] - k_c[E] \tag{4}$$

where

$$E_0 = E + EC$$

can be solved to give:

$$k_{obs} = k_c + k_d = \frac{1}{t} \ln \frac{E_0 - E_e}{E_t - E_e} \tag{5}$$

and

$$\frac{k_c}{k_d} = \frac{E_0 - E_e}{E_e} \tag{6}$$

E_0 = original enzyme activity
E_e = enzyme activity at equilibrium
E_t = enzyme activity at time, t
k_c = rate coefficient for carbamoylation
k_d = rate coefficient for decarbamoylation

From the reaction scheme given in Equation 1 it can be seen that

$$k_c = \frac{k_2 K_1 [CX]}{1 + K_1 [CX]}. \tag{7}$$

The data in Table 1 and Fig. 1 show that the apparent second order rate constant $k_c/[CX]$ ($\equiv k_i$) for both monomethyl and dimethyl carbamoyl cholines are approximately constant over the concentration range used. Thus it is not possible to distinguish between a bimolecular carbamoylation step and a unimolecular carbamoylation step preceded by an association step with only a low affinity constant for the complex, i.e. K_1 is so small that Equation 7 reduces to:

$$k_c = k_2 \cdot K_1 \cdot [CX] \tag{8}$$

Since the results listed in Table 1 show that the values of k_d, the rate constant for decarbamoylation of the carbamoylated acetylcholinesterase, increase with increasing carbamate concentration, the simple scheme of Equations 1 and 2 must be modified to account for this acceleration process. The variation is shown in Fig. 2.

The reaction scheme and kinetic analysis consequently proposed to account for these deviations involves a direct interaction between the carbamate and the carbamoylated enzyme to produce a complex, which breaks down to regenerate enzyme as shown in the following Scheme.

Scheme

$$\begin{array}{ccccc} E + CX & \xrightarrow{k_1} & EC + CX & \xrightarrow{k_3} & E \\ & & \Vert K_4 & & \\ & & ECCX & \xrightarrow{k_5} & E \end{array}$$

This scheme can be analysed kinetically as follows:

$$K_4 = \frac{[ECCX]}{[EC][CX]};$$

$$\frac{d[E]}{dt} = k_3[EC] + k_5[ECCX] - k_i[E][CX]$$

$$= [EC](k_3 + k_5 \cdot K_4[CX]) - k_i[E][CX] \tag{9}$$

Table 1. Rate coefficients for carbamoylation of acetylcholinesterase and the decarbamoylation of carbamoylated acetylcholinesterase

	Dimethyl-carbamoylcholine			Monomethyl-carbamoylcholine		
10^4[CX]	$10^3 k_c$ (s^{-1})	$\frac{k_c}{[CX]}$ ($M^{-1}s^{-1}$)	$10^4 k_d$ (s^{-1})	$10^3 k_c$ (s^{-1})	$\frac{k_c}{[CX]}$ ($M^{-1}s^{-1}$)	$10^4 k_d$ (s^{-1})
—	—	—	3.93*	—	—	8.07*
1.0	0.77	7.7	4.72	—	—	—
2.0	1.05	5.3	5.18	4.40	22.0	13.1
2.5	1.37	5.5	5.32	5.33	21.3	15.0
3.5	1.93	5.5	5.46	—	—	—
4.0	—	—	—	8.68	21.7	19.1
5.0	2.82	5.6	7.02	10.9	21.8	20.6
6.0	3.13	5.2	7.34	—	—	—
7.5	4.61	6.2	8.80	18.1	24.2	24.6
8.5	5.07	5.9	8.28	—	—	—
10.0	6.23	6.2	9.32	23.4	23.4	26.0
15.0	10.0	6.7	11.1	37.4	24.9	28.0

* Measured by decarbamoylation method—see Experimental Section.

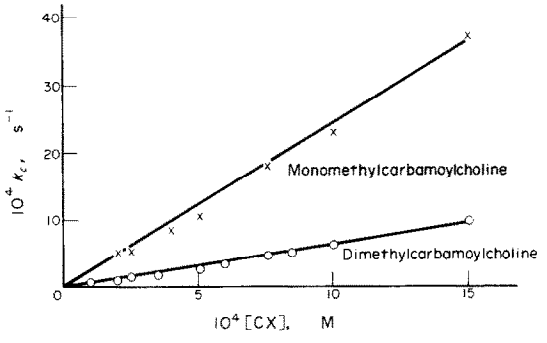


Fig. 1. Variation of the calculated rate coefficients for carbamoylation of acetylcholinesterase with carbamate concentration.

