The Reaction of Carbamates with Cholinesterase

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SUMMARY

It has been shown that both complex formation and carbamylation are of major importance in the reaction between carbamates and cholinesterase. That carbamylation occurs has been shown by demonstration of leaving-group release as the reaction proceeds. Complex formation has been demonstrated by kinetic evidence. Affinity and carbamylation constants have been measured on 11 dimethylcarbamates and 10 methylcarbamates, and correlations with alkaline hydrolyzability have been sought.

INTRODUCTION

Early studies suggested that carbamates inhibited cholinesterase reversibly, because inhibition can be reversed by washing (1), dialysis (2), dilution (3), or addition of substrate (3). In 1951 Goldstein suggested (4) that carbamates are substrates of cholinesterase with exceptionally low turnover rates. Strong support came when Wilson *et al.* (5, 6) showed that the rate at which the inhibited enzyme recovered activity, either spontaneously or under the accelerating influence of hydroxylamine, depended only upon the *N*-substituent of the carbamate. This suggested (but did not prove) that the *O*-substituent was removed in the course of enzyme inhibition. However, it is not universally agreed that inhibition occurs by carbamylation, primarily because steric and not electronic factors are of predominant importance in determining potency of carbamates and because electron-withdrawing substituents have been reported to worsen potency somewhat (2, 7) in reverse of expectation for a simple carbamylation mechanism; consequently it was concluded in 1965 that "the primary mode of inhibition is competitive in view of the relatively slow rates of carbamylation compared to the fast rate of complex formation" (8).

Kitz and Wilson (9) and Main (10) have introduced kinetic procedures that demonstrated the importance of prior complex formation in the analogous reaction of organophosphates and sulfonates with cholinesterase. One part of the present paper involves an extension of Main's treatment to carbamates, and calculations of the rate and equilibrium constants involved. In addition, product analysis in two cases confirms that carbamylation, with concurrent leaving-group release, occurs.

It will be shown that carbamates react with cholinesterase in accordance with the usual enzyme-substrate scheme. If E is cholinesterase, CX the carbamate (with X the *O*-substituent), ECX is reversible complex and EC is carbamylated enzyme:

$$E + CX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ECX \xrightarrow{k_2} EC \xrightarrow{k_3} E \qquad (1)$$

with release of leaving group (X) in the k_2 step, and of carbamyl group in the k_3 step. This formulation is the same as that of Wilson *et al.* (6) but under their conditions the role of complex formation was not the point at issue, and could be neglected. The ratio k_{-1}/k_1 is defined as K_a, the affinity constant.

Portions of this study have been reported in a preliminary communication (11).

METHODS

Kinetic Analysis

The preferred method for measuring cholinesterase activity was that of Ellman (12) which utilizes the yellow-colored thionitrobenzoate ion produced when DTNB [5,5′-dithio*bis*-(2-nitrobenzoic acid)] reacts with thiocholine released by acetylthiocholine hydrolysis. However, in a number of cases the procedure was inapplicable, because at high concentrations certain carbamates appeared to react with the thionitrobenzoate ion. The method could be used for such carbamates only when the carbamates were such good anticholinesterases that they could be used at low concentrations. Because most of the dimethylcarbamates were poor inhibitors, the method was not used for them, and the titrimetric procedure was used (see below). The validity of using two different techniques for cholinesterase assay was verified by examining one compound (carbaryl) by both techniques, and finding excellent agreement between the two results.

Carbamates were made fresh each day as stock solutions in 95% ethanol and diluted before use with 19 volumes of 0.05 M phosphate buffer pH 7.0. Further dilutions were made with 5% ethanol in buffer. Winthrop bovine erythrocyte cholinesterase, 0.5 ml containing 2 units per milliliter of buffer, was pipetted into small test tubes in a water bath at 38°. Appropriate concentrations of carbamates were warmed to 38°, and 1 ml was added to the cholinesterase, mixed, and allowed to react for 3 min. Then 0.5 ml of Sigma acetylthiocholine iodide, 0.01 M and at 38° was added. After 3 min, 2 ml of DTNB (0.5 mg/ml ethanol) was added and the optical density of the yellow solution was measured at 412 mμ on the DU spectrophotometer. A standard was prepared containing buffer in place of the carbamate, and a blank of acetylthiocholine and buffer with the DTNB was subtracted from the readings.

For the pH-stat method, the carbamates were prepared in the same way. However, the phosphate buffer was 10^{-3} M for this method. Four milliliters of the cholinesterase (3 units per milliliter of buffer containing 0.1% gelatin) were pipetted into the cell of the Radiometer TTT lc pH-stat and allowed to warm to 38°. One milliliter of appropriately diluted carbamate, which had been warmed to 38°, was added and allowed to react for 3 min. Then 1 ml of acetylcholine bromide, 6×10^{-2} M and at 38° was added. The hydrolysis of acetylcholine at pH 7.0 was followed in the Radiometer, titrating with 0.005 N NaOH.

The results were computed in an IBM 1604 computer using the weighted regression procedure of Wilkinson (13).

Product Analysis

Bovine erythrocyte cholinesterase (460 units) was allowed to react with 5.5×10^{-6} mmole of ring-tritiated 3,5-diisopropylphenyl methylcarbamate (specific activity 20.8 mC/mmole) in a volume of 4.4 ml of pH 7.4 phosphate buffer, 0.067 M at 38°. At various times, 1 ml was added to 2 ml of ether–hexane (1:3) and shaken; 1.5 ml of the latter was added to a column containing 1 g of activated Florisil, then eluted with ether-hexane and 1-ml fractions were collected. Fractions 3, 4, and 5 contained the 3,5-diisopropylphenol; each fraction was added to 10 ml of a toluene scintillation solution and counted in a Tri-Carb scintillation counter. A parallel experiment without enzyme was conducted to measure nonenzymic hydrolysis, and the data (Fig. 3) were corrected by these amounts. The k_3 value calculated from the data of Fig. 3 uses the intercept on the y axis as the measure of one equivalent of enzyme. The value (58 cpm) shown as "calculated burst" is the equivalent cpm for the calculated moles of enzyme, estimated by using the known activity of the enzyme against acetylcholine and the turnover number of Michel and Krop (14) of 4.9×10^5 min^{-1}. Other, perhaps better, turnover numbers are available from Cohen's studies, such as 2.95×10^5 (15) or 2.78×10^5 or 3.72×10^5 (16). Clearly there is an arbitrary aspect to selection of the number. All that is claimed in this paper is that the observed "burst" is of the same order as the calculated.

