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BIOCHEMICAL AND PHARMACOLOGICAL STUDIES ON N-METHYLATED CARBAMOYLCHOLINES

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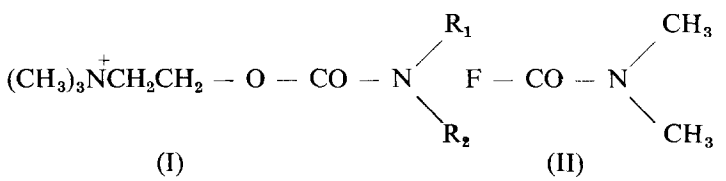
Abstract—N-methyl- and N:N-dimethyl-carbamoylcholines are highly stable compounds in aqueous solution and can only be hydrolysed to yield choline and methylamines when boiled for several hours in alkaline solutions. These esters are not degradated by butyrylcholinesterase, acetylarylesterase and albumin of human serum, or by acetylcholinesterase of electric organ. Cholinesterases are inhibited by concentrations less than 10^{-4} M. The inhibition is progressive and at least partly irreversible, probably owing to carbamoylation of the esterastic site of the enzyme molecule. In comparison with carbamoylcholine the two substituted derivatives are less toxic and have in general weaker pharmacological activities. However, they have more pronounced ganglion stimulating effects and are respiratory stimulants. Potentiation of the response to acetylcholine could not be demonstrated. On the frog rectus both derivatives are approximately four times more potent than carbamoylcholine and five times less potent than acetylcholine. Carbamoylcholine is as potent as acetylcholine on the guinea-pig ileum, while the monomethyl derivative is 40 times, and the dimethyl derivative 150 times less, potent than carbamoylcholine.

CARBAMOYLCHOLINE (I, $R_1 = R_2 = H$) has been extensively studied biochemically and pharmacologically,[1,2] but its carbamoyl N-substituted derivatives have been little investigated[3,4] with the exception of the closely related dibutylcarbamate of ethyl (2-hydroxylethyl) dimethylammonium (dibutoline).[4] The synthesis of N-methylated carbamoylcholines was reported previously[5,6] and their miotic action noticed.[4] It is known that substitution in the aminogroup of carbamoylcholine very greatly reduces the muscarine-like activities and even produces molecules which are antagonistic to acetylcholine, i.e. atropine-like.[4]

In connexion with biochemical studies of a series of N-alkyl- and N:N-diakyl carbamates,[7] it was demonstrated that arylesterases and plasma albumin[8,9] are active in the decomposition of these compounds. Owing to the marked influence of N-alkyl substitution in carbamates on both the biochemical and pharmacological properties and to lack of knowledge of these properties of N-methyl-(MeCbCh) (I, $R_1 = Me$, $R_2 = H$) and N:N-dimethylcarbamoylcholine (Me_2CbCh) (I, $R_1 = R_2 = Me$), these compounds were studied in the present investigation. Some results with N:N-dimethylcarbamoylfluoride (DCF) (II) are inserted for comparison.



(I) (II)

MATERIAL AND METHODS

Compounds tested

The two N-methylated carbamoylcholine chlorides were synthesized and provided by Dr. H. Gysin and Dr. A. Margot of J. R. Geigy, Basle, Switzerland. The melting points for the two compounds, determined with a Kofler block on minute crystals, were: MeCbCh, 173–4 °C; Me_2CbCh, 181–4 °C; Sprinson[6] obtained 178–80 °C and 185–7 °C, respectively. Both compounds were highly hygroscopic. Carbamoylcholine chloride was a commercial product (Doryl). DCF was provided by Dr. G. Schrader of Farbenfabriken-Bayer, Wuppertal-Elberfeld, Germany.

Enzyme preparations

Cholinesterase, arylesterase and albumin were purified preparations from human serum.[10] A purified preparation of acetylcholinesterase from *Torpedo* electric organ was also used.

Biochemical methods

Paper chromatography was carried out according to a method recently described.[11] The solvent used was a *n*-butanol–ethanol–acetic acid–water mixture (8:2:1:3). The ascending technique was used and chromatograms were developed by spraying with ninhydrin for the detection of dimethyl- and monomethylamine, and with dipicrylamine (0·2 g in 50 ml acetone and 50 ml distilled water) for the detection of choline and its derivatives.

Esterase activity was determined with the Warburg technique[12] using acetylcholine chloride as substrate for cholinesterases, and expressed in b_{30} values, i.e. μl CO_2 evolved in 30 min from a bicarbonate–CO_2 buffer with corrections for nonenzymic hydrolysis of the substrate.

