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This issue completes Volume 2
Volume 2, Number 6, November 1966


# MOLECULAR PHARMACOLOGY

## *An International Journal*


EDITOR: Avram Goldstein





A publication of the
American Society for Pharmacology and
Experimental Therapeutics

ACADEMIC PRESS
*New York and London*

# The Reaction of Carbamates with Cholinesterase

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(Received June 7, 1966)

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, $6 \times 10^{-2}$ 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 $5.5 \times 10^{-6}$ 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 $4.9 \times 10^5$ $min^{-1}$. Other, perhaps better, turnover numbers are available from Cohen's studies, such as $2.95 \times 10^5$ (15) or $2.78 \times 10^5$ or $3.72 \times 10^5$ (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) added tc dimethyl 7.4, 0.0( phenate a DU spe Parallel were per 130 min)

*Determin Consta*

The a used to d i.e., whe gave a cc six of the *p*-F, *p*-C the methy naphthyl 2 mg/ml i to 9 ml of temperatu moved ar pyrine (0 pH 8.0). to lower $K_3Fe(CN$ to stand fc developme a blank of mate. A st ing up the trations o was used that 0.1 M of the Na( the hydrol

For two *p*-Me), an the absorp was clearly its parent phenols we *p*-Me, and lengths, th not absorb and hydro method. A 9.5 ml of the absorpt

(Sigma) at 10,000 U/ml was used; 0.2 ml was added to 0.2 ml of $2 \times 10^{-4}$ 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

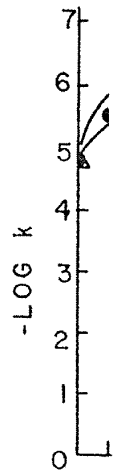


FIG. 1. *dimethylca phosphate*

*O*-S

*m*-Trifluoro
*p*-Chloroph
*p*-Fluoroph
Phenyl
*p*-Methylpl
*p*-Methoxy
3,5-Diisopr
*o*-Isopropox
4-Methylth
phenyl
1-Naphthyl
Sevin)

*p*-Nitrophen
*p*-Cyanophe
*m*-Trifluoro
*p*-Chlorophe
*p*-Fluorophe
Phenyl
*p*-Methylph
*p*-Methyoxy
1-Naphthyl
3,5-Diisopro
1-Isopropyl-
pyrazolyl

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