For *p*-nitrophenate studies, a more concentrated enzyme preparation was needed because of the lesser sensitivity of the optical method employed. Eel cholinesterase

(Sigma) at 10,000 U/ml was used; 0.2 ml was added to 0.2 ml of 2×10^{-4} M *p*-nitrophenyl dimethylcarbamate in phosphate buffer pH 7.4, 0.067 M. The appearance of *p*-nitrophenate was studied at 402 mμ at 38°, using a DU spectrophotometer with microcuvettes. Parallel studies on nonenzymic hydrolysis were performed (Fig. 4 shows the extent at 130 min), and the data were corrected for it.

Determination of k_{OH} (First-Order Rate Constant for Hydrolysis)

The aminoantipyrine method (17) was used to determine k_{OH} in every possible case, i.e., when the phenolic hydrolysis product gave a colored derivative; this was true for six of the dimethylcarbamates (*p*-CH_3O, H, *p*-F, *p*-Cl, *m*-CF_3, 1-naphthyl), and five of the methylcarbamates (*p*-CH_3O, H, *p*-F, *p*-Cl, naphthyl). The carbamate was made up at 2 mg/ml in 95% ethanol, and 1 ml was added to 9 ml of 1 N NaOH at 38° and kept at this temperature. At intervals, 0.5 ml was removed and added to 9 ml of 4-aminoantipyrine (0.1% in phosphate buffer 0.05 M, pH 8.0). One drop of conc. HCl was added to lower the pH to 8.0, then 0.5 ml of $K_3Fe(CN)_6$, 0.5%. The solution was allowed to stand for 5 min to allow for maximum color development, and then read at 505 mμ against a blank of all the reagents except the carbamate. A standard curve was plotted by making up the parent phenol in H_2O at concentrations of 1–10 μg/ml. The same method was used for the methylcarbamates except that 0.1 M phosphate buffer was used instead of the NaOH, at pH 9.0 or 10.0 depending on the hydrolysis rate.

For two dimethylcarbamates (*p*-CN and *p*-Me), and one methylcarbamate (*m*-CF_3), the absorption maximum in the ultraviolet was clearly different for the carbamate and its parent phenol. The maxima found for the phenols were 275 mμ for *p*-CN, 237 mμ for *p*-Me, and 277 for *m*-CF_3; at these wavelengths, the corresponding carbamates did not absorb. The carbamates were prepared and hydrolyzed as in the aminoantipyrine method. At intervals, 0.5 ml was added to 9.5 ml of water at room temperature, and the absorption at the appropriate maximum was measured in the DU spectrophotometer. For *p*-nitrophenyl dimethylcarbamate a similar procedure was followed, but in 0.1 N NaOH, and measuring the yellow *p*-nitrophenate ion at 400 mμ. Hydrolysis of *p*-methyl methylcarbamate was measured by titration, following the hydrolysis with 0.05 N NaOH using an Agla micrometer syringe. All values given are for the first-order rate constant calculated for pH 14.

Syntheses

The carbamates were synthesized in 70–80% yield, either by the reaction of the appropriate phenol with methyl isocyanate in the presence of dibutyl tin dilaurate (18) to give the *N*-methyl derivatives, or by the reaction of the sodium salt of the phenol with dimethylcarbamoyl chloride (19) to give the *N,N*-dimethyl derivatives. All compounds gave the expected absorption peaks in the infrared. Purity and R_F of the carbamates were determined by reverse phase chromatography on paper treated with 5% Silicone (Dow Corning 550 Silicone Fluid in acetone), with pyridine–water 20:80, (v/v) as the mobile phase (20). Initially the Miskus method (21) was used to detect the carbamate spots, but it was found that not all the carbamates and their hydrolysis products reacted with the reagent, 4-nitrobenzediazonium fluoborate. Consequently, the chromatograms were exposed to iodine vapor, a much quicker though nonspecific procedure having the advantage of showing not only the phenol but its parent carbamate, and additionally, other aromatic impurities. The phenols had lower R_F values than the corresponding carbamates but often tended to smear the chromatogram. Chromatography of a few of the carbamates on Florisil (22) to remove traces of impurities showed that the compounds were sufficiently pure to be used directly in the experimental work.

A typical synthesis for each type of carbamate follows:

4-Chlorophenyl methylcarbamate

$$ClC_6H_4OH + CH_3NCO \xrightarrow[\text{dilaurate}]{\text{dibutyl tin}} ClC_6H_4OC(O)NHCH_3$$

A 500-ml 3-necked flask fitted with a condenser and drying tube, dropping funnel, and heavy magnetic stirrer was charged with 12.9 g (0.1 mole) of 4-chlorophenol, 0.2 ml dibutyl tin dilaurate, and 100 ml of anhydrous benzene. The system was flushed with nitrogen, and 8 ml (0.13 mole) of methyl isocyanate in 50 ml of benzene was added over 1 hr to the vigorously stirred mixture. The reaction was refluxed for 2 hr, cooled, and filtered, and the benzene was flashed off via a rotary evaporator. Treatment of the yellowish residue with decolorizing carbon and recrystallization from benzene–2,2,4-trimethylpentane, 1:1, (v/v), gave colorless needles m. 114.9–115.7°; R_F 0.66; $C_{calc.}$ 51.77%, C_{found} 51.92%; $H_{calc.}$ 4.35%, H_{found} 4.28%.

