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FLUORINATED ALDEHYDES AND KETONES ACTING AS QUASI-SUBSTRATE INHIBITORS OF ACETYLCHOLINESTERASE

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Summary

1. The inhibition of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) by compounds containing trifluoromethyl-carbonyl groups was investigated and related to the effects observed with structurally similar, non-fluorinated chemicals.

2. Compounds that in aqueous solution readily form hydrates inhibit acetylcholinesterase in a time-dependent process. On the other hand non-hydrated, carbonyl-containing compounds showed rapid and reversible, time-independent enzyme inactivation when assayed under steady state conditions.

3. *m*-*N*,*N*,*N*-Trimethylammonium-acetophenone acts as a rapid and reversible, time-independent, linear competitive inhibitor of acetylcholinesterase ($K_i = 5.0 \cdot 10^{-7}$ M).

4. The most potent enzyme inhibitor tested in this series was *N*,*N*,*N*-trimethylammonium-*m*-trifluoroacetophenone. It gives time-dependent inhibition and the concentration which inactivates eel acetylcholinesterase to 50% of the original activity after 30 min exposure is $1.3 \cdot 10^{-8}$ M. The bimolecular rate constant for this reaction is $1.8 \cdot 10^{6}$ l · mol^{-1} · min^{-1}. The enzyme-inhibitor complex is very stable as the inhibited enzyme after 8 days of dialysis is reactivated to 20% only. This compound represents a quasi-substrate inhibitor of acetylcholinesterase.

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Introduction

According to Rando [1] enzyme inhibitors that possess a latent reactive group which specifically interacts with a functional group at the active center of an enzyme during its catalytic cycle have been termed k_{cat} inhibitors. Alternatively these same effectors have been viewed as 'suicide substrates' [2]. They are structured in such a way that they become activated by the target enzyme leading to the formation of an enzyme-inhibitor complex. The enzyme thus by its specific mode of action catalyzes its own inactivation.

On the other hand, molecules that resemble the substrate in its transition state geometry may be specifically recognized by enzymes. By virtue of this resemblance they usually bind much tighter to the enzyme than do the parent substrates. Such molecules have been termed 'transition state-analog inhibitors' [3—5] and offer an interesting experimental approach to the design of novel, potent enzyme inhibitors of high specificity as well as to the elucidation of the mechanism of enzyme catalysis.

Serine and cysteine hydrolases such as elastase and papain presumably catalyze their own acylation and deacylation by stabilizing intermediates of tetrahedral character in both steps [6]. On this assumption, aldehydes and ketones, analogous in structure to the acyl portion of the substrates of serine and cysteine hydrolases, have been prepared and examined as potential inhibitors of these enzymes [7—12]. Such studies confirmed that the binding of aldehydes involves the serine hydroxyl located at the active site of serine hydrolases. This serine hydroxyl group is thought to form a hemiacetal linkage resulting in a tetrahedral structure. More recently the concept of transition state-analog inhibitors has been applied to acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) which functions by a double-displacement mechanism similar to the one of the above-mentioned serine and cysteine proteases. Dafforn et al. [13,14] tested a number of ketones, structurally related to acetylcholine, the substrate of acetylcholinesterase, as possible transition state-analog inhibitors. Among all compounds under investigation 4-oxo-pentane-*N*,*N*,*N*-trimethylammonium chloride was the most potent inhibitor which was calculated to bind to the enzyme approximately 120 times as strong as was the estimated binding of acetylcholine [13].

Ketones per se, unless they contain electron-withdrawing substituents, however, exhibit a much weaker tendency to add nucleophiles than do aldehydes, a well-known fact that has recently been restated by Wolfenden [6]. In view of this certainty, it was of interest to us to compare the effect on acetylcholinesterase of molecules carrying an activated carbonyl group to those observed with structurally similar compounds carrying no such chemically activated group. Rama Sastry and Chiou [15,16] studied the hydrolysis of halogen-substituted acetylcholine derivaties. They found that such esters, although activated by α-halogen atoms were no better substrates for acetylcholinesterase than the parent substrate. On the contrary steric effects arising from the bulky halogen atoms on the acyl group impeded the binding of these compounds to the enzyme thus reducing the overall catalytic efficiency.

On the other hand, substitution of hydrogen atoms by fluorine atoms enlarges the Van der Waals' radius of a compound to a small extent only. Con-

sequently aldehydes and ketones bearing a trifluoromethyl group were tested as potential inhibitors of acetylcholinesterase. This paper details the effects obtained with such compounds and compares them to the ones observed with the parent, non-fluorinated molecules. A preliminary account of this work has been published previously [17].