Pharmacological methods

Acute toxicity. The acute toxicity of the compounds was tested by intraperitoneal injection in albino mice weighing about 20 g. The compounds were dissolved in isotonic saline, and the injected volume did not exceed 0·4 ml. The determinations were designed as rough screenings in groups of ten mice.

Experiments in the anaesthetized cat. The effects of the esters were studied in eight cats, anaesthetized with sodium pentobarbital (30–40 mg/kg) administered intraperitoneally. Drugs were injected intravenously through a plastic cannula in a femoral vein. The following parameters were recorded by passing the output of suitably arranged transducers into a Grass Model 5 Polygraph.

Respiration was recorded as pressure differences in the tracheal cannula by means of a pressure transducer (Model PT5, Grass Instrument Corporation). Blood pressure was recorded by means of a Statham Electromanometer from the left carotid artery. Pulse rate was estimated by the use of an interval recorder as described by Goldschmith and Lindgren.[13] The effects on neuromuscular transmission were demonstrated by recording semi-isometric contractions of the gastronemius muscle in response to supramaximal electrical stimulation of the sciatic nerve by means of a Grass stimulator, Model S4. The stimuli were applied to the nerve distally to a crushed region by means of shielded silver electrodes. A force-displacement transducer Model FT10, Grass Instrument Corporation was used for recording the muscle contractions.

Contractions of the nictitating membrane were recorded by means of a force-displacement transducer, FT03. The excitability of the membrane was tested by preganglionic stimulation of the exposed cervical sympathetic trunk for 10 sec with rectangular pulses at a frequency of 30 c/s. Duodenal intraluminal pressure was recorded by means of a Statham electromanometer connected to a balloon, inserted through the oesophagus into the duodenum. The insertion necessitated laparotomy. The balloon and the cannula were filled with water. Femoral arterial blood flow was recorded by photoelectric drop recording with the aid of an interval recorder.[14] The equipment allowed the simultaneous recording of five parameters.

Frog rectus abdominis muscle preparation. The rectus muscle was suspended in a 3 ml bath containing eserinized frog-Ringer solution at room temperature. The preparation was aerated and left in contact with the drugs for exactly 90 sec. The response was compared with that of an acetylcholine standard and expressed as the relative molar potency in percentage units (i.e. as the number of moles of acetylcholine giving a response equivalent to that of 100 moles of the test substance).

Guinea-pig ileum preparation. A test bath of 10 ml was used. An automatic timer regulated the cycle of operations except for the addition of the compounds, which was done manually in a small volume on top of the test bath. The results were expressed as the relative molar potency as defined above.

RESULTS

Stability tests

Aqueous solutions of both MeCbCh and Me_2CbCh were very stable and could be kept at room temperature (22 °C) without any loss in biochemical and pharmacological activities. Even in 0·01 M sodium hydroxide these compounds were stable for several days. Proof of this stability was obtained by paper chromatography (Fig. 1) and the

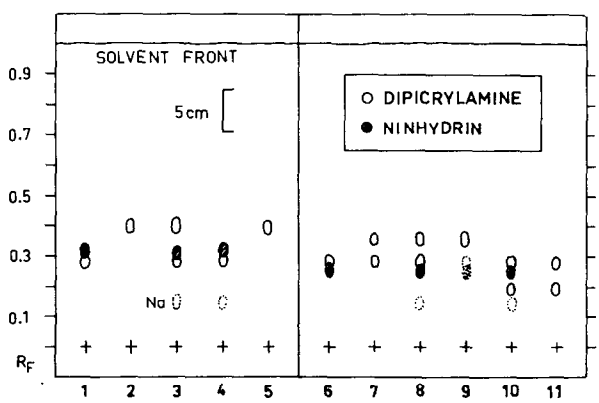


FIG. 1. Paper chromatograms of neutral and alkaline solutions (1 per cent) of MeCbCh and Me_2CbCh. Solvent, *n*-butanol–ethanol–acetic acid–water (8:2:1:3). Alkaline solutions made with 0·01 M NaOH. (1) Choline and Me_2NH; (2) Me_2CbCh, neutral soln.; (3) Me_2CbCh, alkaline soln. boiled for 4 hr: (4) choline and Me_2NH, alkaline soln. boiled for 4 hr; (5)Me_2CbCh, neutral soln. boiled for 4 hr; (6) choline and $MeNH_2$; (7) MeCbCh and choline; (8) MeCbCh, alkaline soln. boiled for 4 hr; (9) MeCbCh, neutral soln. boiled for 4 hr; (10) carbamoylcholine and $MeNH_2$, alkaline soln. boiled for 4 hr; (11) carbamoylcholine and choline.