4-Chlorophenyl dimethylcarbamate

$$CH_3OH + Na \rightarrow CH_3ONa + 1/2\ (H_2)$$

$$CH_3ONa + HOC_6H_4Cl \rightarrow NaOC_6H_4Cl + CH_3OH$$

$$NaOC_6H_4Cl + (CH_3)_2NC(O)Cl \rightarrow ClC_6H_4OC(O)N(CH_3)_2 + NaCl$$

2.3 g (0.1 mole) of freshly cut sodium were added to a solution of 50 ml of anhydrous methanol and 50 ml of toluene in a 1-liter 3-necked flask fitted with a condenser and drying tube, dropping funnel, and heavy magnetic stirrer. Upon completion of the reaction, the volume of the mixture was brought to 500 ml with toluene, and 12.9 g (0.1 mole) of 4-chlorophenol in 50 ml of ether added over 1 hr. The condenser was replaced with a distillation train for vacuum distillation, and 250–300 ml of the solvent mixture was distilled over at a pressure of 18 mm. The flask was recharged with 500 ml of fresh toluene and another 250–300 ml of solvent distilled over. The incorporation of a PU burner as a bleed in the vacuum line was necessary, as the precipitating sodium chlorophenate occasionally caused excessive foaming with subsequent carry-over of the flask contents into the receiver. Judicious control of the bleed prevented this until equilibrium conditions obtained. From there on, the distillation proceeded smoothly. The distillation train was removed, the condenser was returned to reflux position, and 14 ml (0.15 mole) of dimethylcarbamoyl chloride in 50 ml of toluene was added to the warm mixture over 2 hr. After a reflux period of 4 hr, the reaction was cooled, filtered, washed twice with 100 ml of water, twice with 100 ml of 5% (aq.) sodium carbonate, and finally three times with 100 ml of water. The toluene phase was dried over anhydrous sodium sulfate for 24 hr at 4°. The dried solution was then filtered and the toluene was removed by rotary evaporation. Distillation of the yellowish product gave a colorless mobile liquid $b_{0.1\ mm}$ 76.0–77.0°; n_d^{23} 1.5312, R_F 0.56; $C_{calc.}$ 54.15%, C_{found} 54.36%, $H_{calc.}$ 5.03%, H_{found} 5.05%.

Physical constants and analytical data for the remaining compounds follow. 4-nitrophenyl dimethylcarbamate: m. 107.1–107.5°; white flakes; not analyzed as compared with standard sample. 4-Cyanophenyl dimethylcarbamate: m. 64.7–65.1°; white needles; R_F 0.85; $C_{calc.}$ 63.15%, C_{found} 63.43%; $H_{calc.}$ 5.30%, H_{found} 5.38%. 3-Trifluoromethylphenyl dimethylcarbamate: $b_{0.1\ mm}$ 66.5–67.0°; yellow liquid; n_d^{23} 1.4621; R_F 0.20; $C_{calc.}$ 51.51%, C_{found} 51.55%; $H_{calc.}$ 4.32%, H_{found} 4.22%. 4-Fluorophenyl dimethylcarbamate: $b_{0.1\ mm}$ 60.8–61.5°; colorless liquid; n_d^{23} 1.4987, R_F 0.61; $C_{calc.}$ 59.12%, C_{found} 59.16%; $H_{calc.}$ 5.50%, H_{found} 5.48%. Phenyl dimethylcarbamate: m. 43.7–44.2°; white needles; R_F 0.82; $C_{calc.}$ 65.44%, C_{found} 66.40%; $H_{calc.}$ 6.71%, H_{found} 6.78%. 4-Methylphenyl dimethylcarbamate: m. 50.5–51.2°; white needles; R_F 0.62 $C_{calc.}$ 67.02%, C_{found} 67.66%; $H_{calc.}$ 7.31%, H_{found} 6.99%. 4-Methoxyphenyl dimethylcarbamate: m. 64.5–64.9°; white needles; R_F 0.79; $C_{calc.}$ 61.53%, C_{found} 61.62%; $H_{calc.}$ 6.71%, H_{found} 6.72%. 4-Cyanophenyl methylcarbamate: m. 128.3–128.8°; white needles; R_F 0.79; $C_{calc.}$ 61.36%, C_{found} 61.66%; $H_{calc.}$ 4.58%; H_{found} 4.68%. 3-Trifluoromethylphenyl methylcarbamate: m. 69.6–70.2°; white needles; R_F 0.38; $C_{calc.}$ 49.32%, C_{found} 49.29%; $H_{calc.}$ 3.68%, H_{found} 3.66%. 4-Fluorophenyl methylcarbamate: m. 102.0–102.8°; white needles; R_F 0.80; $C_{calc.}$ 56.81%, C_{found} 57.09%; $H_{calc.}$ 4.77%, H_{found} 5.02%. Phenyl methylcarbamate: m. 84.7–85.4°; white needles; R_F 0.72; $C_{calc.}$ 63.57%, C_{found} 63.80%; $H_{calc.}$ 6.00%, H_{found} 6.13%. 4-Methylphenyl methylcarbamate: m. 94.0–94.4°; white needles; R_F 0.76 $C_{calc.}$ 65.44%, C_{found} 65.11%; $H_{calc.}$ 6.71%, H_{found} 6.81%. 4-Methoxyphenyl methylcarbamate: m. 95.3–95.7°; white flakes; R_F 0.75; $C_{calc.}$ 59.66%, C_{found} 59.63%; $H_{calc.}$ 6.12%, H_{found} 6.27%. All solids were recrystallized from hexane, heptane, or isooctane (2,2,4-trimethylpentane) with sufficient benzene to effect solution.