Materials and Methods

Reagents and chemicals. Sepharose 4B was from Pharmacia, Bromma (Sweden), acrylamide and *N,N*-methylenbisacrylamide from Serva, Heidelberg (F.R.G.). All other reagents were standard commerical products of highest purity available. They were purchased either from Fluka AG, Buchs (Switzerland) or from E. Merck AG, Darmstadt (F.R.G.).

Solvents. Unless otherwise stated, all solvents were reagent grade and purchased from E. Merck AG, Darmstadt (F.R.G.).

Syntheses. *m-N,N.N*-Trimethylammonium-acetophenone iodide was prepared from *m*-aminoacetophenone by exhaustive methylation using standard experimental procedures. The product was isolated as crystalline iodide salt.

m-N,N,N-Trimethylammonium-trifluoroacetophenone iodide was prepared by methylation with methyl iodide of the corresponding tertiary amine which in turn was prepared as described by Salvador and coworkers [18,19].

Enzyme. Acetylcholinesterase Type III was purchased from Sigma Chemical Company (St. Louis, MO U.S.A.) or extracted from the electric tissue of *Electrophorus electricus*. The enzyme was routinely purified by the method of biospecific adsorption onto an affinity gel, the preparation of which has been described [20]. The affinity chromatography was carried out as outlined in Ref. 16 except that Triton X-100 was omitted throughout the purification procedure. The purity of the enzyme was assessed by sodium dodecylsulfate gel electrophoresis under reducing conditions [21].

The functional normality of purified acetylcholinesterase was determined using methyl-*O*-ethyl-*S*-(2-diisopropylaminoethyl)phosphonothionate (gas chromatographically pure) as active site titrant. To 2 ml enzyme solution (mainly form D of eel acetylcholinesterase, containing 2586 I.U. and 0.544 mg protein) were added successively 10 μl 5 mM 5,5′-dithio-bis(2-nitrobenzoic acid) and 1.2 μl 40 mM active-site titrant. The release of *N,N*,-diisopropylamino ethanethiol was measured spectrophotometrically. The functional molarity of the above enzyme solution was determined to be $2.85 \cdot 10^{-6}$ M. Thus 1 mg enzyme was equivalent to $1.05 \cdot 10^{-8}$ mol active sites. Correspondingly, a subunit molecular weight of 95 400 was calculated, which is in good agreement with that obtained by Bon et al. [22] for the same pure enzyme form. Pure enzyme was routinely stored at 4° C in a buffer of 10 mM sodium phosphate (pH 7.4), 1 M NaCl and 0.05% (w/v) NaN_3.

Enzyme assay. Acetylcholinesterase activity was estimated at 25° C according to the method of Ellman et al. [23]. Unless otherwise specified the assay mixture contained 1 mM acetylthiocholine, 0.125 mM, 5,5′-dithio-bis(2-nitrobenzoic acid) in a total volume of 1 or 3 ml 100 mM sodium phosphate buffer (pH 7.4). The reaction was started by the addition of 0.01—0.05 I.U. acetyl-

cholinesterase and was followed spectrophotometrically by the increase in absorbance at 412 nm on a Beckman DB-G spectrophotometer equipped with a W + W recorder 3002.

Inhibition experiments. Depending on the nature of the inhibitors employed, both time-dependent or time-independent enzyme inhibition was observed. Time-independent inhibition was reversible and assayed under steady state conditions. Its results were obtained as double-reciprocal plots [24] and their secondary plots [25]. Lines were fitted to the points by the methods of least squares using a Hewlett Packard 9100 A computer. Apparent K_i values were determined according to Cleland [25] and Dixon and Webb [26]. In these experiments the substrate has routinely been varied between 5 and 100 μM.

In the case of time-dependent inhibition, the enzyme (10—50 I.U.) was incubated with the inhibitor in a buffer of 100 mM sodium phosphate (pH 7.4). At indicated times, 1 or 3-μl aliquots of enzyme solution were withdrawn and added to 1 or 3 ml, respectively, of assay solution. This reduced the inhibitor concentration by 1000-fold thus abolishing the non-covalent contribution, if any, to enzyme inhibition.

Water-insoluble compounds were dissolved in methanol and added to the assay and/or incubation mixture in such a way that the final concentration of methanol did not exceed 1.0%.

Results

Acetylcholinesterase is inhibited by several aliphatic and aromatic carbonyl group-containing compounds. Activation of the carbonyl group by three adjacent fluorine atoms resulted in more potent enzyme inhibition.