TABLE 1. STABILITY OF AQUEOUS SOLUTIONS OF MECBCH AND ME_2CBCH MEASURED BY THEIR CHOLINESTERASE INHIBITING ACTIVITIES

(50 mM solutions of the choline esters were kept at 22 °C for various periods of time and tested against human serum cholinesterase with acetylcholine (10 mM) as substrate. Inhibiting activity expressed as the percentage inhibition obtained with dilutions of the stock solutions to give 0·01 mM choline ester in the reaction mixture. Values in brackets refer to 1·0 mM stock solutions in 0·01 M NaOH.)

Inhibitor	Zero time	24 hr	12 days
MeCbCh	63 (62)	64 (68)	65
Me_2CbCh	34	38	31

esterase inhibition technique (Table 1). No hydrolysis products were observed on paper chromatograms, and the esterase inhibition was unaltered with neutral and alkaline solutions, kept for various periods of time. Neutral solutions of the compounds kept for weeks did not alter their pharmacological activities. When the alkaline solutions were boiled for 4 hr the compounds were split into choline and methylamines, detected on paper chromatograms (Fig. 1).

Biochemical studies

Enzymic degradation. No enzymic hydrolysis of the methylated carbamoylcholines was observed with the bicarbonate method using high concentrations of purified preparations of butyrylcholinesterase, acetyl arylesterase and albumin from human serum. When these reaction mixtures were kept for 24 hr at 22 °C with subsequent paper chromatographic analyses of the mixtures, no degradation products were identified on the chromatograms. These same mixtures were tested pharmacologically after 2 weeks and showed the same activities as freshly prepared solutions. These results indicate that the N-methylated carbamoylcholines, as well as the unsubstituted compound known previously, are not hydrolysed by the three enzyme preparations tested.

Cholinesterase inhibition. The inhibitory effects of MeCbCh and Me_2CbCh on acetylcholinesterase of *Torpedo* electric organ and on butyrylcholinesterase of human serum are illustrated in Figs. 2 and 3; for comparison, the results obtained with unsubstituted carbamoylcholine and DCF are also included. The methylated choline esters were much stronger inhibitors than the unsubstituted ester, and less active than DCF, especially with serum cholinesterase. Choline, monomethylamine and dimethylamine had no inhibiting effect when used in the same concentration as those of the esters (1·0 mM) and under the same experimental conditions.

In the experiments with acetylcholinesterase (Fig. 2) the inhibitory effects of carbamoylcholine and DCF were almost independent of incubation time, i.e. the time during which the enzyme was incubated with the inhibitor before the substrate (acetylcholine) was added. Thus, there was little difference in the degree of inhibition obtained when the inhibitor was added to the enzyme before or at the same time as the substrate. DCF was a stronger inhibitor (pI_{50} = 4–4·5) of this esterase than was carbamoylcholine ($pI_{50}<3$).

Butyrylcholinesterase behaved differently towards carbamoylcholine and DCF, the reaction of the latter with this esterase being progressive. DCF was a much stronger

inhibitor than carbamoylcholine. A concentration of the substrate a hundred times higher than that of DCF was unable to reverse the esterase activity when added 6 min after the addition of the inhibitor (Fig. 3, *D*). There was little difference in the degree of inhibition obtained after 6 and 180 min incubation (pI_{50} = 5·5). The reaction of DCF with human serum cholinesterase was therefore a comparatively rapid process of an irreversible type.