RESULTS AND DISCUSSION

Alkaline Hydrolysis Rate

The early report (2) that electrophilic ring substituents worsened anticholinesterase potency, taken with the evidence given below, by product analysis, that carbamylation occurs in cholinesterase inhibition, raised the remote possibility that carbamylation of cholinesterase involved a nucleophilic rather than the anticipated electrophilic attack by the carbamate. If such was the

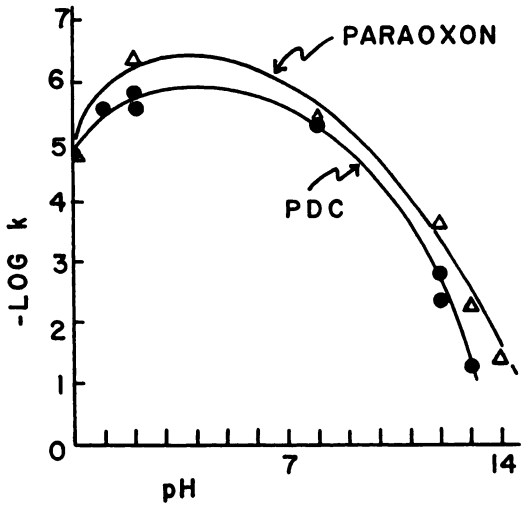

FIG. 1. *pH profile of hydrolysis of p-nitrophenyl dimethylcarbamate (PDC) and diethyl p-nitrophenyl phosphate (paraoxon)*

case, one might expect that, by analogy, hydrolysis of carbamates might be more readily catalyzed by H^+ rather than OH^-. However, the pH profile of hydrolysis showed this not to be the case. The profile for the dimethylcarbamate and the diethyl phosphate derivatives of *p*-nitrophenol were remarkably similar (Fig. 1).

It is known (23) that methylcarbamates hydrolyze much faster than dimethylcarbamates. This can be seen from inspection of Table 1, and Table 2 shows that for the 8 cases where the ratio could be measured, the value of k_{OH} for methlycarbamates was between 4.3×10^4 and 1.59×10^6 times greater than for the corresponding dimethylcarbamate. This has been attributed (23) to the existence in methylcarbamates of a special mechanism involving deprotonation of the NH, followed by prompt cleavage of

TABLE 1
Kinetic constants for methyl- and dimethylcarbamates

O-Substituent	$10^3 \times K_s$ (M)	$10^3 \times$ standard error of K_s	k_2 (min^{-1})	Standard error of k_2	$10^{-2} \times k_i$	k_{OH} (min^{-1} at pH 14)	Hammett Sigma constant
			Methylcarbamates				
m-Trifluorophenyl	1.33	0.17	3.00	0.35	22.6	14,000	0.43
p-Chlorophenyl	3.27	0.58	1.33	0.21	4.1	3,700	0.23
p-Fluorophenyl	17.9	4.24	1.87	0.48	1.0	788	0.06
Phenyl	2.90	0.27	1.56	0.072	5.4	1,050	0
p-Methylphenyl	1.30	0.15	1.50	0.053	11.6	690	−0.17
p-Methoxyphenyl	2.71	0.50	1.36	0.22	5.0	185	−0.27
3,5-Diisopropylphenyl	0.0034	0.0007	1.38	0.082	4070	—	—
o-Isopropoxyphenyl	0.0099	0.0023	1.05	0.13	1062	—	—
4-Methylthio-3,5-dimethylphenyl	0.0067	0.0013	1.24	0.16	1856	—	—
1-Naphthyl (= carbaryl or Sevin)	0.0106	0.0023	1.33	0.086	1250	2,240	—
			Dimethylcarbamates				
p-Nitrophenyl	0.328	0.10	0.25	0.033	7.5	0.076	0.78
p-Cyanophenyl	1.11	0.22	0.54	0.066	4.8	0.0248	0.66
m-Trifluorophenyl	0.44	0.10	0.63	0.058	14.3	0.0088	0.43
p-Chlorophenyl	1.88	1.07	0.60	0.180	3.2	0.011	0.23
p-Fluorophenyl	72.9	18.0	2.03	1.21	0.3	0.008	0.06
Phenyl	1.49	2.2	0.22	0.097	1.5	0.0071	0
p-Methylphenyl	6.44	1.1	0.51	0.078	0.8	0.0040	−0.17
p-Methyoxyphenyl	0.36	0.14	0.13	0.015	3.5	0.0043	−0.27
1-Naphthyl	0.093	0.023	2.43	0.91	261.3	0.0040	—
3,5-Diisopropylphenyl	0.058	0.012	0.75	0.086	129.8	—	—
1-Isopropyl-3-methyl-5-pyrazolyl (= Isolan)	0.008	0.0003	1.31	0.020	1641.2	—	—

TABLE 2
Ratio of values for N-methyl:N-dimethyl for phenyl carbamates

Phenyl substituent	k_i	K_a	k_2	k_{OH}
m-CF_3	1.58	3.01	4.77	1,590,000
p-Cl	1.29	1.74	2.23	337,000
p-F	3.50	0.25	0.92	98,500
H	3.79	1.95	7.18	148,000
p-CH_3	14.5	0.20	2.93	172,500
p-CH_3O	1.46	7.5	10.86	43,000
[Naphthyl]	4.77	0.11	0.55	560,000
3,5-Diisopropyl	31.95	0.06	1.84	—

the residue to methly isocyanate and the substituted phenate.

Both methyl- and dimethylcarbamates show a marked dependence of their k_{OH} upon Hammett's Sigma constant, in the expected direction. Figure 2 shows data for the dimethyl series for which the correlation coefficient for *p*-substituted compounds was 0.83; for the methyl series, the coefficient was 0.86. It might seem surprising that the methyl series showed a similar dependence, for one would suppose the electronegativity of the N was involved (i.e., its susceptibility to parting from the proton) in contrast to the electronegativity of the carbonyl carbon in the dimethyl series (i.e., its susceptibility to OH^- attack), and since the N is one atom further from the ring than is the carbonyl carbon, one might expect a lesser dependence on ring substituents for the methyl series. Presumably the fact is that the deprotonation of methylcarbamates involves a concerted mechanism with simultaneous deprotonation and O—C cleavage, so that electronic effect at the O—C bond is of importance in this series as well as the dimethyl series.