The simplest compounds tested in this series were trifluoroacetaldehyde hydrate and trifluoroacetaldehyde-methylhemiacetal. Fig. 1 shows the inhibition by trifluoroacetaldehyde hydrate and gives evidence that the inhibition is time-dependent and levels off after reaching a state of equilibrium. The extent of the inhibition increased with increasing inhibitor concentration. Upon dilution enzyme activity was restored in a time-dependent process too (Fig. 1). Acetaldehyde (1 or 5 mM) inhibited the enzyme to a small extent only. At 20 mM aldehyde enzyme activity was decreased by 37%. The inhibition was rapid, time-independent, and it was readily reversed upon dilution (inset Fig. 1). Similar results to those shown in Fig. 1 were obtained with trifluoroacetone hydrate. On the other hand neither acetone (100 mM) nor hexafluoroacetone (20 mM) inhibited the enzyme to any extent. The results of the time-dependent inhibition experiments are summarized in Table I, which lists the concentration of inhibitor necessary to yield a 50% enzyme inhibition after 30 min exposure time. In order to compare these results with those of known compounds that inhibit acetylcholinesterase in a time-dependent process [27], the negative logarithm of the I_{50} values and the apparent bimolecular rate constants are also listed. The simplest aromatic trifluoroketone is trifluoroacetophenone. This compound for reasons of solubility was added to the enzyme in a solution of methanol. Its inhibition was a rapid reversible time-independent process with a competitive kinetic pattern (Fig. 2). Trifluoroacetophenone inhibited the enzyme 100-times stronger than did acetophenone.

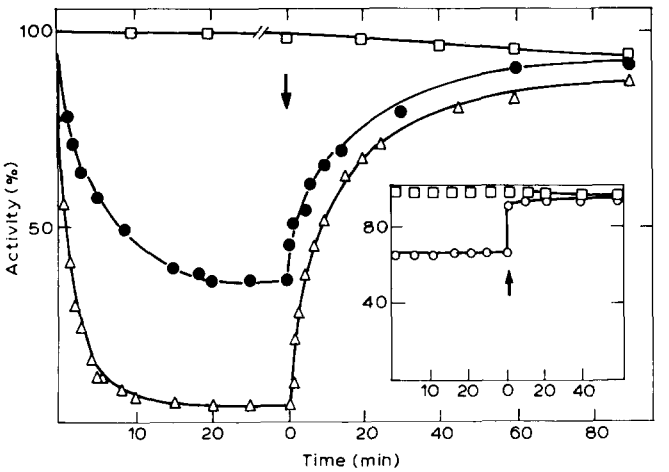


Fig. 1. Time course of inhibition of acetylcholinesterase by trifluoroacetaldehyde-hydrate. To 20 I.U. of the enzyme in 100 mM sodium phosphate buffer (pH 7.4) trifluoroacetaldehyde-hydrate (final concentration 1 mM (●———●) or 5 mM (△———△)) was added. At time indicated 3 μl were withdrawn and added to 3 ml of the assay mixture described under Materials and Methods. After 30 min (arrow) the enzyme and inhibitor-containing incubation mixture was diluted 200-fold by the addition of the above buffer. Reappearance of enzyme activity was then determined as follows: diluted enzyme solution (0.6 ml) was added to 2.4 ml of an assay mixture in which the concentrations of acetylthiocholine and 5,5′-dithio-bis-(2-nitrobenzoic acid) was increased by a factor of 1.25 which corrected the dilution imposed by the addition of 0.6 ml enzyme to 2.4 ml of substrate/5,5′-dithiobis(2-nitrobenzoic acid) solution. □———□, control of acetylcholinesterase activity measured under identical conditions but in absence of added inhibitor. The inset shows the time course of enzyme inhibition observed in the presence of 20 mM acetaldehyde hydrate and the effect of 200-fold dilution (arrow).

Introducing a dimethylamino group in the meta position further increased the power of inhibition. *m*-Dimethylaminotrifluoroacetophenone was added to the enzyme in aqueous solution and showed a time-dependent inhibition pattern (Table I). On the other hand the non-fluorinated, parent compound under identical conditions gave a time-dependent linear, compe titive inhibition.

TABLE I

CONCENTRATION OF TRIFLUORO-CARBONYL CONTAINING-COMPOUNDS GIVING 50% ENZYME INHIBITION WITHIN 30 min EXPOSURE TIME AND APPARENT BIOMOLECULAR RATE CONSTANTS

The pI_{50} value is the negative logarithm of the inhibitor concentration that inactivates acetylcholinesterase to 50% of the control activity within a 30 min exposure time [27]. k_2 is the apparent bimolecular rate constant [27] calculated from the I_{50} value according to the relationship $k_2 = 0.695/(I_{50} \cdot t)$ in which t = 30 min.