since

$$[E]_0 = [E] + [EC] + [ECCX]$$

$$[E]_0 = [E] + [EC](1 + K_4 \cdot [CX]) \quad (10)$$

Combining Equations 10 and 9 gives:

$$\frac{d[E]}{dt} = \frac{k_3 + k_5 \cdot K_4 \cdot [CX]}{1 + K_4[CX]}([E]_0 - [E]) - k_i[E][CX] \quad (11)$$

Equating coefficients in Equations 4 and 11, it can be seen that:

$$k_d = \frac{k_3 + k_5 \cdot K_4[CX]}{1 + K_4 \cdot [CX]} \quad (12)$$

If K_4 is small, as is the case with K_1, then Equation 12 reduces to

$$k_d = k_3 + k_5 K_4[CX] \quad (13)$$

If the curvature of the $k_d - [CX]$ plot is not very great, then Equation 13 is adequate and it is not possible to separate k_5 and K_4.

However, the slope of the $k_d - [CX]$ plots decreases as the carbamate concentration is increased showing that there is significant association i.e. K_4 is not negligible. It should therefore be possible to calculate k_5 and K_4.

Values of k_5 and K_4 were calculated by obtaining a least mean squares fit of Equation 12 to the experimental values of k_d in Table 3 using an iterative method [10].

Values of k_d, which are calculated by combination of Equations 5 and 6, viz.

$$k_d = \frac{E_c}{E_0} \cdot k_{obs} \quad (14)$$

are reproducible to ca. $\pm 10\%$. The errors on k_d are proportional to k_d and weights were taken as proportional to $1/k_d^2$. The values of k_3 shown in Table 2 were assumed to be without error so that the estimate of error on k_5 and K_4 is reduced.

Table 2 shows the calculated values of k_5 and K_4 together with standard errors. Best fit calculated values of k_d are shown in Table 3 together with the observed k_d values.

It should be noted that since the errors are large and the values of k_5 and K_4 heavily negatively corre-

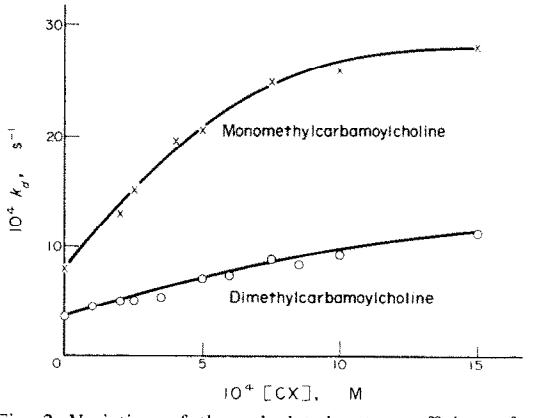


Fig. 2. Variation of the calculated rate coefficients for decarbamoylation of carbamoylated acetylcholinesterase with carbamate concentration.

Table 2. Rate and equilibrium constants for the reactions of carbamates with acetylcholinesterase using the scheme illustrated

Carbamate	k_i ($M^{-1}s^{-1}$)	k_3 (s^{-1})	K_4 (M^{-1})	$10^3 k_5$ (s^{-1})	k_5K_4 ($M^{-1}s^{-1}$)
Physostigmine	5.5×10^4	8.1×10^{-4}	$3.6 \pm 1.0 \times 10^5$ (3.5×10^5)	11.2 ± 2.7 (11.6)	408 ± 18 (405)
Mobam	3.2×10^3	8.1×10^{-4}	$1.25 \pm 0.53 \times 10^4$ (2.0×10^4)	22.0 ± 8.3 (14.5)	275 ± 11 (290)
Decarbofuran	3.1×10^3	8.1×10^{-4}	$4.5 \pm 1.7 \times 10^4$ (6.0×10^4)	6.6 ± 1.8 (5.3)	298 ± 31 (318)
Pyridostigmine	3.3×10^3	4.0×10^{-4}	$5.0 \pm 1.6 \times 10^4$ (7.6×10^4)	6.7 ± 1.7 (5.3)	331 ± 29 (403)
Dimethylcarbamoylcholine	6.5	3.9×10^{-4}	$1.9 \pm 1.2 \times 10^2$ (3.1×10^2)	$3.8 \pm (1.9)$ (2.6)	0.70 ± 0.11 (0.81)
Monomethylcarbamoylcholine	23.0	8.1×10^{-4}	$1.04 \pm 0.23 \times 10^3$ (1.0×10^3)	4.3 ± 0.5 (4.5)	4.45 ± 0.45 (4.50)

lated it is unlikely that the errors are normally distributed.