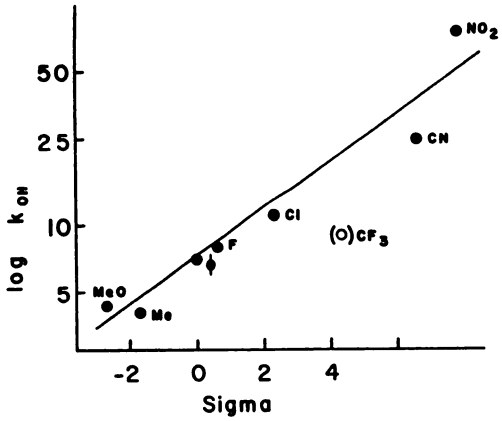

FIG. 2. *Dependence of k_{OH} of dimethylcarbamates upon Hammett Sigma constant of the phenolic substituent*

All are *p*-substituents except for *m*-CF_3, shown parenthetically.

Demonstration of Leaving Group

The best direct evidence for the carbamylation theory would be to prove that in the course of inhibition, there is indeed a leaving group and that it is the aromatic group X. This is demonstrated in Fig. 3 for the case of ring-tritiated 3,5-diisopropylphenyl methylcarbamate, in large excess over bovine erythrocyte cholinesterase: 3,5-diisopropylphenol is produced first in a "burst" which is approximately stoichiometric with enzyme concentration, and then progressively and linearly, i.e., with zero-order kinetics. The interpretation is that the "burst" represents carbamylation of enzyme at a rapid rate determined by k_2, and that further reaction must await regeneration of enzyme, determined by the slow k_3, permitting recycling and hence more phenol production. From Fig. 3 one can calculate a k_3 of 0.055 min^{-1}, which is similar to the value of 0.037 min^{-1} calculated below from recovery data.

In an experiment such as the above where prompt achievement of a steady state (i.e., cycling at the maximum possible velocity, dictated by k_3) is desired, one should add enough inhibitor to assure that no free enzyme is present, so that all that is not carbamylated is complexed. To achieve 99% of this goal one would have to add a carbamate concentration of 100 K_a. In fact, the K_a for 3,5-diisopropylphenyl methylcarbamate was not measured until two years after the leaving-group experiment, and it was then found to be about 3.4×10^{-6}; in the experiment of Fig. 3, 1.2×10^{-6} M carbamate was used, so one can say something less than half-saturation was achieved.

Subsidiary evidence would be desirable that it is in fact cholinesterase that is re-

sponsible for release of the phenol, rather than other esteratic activity which might be present in the purified preparation. Unfortunately, it was impossible to block the cholinesterase sites with acetylcholine, because of its extremely rapid destruction by the concentrated enzyme. For instance, when 10^{-2} M acetylcholine was added, no marked effect on phenol production was noted, but

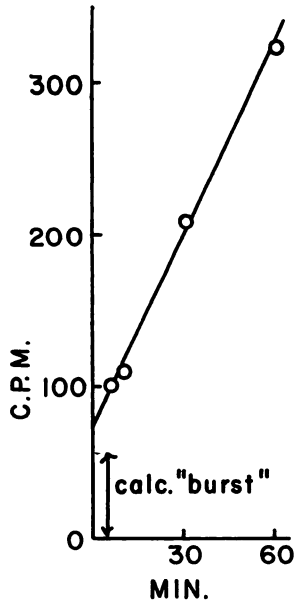

FIG. 3. *Radioactive 3,5-diisopropylphenol produced by reaction of ring-^{3}H 3,5-diisopropylphenyl methylcarbamate with bovine erythrocyte cholinesterase*

See Methods for basis of "calculated burst."

one may calculate that the initial degradation rate at 10^{-2} M is 20.7 μmoles min^{-1} with this enzyme concentration, and only 20 μmoles of acetylcholine was added. However, addition of the potent selective anticholinesterase *O,O*-diethyl *S*-triethylammoniumethyl phosphorothiolate, at 3×10^{-7} M, 15 min before adding the carbamate, completely prevented phenol release from the tritiated carbamate.

Figure 4 shows another case of release of leaving group: *p*-nitrophenol production when *p*-nitrophenyl dimethylcarbamate is allowed to react with electric eel cholinesterase. This carbamate, used because of the ease of measurement of *p*-nitrophenol, is an extremely poor cholinesterase inhibitor. Hence a large concentration of carbamate (10^{-4} M) is required for reaction. Clearly there is no "burst" in this case: the calculated burst (stoichiometric with enzyme) would correspond to an optical density increase of 0.176, which would be very easy to see. The absence of burst could in principle be due to k_2 being slower than k_3 or $(k_1 - k_{-1})$ being slower than k_3. Since k_3 is necessarily the same for all dimethylcarbamates, and k_2 differs relatively little among the carbamates (Table 1) it is likely that in fact an unusually low value for $(k_1 - k_{-1})$ is responsible for the absence of burst. The overall k value for *p*-nitrophenate release, calculated from Fig. 4, is 0.0020 min^{-1} which may therefore be the value of $k_1 - k_{-1}$, and is indeed appropriately slow.

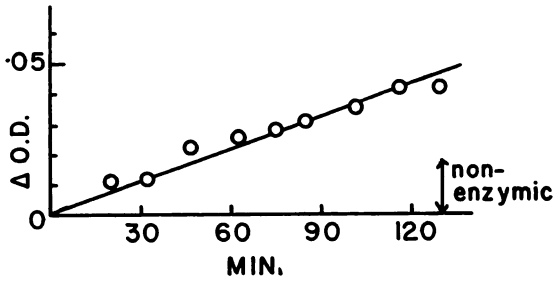

FIG. 4. *p-Nitrophenate produced by reaction of p-nitrophenyl dimethylcarbamate with eel cholinesterase*

The "calculated burst" if stoichiometric *p*-nitrophenate was released would correspond to an optical density of 0.176. The length of the arrow shows the contribution of nonenzymic hydrolysis at 130 min. Each point has been corrected for nonenzymic hydrolysis.

The absence of burst in this case is the more puzzling in view of the fact that Bender and Stoops (24) found that the closely related *o*-nitrophenyl dimethylcarbamate reacted with eel cholinesterase to give a burst, implying (as they point out) that $k_2 > k_3$.