Compound	I_{50} (M)	$l \cdot mol^{-1} \cdot min^{-1}$	
		pI_{50}	k_2
Trifluoroacetaldehyde methylhemiacetal	$6.3 \cdot 10^{-4}$	3.2	$1.5 \cdot 10^2$
Hydrates of			
Trifluoroacetaldehyde	$6.3 \cdot 10^{-4}$	3.2	$1.5 \cdot 10^2$
Trifluoroacetone	$6.3 \cdot 10^{-4}$	3.2	$1.5 \cdot 10^2$
Hexafluoroacetone	(no inhibition at 20 mM)		
N,*N*-Dimethyl-amino-*m*-trifluoroacetophenone	$1.0 \cdot 10^{-7}$	7.0	$2.3 \cdot 10^5$
N,*N*,*N*-Trimethylammonium-*m*-trifluoroacetophenone	$1.3 \cdot 10^{-8}$	7.9	$1.8 \cdot 10^6$

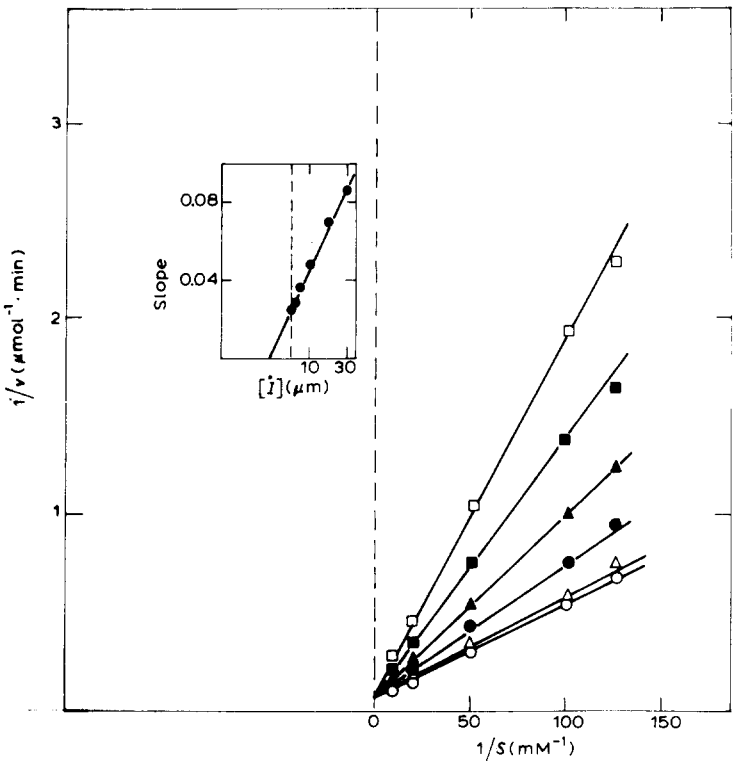


Fig. 2. Double-reciprocal plot showing the inhibition of acetylcholinesterase by trifluoroacetophenone at: ○, zero; △, 1 μM; ●, 5 μM; ▲, 10 μM; ■, 20 μM, and □, 30 μM inhibitor concentration, respectively. The inset shows the secondary plot of slopes vs. inhibitor concentration.

Quaternization of the nitrogen atom had no effect on the type of inhibition observed, but further increased the inhibitory power of these compounds. Fig. 3 shows the double-reciprocal plot of the inhibition data obtained with *m*-trimethylammonium-acetophenone and Table II summarizes the data obtained with the compounds showing a time-independent enzyme inhibition. Fig. 4 gives the time course of acetylcholinesterase inactivation by trimethylammonium-trifluoroacetophenone. It also shows that under identical assay conditions the inhibition by the non-fluorinated compounds is abolished upon dilution. Contrary to the afore-mentioned trifluorocarbonyl compounds, no rapid reactivation upon dilution was obtained with enzyme inhibited by *m*-dimethylamino- and *m*-trimethylammonium-trifluoroacetophenone. In fact the latter compound formed an extremely stable enzyme-inhibitor complex. Dialysis at 4° C with two daily buffer changes after 8 days resulted in 20% enzyme reactivation only.