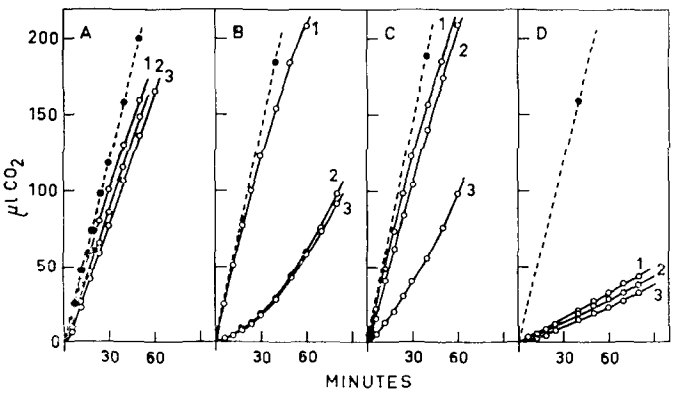


FIG. 2. Inhibition of acetylcholinesterase of electric organ by carbamoylcholines and DCF. Substrate, 10 mM acetylcholine chloride. Inhibitor, 1·0 mM carbamoylcholine chloride, its N-methyl derivatives, or DCF. Inhibitor-concentration values refer to those during esterase determination; the concentrations during enzyme-inhibitor incubation, before the addition of substrate, were five times higher. *A*: carbamoylcholine; *B*: MeCb Ch; *C*: Me_2CbCh; *D*: DCF; ●---●, control; (1) inhibitor and substrate simultaneously mixed with the enzyme; (2) and (3): enzyme incubated 6 and 180 min, respectively, with the inhibitor before the addition of substrate.

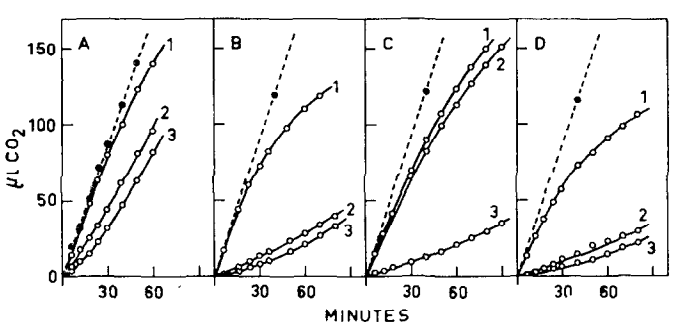


FIG. 3. Inhibition of human serum cholinesterase by carbamoylcholines and DCF. Experimental conditions and keys as in Fig. 3, except for the concentration of DCF, which was 0·01M.

The inhibitory properties of the methylated carbamoylcholines were intermediate between those of carbamoylcholine and DCF. The reaction rates with both acetyl- and butyrylcholinesterase were much higher for MeCbCh than for the Me_2CbCh. The inhibiting effects were stronger for butyrylcholinesterase than for acetylcholinesterase. After 180 min incubation, depending on the degree of inhibition, added substrate could reverse the esterase activity only partly or not at all.

These results indicate that the esterase inhibition by the methylated carbamoylcholines was irreversible. Dialysis of the reaction mixtures of esterase and MeCbCh also failed to reactivate the inhibited enzyme especially after long periods of incubation. In addition, esterase inhibition at various substrate concentrations, evaluated graphically in Fig. 4, was shown to be non-reversible. With reversible inhibition (competitive

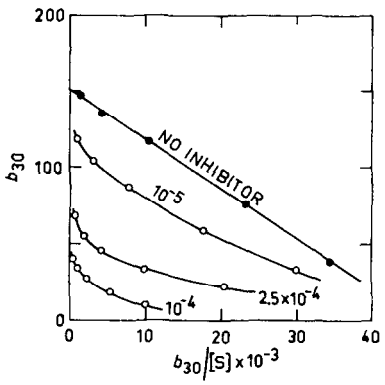


FIG. 4. Graphical evaluation of the inhibition of human serum cholinesterase by Me_2CbCh as function of substrate concentration. The esterase was incubated for 50 min with the inhibitor before the addition of acetylcholine. Esterase activities (b_{30}) were extrapolated values of hydrolysis rates 30 min after the addition of the substrate (S).

or non-competitive) these lines should be straight. The substituted carbamoylcholines probably reacted with the esteratic site of the enzyme molecule, because choline, in contrast to acetylcholine (Figs. 2 and 3), could not protect the enzyme from inhibiton, as was shown in an experiment (Table 2) with butyrylcholinesterase and MeCbCh.

TABLE 2. EFFECT OF CHOLINE ON THE CHOLINESTERASE INHIBITION BY MeCbCh

(Substrate, 10 mM acetylcholine chloride. Concentrations of choline chloride and MeCbCh during esterase determination, 10 mM and 2 mM, respectively. Controls performed in the absence of both choline and MeCbCh. In the presence of choline and MeCbCh the compound marked with an asterisk was added 6 min after the addition of the other. Activity values refer to b_{30} extrapolated from equilibrium curves.)

Additions	Acetylcholinesterase	Butyrylcholinesterase
Nil (control)	120	89
Choline	102	75
MeCbCh	28	7
Choline + MeCbCh	30	8
MeCbCh + choline	26	8

Pharmacological studies

Acute toxicity. The results of the toxicity determinations are summarized in Table 3.