Recovery of Inhibited Enzyme

Wilson *et al.* (5) have shown the recovery of eel acetylcholinesterase at 25° and pH 7, when inhibitor was "diluted away from" the enzyme-inhibitor mixture, but they did not show graphical data. Figure 5 shows recovery

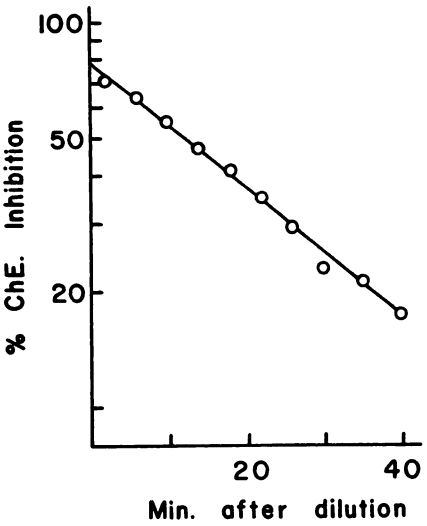

FIG. 5. *Recovery from inhibition of bovine erythrocyte cholinesterase inhibited by 3,5-diisopropylphenyl methylcarbamate*

Dry enzyme (125 units) was added to 0.25 ml of 5×10^{-7} M inhibitor in buffer and held at 38° for 15 min, then (zero time on graph) diluted 1000-fold with buffer at 38°. Assay was by Ellman method. Half-life, from graph = 19 min, $k_3 = 0.0365$ min^{-1}.

of bovine erythrocyte cholinesterase at 38° and pH 7, using a similar technique. The characteristic first-order recovery from inhibition is apparent, and is interpreted as decarbamylation, controlled by k_3. Our value of k_3 for this methylcarbamate of 0.0365 min^{-1} at 38° is compatible with Wilson's value of 0.018 at 25°.

Kinetics of Inhibition

The reaction of carbamates with cholinesterase, as judged by inhibition of the enzyme, is linear with time at first, but in every case the inhibition reaches an apparent equilibrium. Examples are shown in Fig. 6. The apparent equilibrium is in fact a steady state, achieved when the rate of formation of carbamylated enzyme equals its rate of breakdown by the k_3 step. The subsequent results described herein are for a 3-min inhibition time, which is within the first-order phase of inhibition.

In comparing Fig. 6 with Fig. 3, the time to reach steady state may seem long in Fig. 6, e.g. 20 min for EC to reach steady state at the highest carbaryl concentration, which is about 0.5 K_a, and short in Fig. 3 for 3,5-diisopropylphenyl carbamate, when linear production of X (associated with a steady-state level of EC) occurs rapidly. The difference is in fact small, for in Fig. 3 linear production of X occurs at about 6 min or less, and the difference between the 6 min and 20 min is accountable in terms of the 3-fold better affinity of the 3,5-diisopropylphenyl carbamate (Table 1).

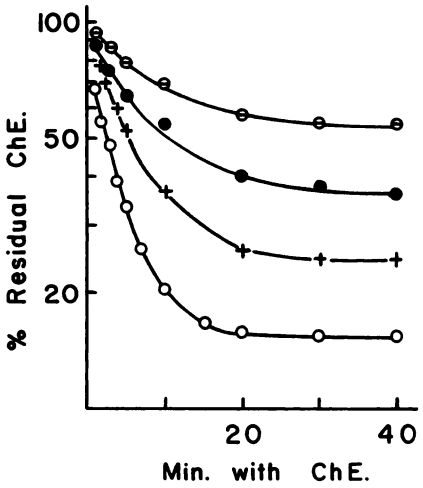

FIG. 6. *Progress of inhibition of bovine erythrocyte cholinesterase (38°) by carbaryl (Sevin) at various concentrations, to show achievement of steady states*

Concentrations are (top to bottom) 5×10^{-7} M, 10^{-6} M, 2.5×10^{-6} M, and 5×10^{-6} M. Ordinate is on log scale.

Kitz and Wilson (9) and Main (10) have described, for the parallel case of inhibition by sulfonates and phosphates, kinetic procedures for evaluating K_a and k_2 in Eq. 1; in both cases, k_3 was negligibly small, and this feature will be discussed below. The basic equation of Main is:

$$\frac{1}{i} = \frac{t}{2.3\Delta \log V} \cdot \frac{k_2}{K_a} - \frac{1}{K_a} \qquad (2)$$

where i is inhibitor concentration, t the inhibition time (which Main writes as Δt; but here we are discussing the special case when the first time considered is zero), and $\Delta \log V$ is the difference in the logarithms of the velocities of inhibited and uninhibited enzyme. Thus a plot of $1/i$ against $t/2.3\Delta$

$\log v$ is linear, with an x intercept of $1/k_2$ and a y intercept of $-1/K_a$.

It is of major importance, in order for the data to be interpreted in terms of the Main equation, that the inhibition be first order initially. Representative data which demonstrate this to be the case are given in Fig. 7 for all five compounds which were so tested, using time intervals short enough so that the subsequent leveling-off does not intrude.

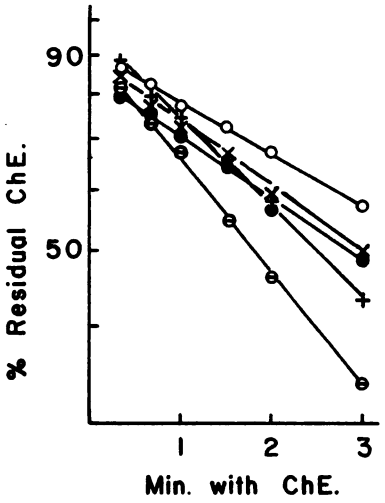

FIG. 7. *Progress of inhibition of bovine erythrocyte cholinesterase (38°)*

Inhibition by: O,1-naphthyl dimethylcarbamate at 5×10^{-6} M; × carbaryl at 5×10^{-6} M; ●, *p*-methylphenyl methylcarbamate at 5×10^{-4} M; + phenyl methylcarbamate at 5×10^{-4} M; ⊖ *p*-chlorophenyl methylcarbamate at 1×10^{-3} M. Short times were selected to show first-order inhibition. Ordinate is on log scale.