The specificity of interaction of *m*-trimethylammonium-trifluoroacetophenone with different cholinesterases and other serine hydrolases was also investigated. Besides eel acetylcholinesterase this inhibitor inactivated acetylcholinesterase from human erythrocytes, serum cholinesterase and the enzyme from plaice body muscle. The concentrations yielding 50% enzyme inhibition after 30 min exposure are summarized in Table III. On the other hand neither

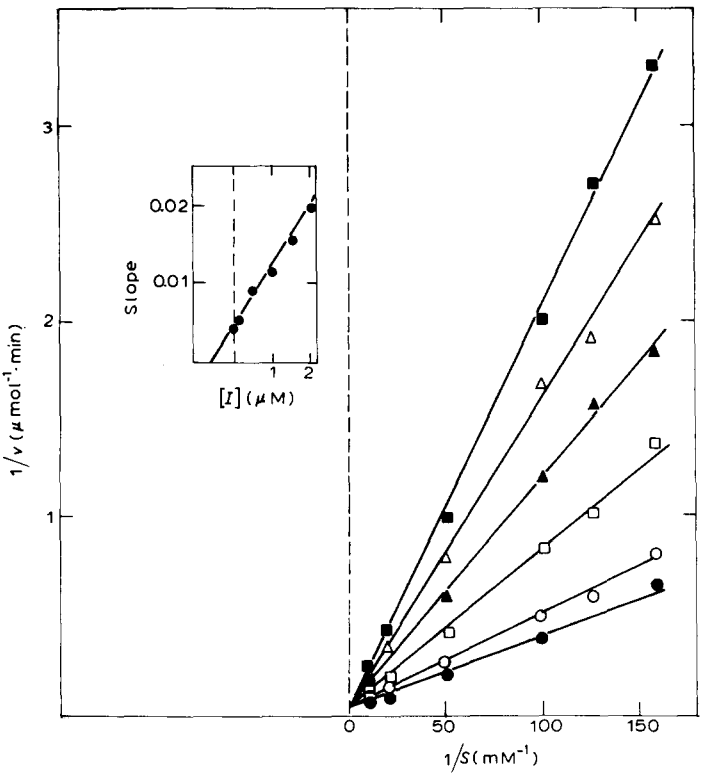


Fig. 3. Double-reciprocal plot showing the inhibition of acetylcholinesterase by *m*-trimethylammonium-acetophenone at: ●, zero; ○, 0.1 μM; □, 0.5 μM; ▲, 1 μM; △, 1.5 μM, and ■, 2.0 μM inhibitor concentration, respectively. The inset shows the secondary plot of slopes vs. inhibitor concentration.

trypsin, chymotrypsin nor elastase were inhibited by this compound. In order to relate the effects of these novel inhibitors to those derived from organophosphorus compounds, the amount of remaining activity found after 30 min incubation with the inhibitor is plotted as a function of the logarithm of the inhibitor concentration (inset Fig. 5). From this figure, it was calculated that *m*-trimethylammonium-acetophenone exceeds diisopropylphosphorofluoridate as inhibitor of acetylcholinesterase by a factor of 154. When tested in vivo

TABLE II

SUMMARY OF LINEAR, COMPETITIVE INHIBITION DATA OBTAINED WITH THOSE COMPOUNDS THAT INHIBITED ACETYLCHOLINESTERASE IN A TIME-INDEPENDENT, REVERSIBLE WAY

Compound	Inhibition constants derived from Dixon plots (M)
Acetophenone	$1.6 \cdot 10^{-3}$
Trifluoroacetophenone	$1.8 \cdot 10^{-5}$
m-Dimethylaminoacetophenone	$9.0 \cdot 10^{-6}$
m-Trimethylammonium-acetophenone	$5.0 \cdot 10^{-7}$

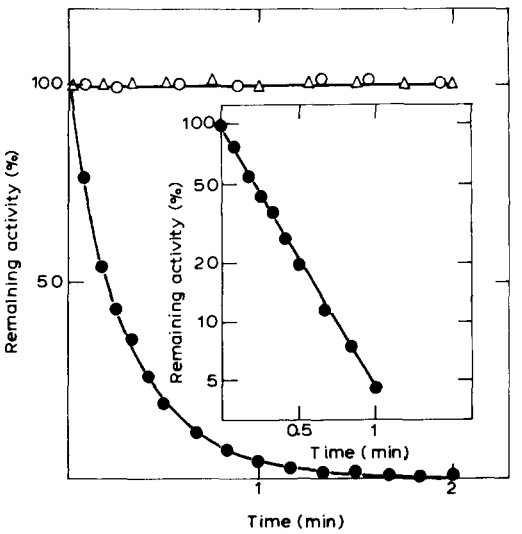


Fig. 4. Time course of inhibition of acetylcholinesterase by trimethylammonium-trifluoroacetophenone (•). The enzyme (1 I.U.) was incubated with inhibitor (final concentration 1.0 μM and the inactivation was monitored by determining the remaining activity at times indicated. When assayed under identical conditions, *m*-trimethylammonium-acetophenone (1 μM final concentration (△)) gave no inhibition. Control for enzyme activity measured under identical conditions in absence of inhibitor (○). The inset shows semilogarithmic plot of the time course of enzyme inactivation (•).