Experiments in the anaesthetized cat

Effects on respiration. Both the monomethyl and the dimethyl analogue were powerful respiratory stimulants. The effect rapidly followed intravenous injection, and then

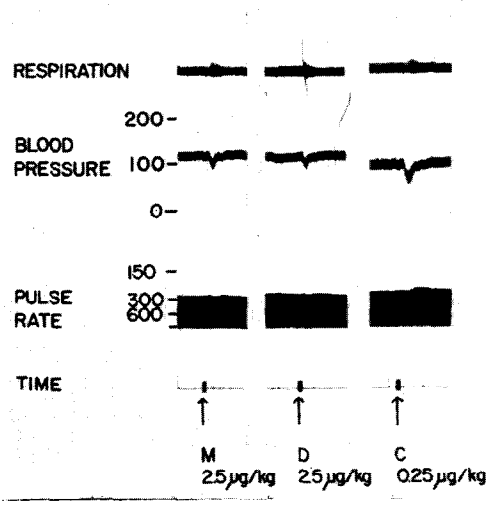


FIG. 7. Effects of MeCbCh (*M*), Me_2CbCh (*D*) and carbamoylcholine (*C*) on respiration, blood pressure and pulse rate. Time in minutes. Interval between injections 15 min. Cat 1·5 kg. Sodium pentobarbital anaesthesia.

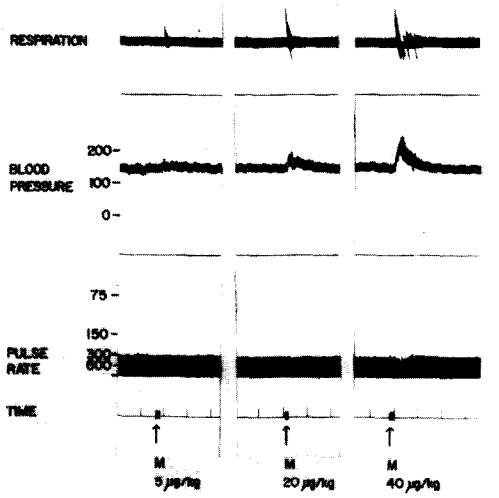


FIG. 9. Effects on respiration, blood pressure and pulse rate of increasing doses of MeCbCh (*M*) following atropinization (1 mg per kg). Time in minutes. Interval between injections 15 min. Cat 1·5 kg. Sodium pentobarbital anaesthesia.

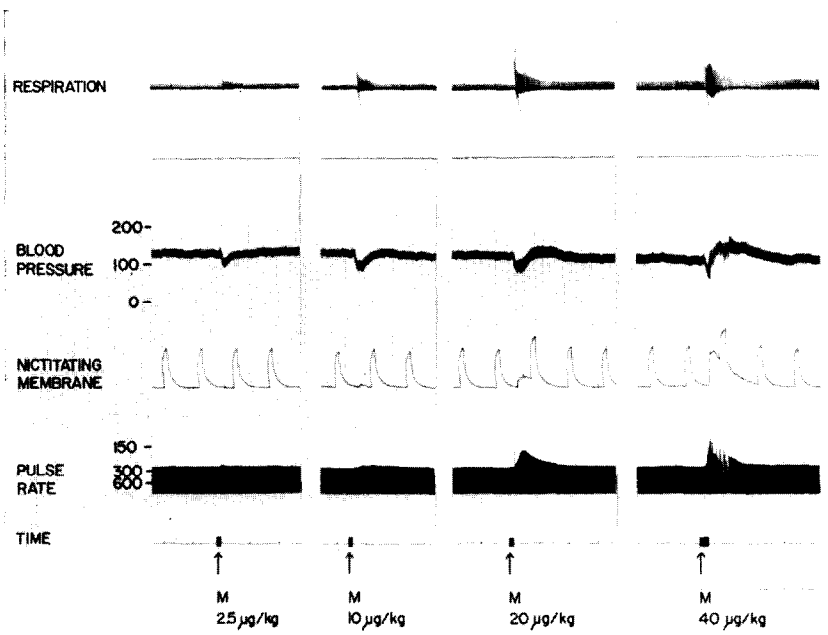


FIG. 8. Effects of increasing doses of MeCbCh (*M*) on respiration, blood pressure, nictitating membrane and pulse rate. Time in minutes. Interval between injections 15 min Cat 1·5 kg. Sodium pentobarbital anaesthesia.

contractions of the nictitating membrane and an increase in the response after stimulation of the sympathetic trunk (Fig. 8). Doses up to 100 μg/kg were tested, and no signs of blockade of the response to preganglionic stimulation were seen. The effects on the nictitating membrane were completely abolished by hexamethonium.