Equation 12 can be rearranged to yield

$$\frac{k_d - k_3}{[CX]} = k_5K_4 - k_dK_4 \qquad (15)$$

and thus it is also possible to evaluate k_5 and K_4 graphically. Values obtained from the graphical method are included in Table 2 in parentheses. It can be seen that there are some quite large variations over the data obtained by the statistical method. The graphical method, although much more convenient to use, suffers the normal inconvenience of a Hofstee plot, i.e. lack of surveyability since both y and x contain k_d, and also that when $k_d - k_3$ is small, considerable errors will be observed in the function

$$\frac{k_d - k_3}{[CX]}$$

The scheme derived is a general one, but in the limiting case of very low carbamate concentrations, it can be seen from Table 1 that the rate coefficient for decarbamoylation, calculated from Equation 2 approaches the value for the rate coefficient for decarbamoylation measured in the absence of carbamate. Thus the kinetic schemes of earlier workers can be justified in this limiting case.

DISCUSSION

It can be seen that excellent agreement is obtained between the rate constants k_3 for decarbamoylation of the monomethylcarbamoylated acetylcholinesterases produced from the various carbamates studied, thus indicating that k_3 is a spontaneous decarbamoylation rate constant. A similar agreement is found for the rate constant for decarbamoylation of dimethylcarbamoylated acetylcholinesterase produced from the two dimethylcarbamoyl compounds studied. No such agreement is found amongst the values of k_5, which also involves a decarbamoylation reaction. Furthermore, it might be expected that the same order of reactivity for decarbamoylation of monomethylcarbamoylated acetylcholinesterase relative to dimethyl-carbamoylated acetylcholinesterase would be observed as for k_3. However, in the case of k_5, decarbamoylation is occurring in the presence of the carbamate itself and therefore the nature of the ester group of the intact carbamate may play an important part in the decarbamoylation reaction. For the

Table 3. Comparison of observed and calculated rate coefficients for the decarbamoylation of carbamoylated acetylcholinesterase

Physostigmine	10^7 [CX]	0.5	0.6	0.7	0.8	1.0	2.0	3.0	4.0		
	$10^4 k_d$ obs.	10.2	10.2	10.6	11.1	11.7	15.2	18.4	21.4		
	$10^4 k_d$ calc.	10.0	10.3	10.7	11.0	11.8	15.2	18.3	21.3		
Mobam	10^6 [CX]	1.0	1.5	2.0	2.5	3.5	5.0	7.5			
	$10^4 k_d$ obs.	10.5	12.1	13.5	14.6	16.9	20.4	26.4			
	$10^4 k_d$ calc.	10.7	12.0	13.3	14.5	17.0	20.6	26.3			
Decarbofuran	10^6 [CX]	1.0	1.5	2.0	2.5	3.5	5.0	6.0	7.5		
	$10^4 k_d$ obs.	9.9	11.9	13.0	14.3	16.2	19.1	20.0	22.6		
	$10^4 k_d$ calc.	10.6	11.8	12.9	14.0	16.0	18.8	20.4	22.7		
Pyridostigmine	10^6 [CX]	0.1	0.5	0.75	1.0	1.5	2.0	3.5	5.0	7.5	10.0
	$10^4 k_d$ obs.	4.10	5.10	5.68	7.21	8.94	10.4	13.2	16.2	21.6	24.2
	$10^4 k_d$ calc.	4.31	5.52	6.25	6.96	8.34	9.67	13.3	16.5	21.0	24.8
Dimethylcarbamoylcholine	10^4 [CX]	1.0	2.0	2.5	3.5	5.0	6.0	7.5	8.5	10.0	15.0
	$10^4 k_d$ obs.	4.72	5.18	5.32	5.46	7.02	7.34	8.80	8.28	9.32	11.1
	$10^4 k_d$ calc.	4.52	5.12	5.41	5.97	6.79	7.31	8.05	8.53	9.22	11.3
Monomethylcarbamoylcholine	10^4 [CX]	2.0	2.5	4.0	5.0	7.5	10.0	15.0			
	$10^4 k_d$ obs.	13.1	15.0	19.1	20.6	24.6	26.0	28.0			
	$10^4 k_d$ calc.	14.1	15.3	18.3	20.0	23.3	25.8	29.3			

dimethyl and monomethylcarbamoylated enzymes where the ester group is the same, i.e. choline, the error on k_5 for dimethyl-carbamoylcholine is too large to give a meaningful comparison of the k_5 ratio with the corresponding ratio of k_3 values for the simple decarbamoylation. To substantiate this explanation, it would be necessary to examine the dimethylcarbamoyl analogues of physostigmine, mobam and decarbofuran, and the monomethylcarbamoyl derivative of pyridostigmine.

The larger k_5 value relative to k_3 is probably a consequence of a conformational change of the carbamoylated enzyme or a change in the micro environment of the carbamoylated site. A study involving quaternary ammonium compounds is being undertaken in order to investigate this difference.

Acknowledgements We wish to thank Dr. F. Berends, Medisch Biologisch Laboratorium, TNO, Holland, for valuable comments on this work, and Mr. S. Callaway, CDE, Porton Down, for help with the statistical analysis.

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