m-trimethylammonium-trifluoroacetophenone also proved to be a strong anticholinesterase agent. The inhibitor was dissolved in pyrogen-free, physiological saline solution and injected intraperitoneally into mice. At doses above the LD_{50} the animals, within the first few minutes after administration of the toxic agents, developed the typical signs observed with anticholinesterase agents. Fig. 6 shows dose-response curves obtained with the aromatic trifluoroketones. Mortality is plotted on a Hazen probability scale as outlined by Ther [28]. From such plots the dose killing 50% of the mice injected (LD_{50}) are obtained. Table IV summarizes these values and compares them to those obtained with the non-fluorinated compounds.

TABLE III

INHIBITION OF CHOLINESTERASES BY *N,N,N*-TRIMETHYLAMMONIUM-*m*-TRIFLUOROACETOPHENONE AND COMPARISON TO INHIBITION BY DIISOPROPYLPHOSPHOROFLUORIDATE

A, *N,N,N*-trimethylammonium-*m*-trifluoroacetophenone; B, diisopropylphosphorofluoridate.

Enzyme source	I_{50} (M)	
	A	B
Electric eel	$1.3 \cdot 10^{-8}$	$2.0 \cdot 10^{-6}$
Human erythrocytes	$2.0 \cdot 10^{-8}$	$3.6 \cdot 10^{-7}$
Human serum	$5.0 \cdot 10^{-7}$	$6.3 \cdot 10^{-9}$
Plaice body muscle	$1.3 \cdot 10^{-8}$	$7.1 \cdot 10^{-10}$

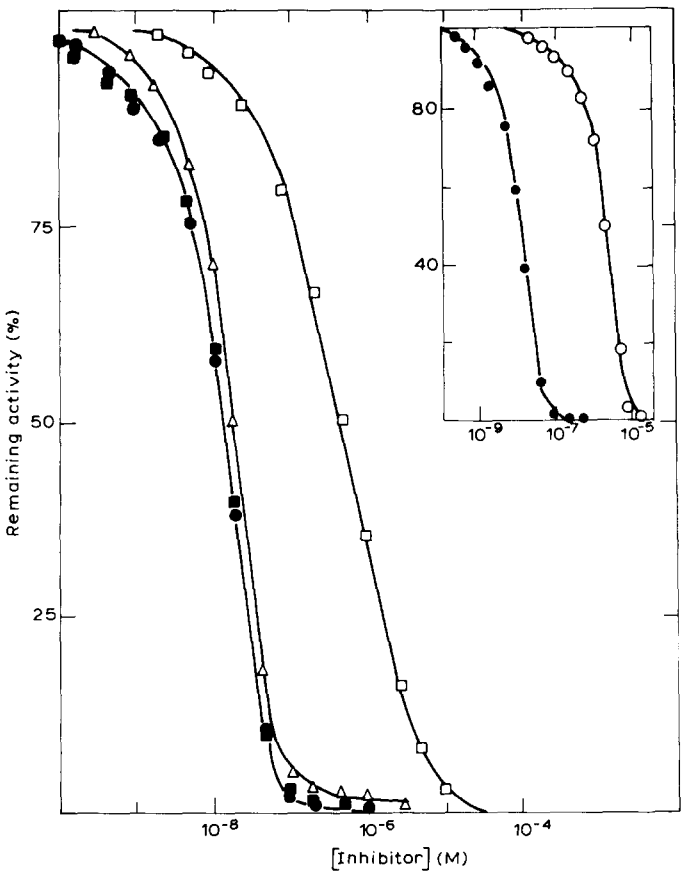


Fig. 5. Comparison of inhibition of cholinesterases by *m*-trimethylammonium-trifluoroacetophenone. The enzymes were incubated with increasing concentrations of inhibitor, and after 30 min enzymatic activity was determined essentially as described in the legend to Fig. 1. Acetylcholinesterase from the electric eel (●) and human erythrocytes (△). Cholinesterases from plaice body muscle (■) and human serum (□). The inset compared the inhibition of eel acetylcholinesterase by the above trifluoroketone to that by diisopropylphosphorofluoridate (○).

TABLE IV

TOXICITIES IN MICE OF REPRESENTED COMPOUNDS WHICH INHIBIT ACETYLCHOLINESTERASE

Water-soluble toxic agents were dissolved in pyrogen-free, physiological saline solution, the water-insoluble compounds in dimethylsulfoxide. The solutions were injected intraperitoneally into mice, the LD_{50} was obtained from results shown in Fig. 6. i.p., intraperitoneally.