The kinetic findings were indeed fully compatible with the Main equation; a sample plot is shown in Fig. 8. This provides supporting evidence for a parallelism between phosphorylation and carbamylation of cholinesterase, and permits one to evaluate K_a and k_2 numerically, as the reciprocal of the intercepts on the ordinate and abscissa, respectively (values were in fact evaluated by calculation rather than by eye; see under Methods). The results are shown in Table 1.

At first it may seem paradoxical that with a k_2 which is typically in the region of 1, the half-life of the first-order part of the inhibition reaction should be about 1.5 min (Fig. 7); the familiar relation half-life = $\ln 2/k$ for first-order reactions suggests a half-life of 0.69 for $k = 1$. But this relation is not valid for the present situation. If one takes the condition for Eq. 2 that $K_a = i$ and $k_2 = 1$, he will find that the half-life (i.e., when $\Delta \log V = 0.3$) is 1.4 min, in excellent agreement with Fig. 7.

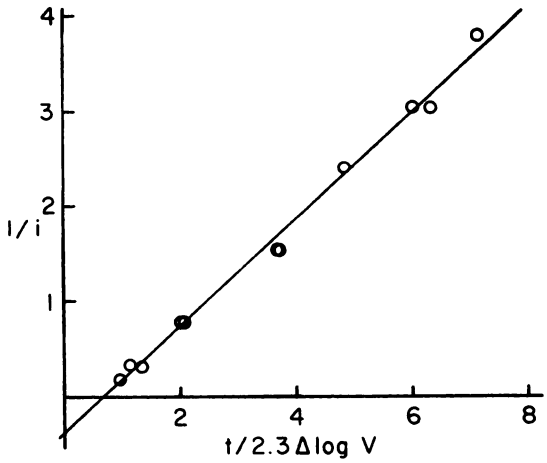

FIG. 8. *Main plot for inhibition of bovine erythrocyte cholinesterase by phenyl methylcarbamate at 38°*

Various concentrations at $t = 3$ min. Ordinate: 10^{-3} times reciprocal of molar concentration.

The concentrations used for Table 1 were such that in all cases K_a/i was in the region of 1. For the highest value of i in each case, the value of K_a/i was 1 or more in eleven cases, 0.3 or more in nine cases, and 0.14 for one case, *p*-fluorophenyl dimethylcarbamate.

In the organophosphates which Main dealt with, the k_3 step was negligibly small; but in carbamates, k_3 is at least measurable; thus Wilson (6) reports values of 1.8×10^{-2} min^{-1} for methyl- and 2.6×10^{-2} min^{-1} for dimethylcarbamates. Let us consider how inclusion of the k_3 step in the original Main argument modifies the outcome. One must include the consideration that CE is broken down as well as formed, i.e.

$$\frac{d[CE]}{dt} = k_2[C \times E] - k_3[CE] \qquad (3)$$

This consideration may be introduced into the following rewritten form of the Main equation:

$$\frac{1}{i} = \frac{\Delta t \cdot \frac{k_2}{K_a}}{2.3 \log \frac{[E] - [EC_1]}{[E] - [EC_2]}} - \frac{1}{K_a} \quad (4)$$

where i is inhibitor concentration, [E] is the initial enzyme concentration and [EC_1] and [EC_2] are the concentrations of EC at times Δt min apart. The insertion of the above consideration gives:

$$\frac{1}{i} = \frac{\Delta t \left[\frac{k_2 + k_3}{K_a} + \frac{k_3}{i}\right]}{2.3 \log \frac{[E] - [EC_1] - \frac{k_3[EC_1]}{z}}{[E] - [EC_2] - \frac{k_3[EC_2]}{z}}} - \frac{1}{K_a} \quad (5)$$

where z is $(ik_2)/(i + K_a)$. It should be noted for both equations that $\Delta \log V$ in graphs such as Fig. 8 is a measure of log $([E] - [EC_1])/([E] - [EC_2])$, and consequently measures the change in free enzyme, under assay conditions such that all ECX is converted to free enzyme, by using a very large excess of substrate.

Comparison of Eqs. 4 and 5 shows that when k_3 is sufficiently small compared to i and k_2, the terms k_3/i and k_3 [EC]/z may be neglected, and one achieves the Main equation but with $(k_2 + k_3)$ in place of k_2 in the numerator. Therefore the reciprocal of the x intercept in Fig. 2 gives not k_2 but $(k_2 + k_3)$. Since k_3 is about 20–40 times smaller than k_2, the correction is not large.

Main has shown for organophosphates that the apparent bimolecular rate constant (k_i) which emerges if one ignores complex formation is in fact given by k_2/K_a, and so is made up of a rate constant and an equilibrium constant. But k_i constitutes a useful overall measure of inhibitory potency; Main suggests that it be called the bimolecular reaction constant.

Using the Main equation, the values of k_i, K_a, and k_2 were computed for a 3-min incubation of inhibitor with enzyme. However, when certain of these values were measured for lesser incubation times (1 or 2 min) the values of K_a or k_2 varied severalfold, but that of k_i remained constant. In terms of Fig. 8, it will be seen that k_i represents the slope of the line, whereas K_a and k_2 are derived from intercepts. In view of the double-reciprocal nature of the plot, and the nearness of the intercepts to the origin, our confidence in the values of K_a and k_2 is small enough that we are reevaluating them by a somewhat different approach; the values are included here as rough guides only. The k_i values, by contrast, are measures of a slope which is large enough to be accurately measured, and can be relied on as excellent estimates within the indicated standard error.

Structure and Inhibition

Table 1 shows the K_a (affinity constant) and k_2 (carbamylation constant) and k_i for each of eleven dimethylcarbamates and ten methylcarbamates, along with the Hammett's substituent constant for the phenyl substituent (where known).