Duodenal intraluminal pressure. Carbamoylcholine produced an increase in the duodenal intraluminal pressure of considerably longer duration than did the substituted analogues, which also had a stimulating effect although slight.

Effect on femoral arterial blood flow. At doses exceeding 20 μg/kg i.v. the two substituted analogues produced an increase in femoral arterial blood flow, which coincided with the elevation of blood pressure and was followed by a decrease of short duration. When the blood pressure had reached a normal level, the blood flow was also usually normalized. Sometimes an initial decrease in blood flow was noticed. This appeared at the same time as the initial fall in blood pressure, and was most evident for the monomethyl derivative.

Effects on the isolated guinea-pig ileum and frog rectus. The results are summarized in Table 4. Carbamoylcholine was as potent as acetylcholine on the ileum, while the methylated compounds were much less potent. The effects on the guinea-pig ileum were completely abolished by atropine. On this preparation a bell-shaped dose–response curve was obtained for the two substituted analogues. On the frog rectus both the substituted derivatives were approximately four times more potent than carbamoylcholine and five times less potent than acetylcholine.

TABLE 4. RELATIVE MOLAR POTENCY (ACETYLCHOLINE = 100) OF CARBAMOYLCHOLINE AND THE TWO N-METHYLATED DERIVATIVES ON THE GUINEA-PIG ILEUM AND FROG RECTUS

Compound	Relative molar potency (Acetylcholine = 100)	
	Guinea-pig ileum	Frog rectus
Carbamoylcholine	100	5
MeCbCh	2·5	20
Me_2CbCh	0·7	20

DISCUSSION

The results of the pharmacological studies with the N-methylated carbamoylcholines presented in the present paper confirm previous observations[4] that substitution in the amino group of carbamoylcholine greatly reduces the muscarine-like activity. Me_2CbCh has almost pure nicotine-like activity and the monomethyl derivative is intermediate. The stimulating effect on respiration observed with the methylated derivatives was previously demonstrated with other choline esters.[15] The mechanism underlying this effect, however, has not been further investigated. Nor was it possible to explain the decrease in the response of the guinea-pig ileum produced by increasing doses of the methylated derivatives. Similar bell-shaped dose–response curves have been obtained with other quaternary ammonium compounds, and the phenomenon was recently discussed by van Rossum and Ariëns[16]. Possibly this decrease might be due to the reaction of the receptor with the carbamate moiety at high doses instead of with the choline radical of the molecule.

The methylated carbamoylcholines are moderate cholinesterase inhibitors and less active in this respect compared with the most active of known carbamates (e.g. physostigmine and the prostigmine analogues). The inhibitory mechanism is probably a carbamoylation of the active sites of the esterase molecule; the esteratic site is most likely to be involved. The results obtained with DCF[7,17] and a number of other N-alkyl and N:N-dialkylcarbamates[7] have indicated that the inhibition of cholinesterases by these agents involves the formation of carbamoyl derivatives of the esterases. The inhibitory process observed with the methylated carbamoylcholine is probably similar. Quantitative differences exist between various derivatives in this group of esterase inhibitors. The more easily the carbamate link can be broken by hydrolysis the more rapidly and strongly is the enzyme inhibited. The irreversible nature of this inhibitory reaction is analogous to the phosphorylation of esterases by organic phosphate esters.

In vivo no potentiation of the response to acetylcholine could be demonstrated with the methylated carbamoylcholines. This is probably due to the protective effect of the substrate (acetylcholine) on the esteratic site against inhibition (choline had no such effect), to the relatively low affinity of the compounds to the esterase, and to the fact that the reaction (carbamoylation) with the esterase is a comparatively slow process.

The biochemical studies indicated that the compounds studied are extremely stable in aqueous solutions and no esterase preparations tested were able to hydrolyse them at a measurable rate. However, the effects of the compounds *in vivo* are of comparatively short duration and no cumulative effect could be demonstrated. Plasma esterases are only occasionally involved in the hydrolysis of these compounds, as has also been noticed previously with other carbamates.[7,8] Other systems must be involved in the detoxication, the nature of which is not known.

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