Compound	LD_{50} i.p. (mg/kg body wt.)
Acetophenone	>2000 *,***
Trifluoroacetophenone	330
m-Dimethylamino-acetophenone	>2000 *,***
m-Dimethylamino-trifluoroacetophenone	260
m-Trimethylammonium-acetophenone	100
m-Trimethylammonium-trifluoroacetophenone	1.6 **

* No obvious signs of anticholinesterase poisoning up to 2000 mg/kg body wt. the highest concentration tested.

** Because of this toxicity, due care should be taken in handling this compound.

*** Water-insoluble compounds.

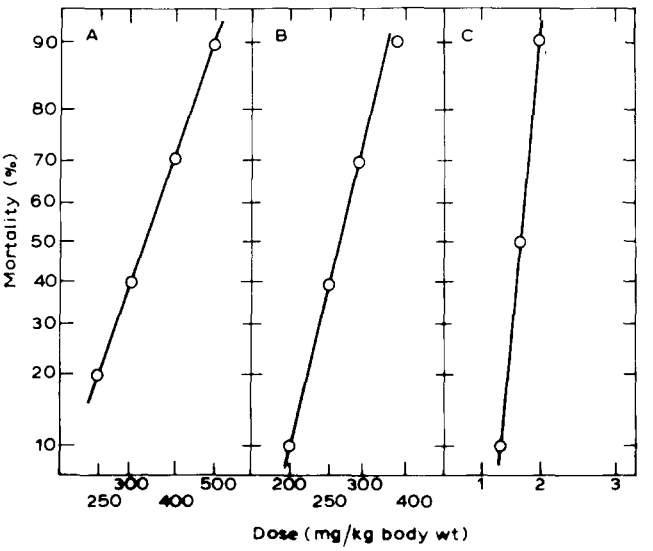


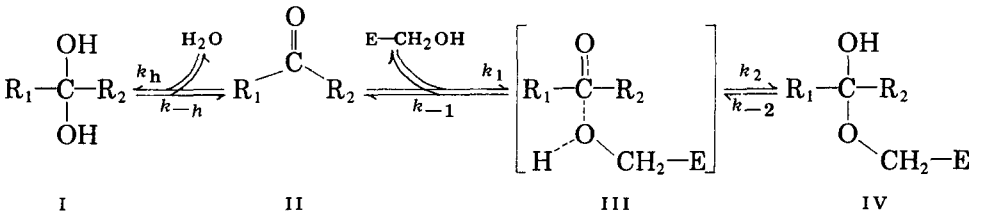
Fig. 6. Dose vs. response curve relating mortality to the dose of aromatic trifluoroketones. The represented compounds were injected intraperitoneally into mice. Experimental conditions are those listed under Table IV. Mortality is plotted on a Hazen probability scale as outlined by Ther [26]. (A) Trifluoroacetophenone; (B) *m*-dimethylamino-trifluoroacetophenone (C) *m*-trimethylammonium-trifluoroacetophenone.

Discussion

The present studies are based on the fact that an activated carbonyl group acts as a stronger electrophile than the non-activated carbonyl group. The results demonstrate that acetylcholinesterase interacts more strongly with compounds carrying such an activated carbonyl function than with the parent aldehydes or ketones. Activation per se is a necessary but not sufficient criterion for enzyme inhibition, as stereochemical requirements have to be met also. The studies by Chiou and Rama Sastry [16] revealed that halogenation of the acyl moiety of acetylcholine significantly influenced its hydrolysis rate by acetylcholinesterase. Fluoroacetylcholine was the only monohalogen derivative of the substrate that was hydrolyzed as fast as acetylcholine. The bulkiness of the chlorine, bromine and iodine atoms hindered these derivatives from effectively interacting with the catalytic site on acetylcholinesterase. On the other hand, both the covalent and the van der Waals' radii of the fluorine atom are only slightly longer than those of hydrogen [29]. This increase in size appears small enough to allow trifluoromethyl aldehydes and ketones to be accomodated on the esteratic site of acetylcholinesterase. The two factors, activation and spatial requirements, as they are combined in trifluoroaldehydes and ketones, make such compounds novel and powerful inhibitors of acetylcholinesterase. The compounds under investigation acted either as time-dependent or time-independent inhibitors. These two modes of inhibition can directly be related to the state of hydration of the aldehydes and ketones. In aqueous solution these compounds are subject to a hydration equilibrium. Guthrie [30] determined the equilibrium constants for the addition of water to

fluorinated aldehydes and ketones. From his work and additional data compiled from the literature [31] it is evident that the fluorinated compounds in aqueous solution are hydrated between 97 and 100%. Acetaldehyde is hydrated to 51% whereas the hydration of the other, non-fluorinated ketones is well below 1% [30].