One of the pieces of evidence which at one time cast doubt upon the carbamylation as the mechanism of carbamate inhibition, thus supporting indirectly the alternative view that the complex was the form of the inhibited enzyme, was the report that electrophilic ring substituents lessened anticholinesterase activity (2, 25). This is the opposite of what one expects from a carbamylation reaction, and the opposite of what one finds in the organophosphates in which one sees positive correlations between Sigma values, anticholinesterase activity (ability to phosphorylate cholinesterase), and alkaline hydrolyzability (which may be considered as the ability to phosphorylate hydroxyl ion). Figure 9 shows that for dimethylcarbamates, electrophilic substituents *do* improve anticholinesterase activity. Thus k_i is positively correlated with Sigma (correlation coefficient $r = 0.78$) and with k_{OH} ($r = 0.866$). However, for methylcarbamates no such correlation exist; in this series, for k_i and Sigma the value of r is -0.47, and for k_i and k_{OH} the value of r is -0.09. Graphical representation confirms this noncorrelation. This difference between the *N*-methyl and *N*-dimethyl series suggests the possibility of different mechanisms of carbamylation.

Carbamylation or Complex Formation?

The data permit one to compute, for any given set of conditions, the amount of inhibited enzyme that is in the reversible form (ECX) and the irreversible form (EC). Rewriting Eq. 3, of Main (10), in appropriate terms:

$$ECX = \frac{(E - EC)CX}{CX + K_a} \qquad (6)$$

where E and CX are initial concentrations of enzyme and inhibitor (which is in large excess), and ECX and EC are concentrations of complexed and carbamylated enzyme at any time. From this equation one can calculate that when K_a = CX, one will get almost immediate 50% inhibition with all the inhibited enzyme in the complexed form. Thereafter, inhibition will be progressive, and when 90% is inhibited, one-ninth of the inhibited enzyme will be in the complexed form (ECX), and the rest of inhibited enzyme will be carbamylated (EC). When K_a = 100 CX, one will get almost immediate 1% inhibition, all as complexed form. Thereafter, progressive inhibition will occur and at 90% inhibition, only 1/900 of the inhibited enzyme will be in the complexed form.

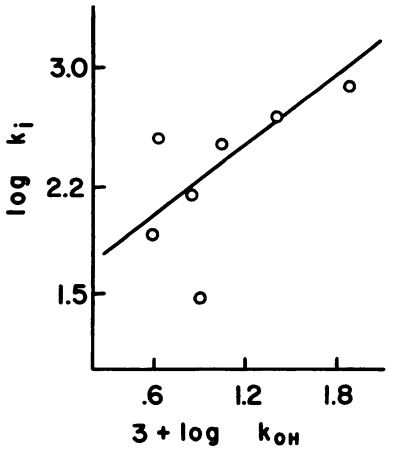

FIG. 9. *Correlation of log k_i with log k_{OH} for unsubstituted and p-substituted phenyl dimethylcarbamates*

The logarithmic scales are for convenience only; the correlation coefficients in the text are calculated for actual values of the *k*'s.

In customary assay techniques (for instance, in this study) substrate concentrations are very much larger than inhibitor concentrations. One then only observes inhibition caused by carbamylation, because the substrate displaces the carbamate–enzyme complex. In confirmation, we performed a study with 10^{-5} M carbaryl, which because it has one of the highest affinities (i.e., lowest K_a) might be particularly difficult to displace with substrate. It was found that only at substrate concentrations below 2.5×10^{-4} M did total inhibition increase with decreasing substrate concentrations; at all higher concentrations tested (i.e., up to 7.5×10^{-3} M) inhibition was independent of substrate concentration. It will be recalled that the usual substrate concentration used is 2.5×10^{-3} M. Winteringham and Disney (26) have compared high (10^{-2} M) and unusually low (5×10^{-5} M) substrate concentrations for studying carbamate inhibition. One would predict from the above calculations that the inhibition results with high and very low substrate concentration would differ substantially when the inhibitor concentration is close to the K_a, for then measurable amounts of complexed enzyme exist, but would not differ significantly if the inhibitor concentration is much less than K_a, for then little complexed enzyme exists. Winteringham and Disney found a small difference for DFP (diisopropyl phosphorofluoridate) and a large difference for 2-isopropoxyphenyl methylcarbamate, using the inhibitors at 10^{-6} M. Unfortunately, one cannot make calculations from their data because they used mixed cholinesterases (whole blood). The data of Table 1 suggest that methylcarbamates have poor (low) k_2 values, compensated by very low K_a values. Consequently, one would expect the inhibitor concentration to be close to the K_a, and therefore expect that the observed inhibition would depend markedly on substrate concentration (as Winteringham and Disney found), but only with unusually low substrate concentrations.

The data in this paper support the formulation of Eq. 1 and show that carbamates are substrates for cholinesterase, with high affinity, low carbamylation rates, and even

lower decarbamylation rates. One cannot neglect the complex-formation step; on the contrary, virtually all the differences in anticholinesterase activity among the 13 carbamates described herein were due to differences in complexing ability. This fact accounts for the ample evidence that in designing carbamates, the major factor to consider is the "fit" upon the enzyme surface (7, 8, 27). The remarkable insensitivity of k_2 (the carbamylation step) to ring substituents was not expected, but finds a parallel in the insensitivity of alkaline hydrolysis rates of acetanilides to ring substituents (28). In that case, it was shown that any given change in substituent improved formation of the hydroxylated intermediate, but worsened the probability that the intermediate would form product rather than revert to the parent. The same explanation might hold for the reaction between carbamates and cholinesterase.

However, the dominance of the complex in determining potency does not permit one to say that "the primary mode of inhibition is competitive" (8) or that carbamylation is "of secondary importance in the inhibition process" (29). On the contrary, the form of inhibited enzyme as usually measured is almost exclusively carbamylated cholinesterase; the primary mechanism of inhibition, when so measured, is therefore carbamylation. The truth of this statement emerges (quite apart from the elaborate arguments in this paper) from the progressive nature of the inhibition. However, this progressive inhibition levels off before completion, a process not seen in the organophosphates. The effect is due to the decarbamylation rate being relatively fast as compared with dephosphorylation.

ACKNOWLEDGMENTS

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