Time-dependent inhibition was obtained with those compounds the carbonyl group of which is not hydrated to any extent in aqueous solution. Trifluoroacetophenone appeared to be an exception and will be discussed presently. Time-dependent inhibition resulted if the carbonyl compound was present as hydrate or hemiacetal. The interaction of aldehydes and ketones with the serine hydroxyl at the active site of acetylcholinesterase (E-CH_2-OH) can be schematized in the following way:



I II III IV

Scheme I

From this scheme it can be deduced that compounds carrying an activated carbonyl function for which $k_h > k_{-h}$ are readily hydrated and for the same reason show an increased rate of formation of the hemiketal linkage ($k_2 > k_{-2}$). Conversely non-activated carbonyl groups are hydrated to a small extent only ($k_h < k_{-h}$) and show little tendency to form the covalent enzyme adduct ($k_2 < k_{-2}$). In hydrated trifluoromethyl ketones and aldehydes the rate of formation of II from I is slow and becomes the rate limiting step in the production of IV. These compounds thus show a time-dependent inactivation of acetylcholinesterase.

As mentioned above trifluoroacetophenone is an apparent exception in that it shows a rapid reversible time-independent enzyme inactivation despite the fact that in water it is more than 98% hydrated [31]. It should be noted, however, that for reasons of solubility this compound was added to the enzyme in a methanol solution in which the compound still is present in the carbonyl form. In the resulting competition between the enzyme's serine hydroxyl group and the water molecules the reaction becomes kinetically controlled. The enzyme thus preferentially forms the hemiketal complex before the water molecules can react.

Hexafluoroacetone was unable to inhibit acetylcholinesterase. This can be attributed to the fact that the constant of hydration K_H of this molecule is $1.2 \cdot 10^6$ [30], a magnitude that reduces the amount of unhydrated ketone, which would inhibit the enzyme, to practically zero. Furthermore, hexafluoroacetone hydrate at pH 7.4 is deprotonated to 75% [32]. This additional factor contributes to the inability of hexafluoroacetone hydrate to inhibit the enzyme.

It is of interest to note that the inhibition of acetylcholinesterase by *m*-dimethylamino- and *m*-trimethylammonium-trifluoroacetophenone could not be readily reversed upon dilution or dialysis. It is safe to assume that k_1 i.e. the rate of formation of the enzyme-inhibitor complex III is by far the fastest step (Scheme I). The unusual stability of complex IV then implies that $k_2 \gg k_{-2}$. The fact that *m*-trimethylammonium-trifluoroacetophenone is the most potent inhibitor in this series can be rationalized in terms of a chemical and a biological factor. Of all substituents in the benzene ring the *m*-trimethylammonium group exerts the strongest influence in polarizing the carbonyl group of acetophenones [33]. In addition the quaternary ammonium group by its specific binding affinity to the anionic site of the enzyme also contributes to the stability of the complex. When tested in vivo, our inhibitor was slightly more toxic than diisopropylphosphorofluoridate, for which the LD_{50} intraperitoneally was reported to be 9.0 mg/kg body weight [27].

As pointed about above, *m*-dimethylaminotrifluoroacetophenone is a better inhibitor than trifluoroacetophenone. According to one editorial reviewer's comment, this fact is inconsistent with the general trend expected from our results. We deliberately refrain from speculating on possible explanations at this time because in an inhibition process like the one in question, involving both non-covalent and covalent interactions, there are a number of other factors superimposed on purely electronic effects, such as hydrophobic, van der Waals, hydrogen bonding and hydration interactions. Therefore the inhibitory power need not necessarily be consistent with Taft's rules of polar substituent effects.

In view of the observation that the carbonyl form of *m*-trimethylammonium-trifluoroacetophenone specifically binds to the catalytic site of acetylcholinesterase and in the absence of any evidence to the contrary, it is most reasonable to assume the formation of a hemiketal linkage to the reactive serine residue. This working hypothesis is further sustantiated by the recent findings of Berndt et al. [35] who noted the formation of a similar hemiketal adduct with chicken liver carboxylesterase. All our compounds investigated represent quasi-substrate enzyme inhibitors. We thus conclude our work with the explicit postulate that the true transition state-analog inhibitors of serine hydrolases are those compounds that undergo a change in bond order during their interaction with the enzyme.

Acknowledgement

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