Biochemical Pharmacology, Vol. 21 pp. 1187–1196. Pergamon Press, 1972. Printed in Great Britain.

INFLUENCE OF ACETYL-β-METHYLCHOLINE, CARBAMOYLCHOLINE, AND BIS-PYRIDINIUM COMPOUNDS ON THE ACTIVITY OF ACETYLCHOLINESTERASE

HELMUT KUHNEN

Institut für Aerobiologie der Fraunhofer-Gesellschaft zur Förderung der angewandten Forschung e.V., Grafschaft/Sauerland W. Germany

(*Received* 28 *July* 1971; *accepted* 8 *November* 1971)

Abstract—The decomposition rate of the substrate acetyl-β-methylcholine by AChE in presence of the antagonists TMB 4,* toxogonin,† and HS 6‡ was measured *in vitro*. The results are compared with those of previous studies using acetylcholine as substrate. Furthermore the reactivating potency of toxogonin on the carbamoylcholine-inhibited enzyme was investigated.

The antagonists showed different effects on the substrate decomposition rates of the enzyme, depending on the nature of the substrate. It is concluded that the antagonists as well as the substrates react not only with the active centre of AChE but also with a secondary binding site. In the reactivating reaction of carbamoyl-inhibited AChE, a reaction at a secondary binding site is also involved.

IN PRECEDING papers[1,2] the activating and inhibitory effects of alkane and dimethyl-ether bis-pyridinium compounds on the structure-bound acetylcholinesterase (AChE) was reported. These derivatives owe their activity against the enzyme to their bis-quaternary structure. The influence of substituents, e.g. oxime or carbamide groups, is only an enhancement or a decrease of the original effect.

It was suggested that the substrate as well as the antagonists may be bound not only at the active centre but also on another site of the enzyme, with, as a result, a mixed competitive–noncompetitive mechanism of action. The present work reports the results of investigations on the activity of AChE toward the substrate acetyl-β-methylcholine (MeCh) under the influence of three bispyridinium compounds toxogonin, TMB 4, and HS 6 (formulas are listed in Table 1). The reaction of toxogonin against the competitive inhibitor carbamoylcholine has also been studied.

METHODS

Isolated membranes, freshly prepared from bovine red cells, were used as a source of the structure bound enzyme. Erythrocytes from fresh bovine blood are washed with Tyrode solution ($0{\cdot}15.10^{-1}$ M NaCl, 1.10^{-2} M $MgCl_2$, ionic strength $I = 0{\cdot}18$) by centrifuging several times for 20 min at 1500 *g*. Then the red cells are haemolysed. After that the membranes are centrifuged for 30 min at 70,000 *g* in an ultracentrifuge at 4°. Washing of the membranes in Tyrode solution and centrifugation is repeated until the precipitate becomes milky-white, and the supernatant fluid has no more traces of haemoglobin. The membranes from 1 l. of whole blood are made up to a total

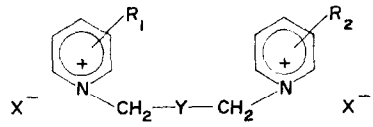
* [1,1′-trimethylene bis-(4-formylpyridinium bromide)] dioxime.

† Obidoxime = [1,1′-oxydimethylene bis-(4-formylpyridinium chloride)] dioxime.

‡ [1,1′-oxydimethylene (2-formyl-3-carbamyl-pyridinium chloride)].

volume of 100 ml. This solution remains stable without loss of enzymatic activity for at least 6 weeks, when it is kept in a refrigerator at 4°. The structure-bound enzyme in the isolated membrane is a true AChE. It has a specific activity against acetylcholine and acetyl-β-methylcholine. The substrates butyrylcholine and benzoylcholine are not decomposed by this enzyme. The substrate decomposition rate was measured by the pH-stat method (Kombititrator 3 D, Metrohm). The rate of formation of acetic acid (which equals the substrate decomposition rate) is recorded automatically as a function of time, thus giving the enzymatic activity in the time units. All experiments were carried out in Tyrode solution. The temperature was kept at 37° and the pH at 7·4. In a first series of studies the antagonists toxogonin, TMB 4, and HS 6 were examined at four concentrations (3.10^{-3}, 3.10^{-4}, 3.10^{-5} and 3.10^{-6} M) in the presence of five concentrations (1.10^{-1}, 1.10^{-2}, 2.10^{-3}, 1.10^{-3} and $6{\cdot}67.10^{-4}$ M) of the substrate MeCh.

TABLE 1



Antagonist	—Y—	R_1	R_2	X^-
Toxogonin	—O—	4 —CH=NOH	4 —CH=NOH	Cl
TMB 4	—CH_2—	4 —CH=NOH	4 —CH=NOH	Br
HS 6	—O—	2 —CH=NOH	3 —CO—NH_2	Cl

In a second set of experiments the reactivating potency of toxogonin on carbamoylcholine-inhibited AChE was investigated. The inhibitor was used in concentrations of 3.10^{-3}, 3.10^{-4}, 3.10^{-5} and 3.10^{-6} M. In each determination the enzyme was preincubated for 5 min with the chosen inhibitor, and incubated 5 min longer with the chosen reactivator. Then the substrate decomposition rate was measured. In these experiments acetylcholine (ACh) was used as substrate. The investigations were carried out under the same conditions and by the technique of cumulative substrate doses as described previously.[2] All concentrations are given here in moles per litre (M).

RESULTS

Plots of the decomposition rate of MeCh versus log substrate concentrations curves are shown in Figs. 1–3. Each point represents the average of six determinations. The calculated standard error of the mean does not exceed the size of the symbols. Presentation of these results in Lineweaver–Burk plots is not possible, since the Michaelis–Menten equation is clearly not valid for these AChE–antagonist–substrate systems. For the concentrations used, MeCh shows no substrate inhibition of the enzyme as does ACh. The enzymatic activity in presence of the antagonists also depends on the nature of the substrate used. In previous studies[2] it was found that the compounds toxogonin and TMB 4 show, with ACh as substrate, both activating and inhibitory actions on the enzyme, depending upon the concentration of the antagonist and the

substrate. Under the experimental conditions of the present investigations the antagonists showed, at concentrations of 3.10^{-3} and 3.10^{-4} M, a weak decrease of the enzymatic activity. At concentrations of 3.10^{-5} and 3.10^{-6} M the compounds are without any significant influence on the rate of decomposition of the substrate MeCh (see Figs. 1 and 2). On the other hand 3.10^{-5} and 3.10^{-6} M concentrations of HS 6 activate AChE toward MeCh. This is interesting in comparison with the finding that the decomposition rate of ACh is decreased markedly by HS 6 at all concentrations investigated (see Fig. 3).

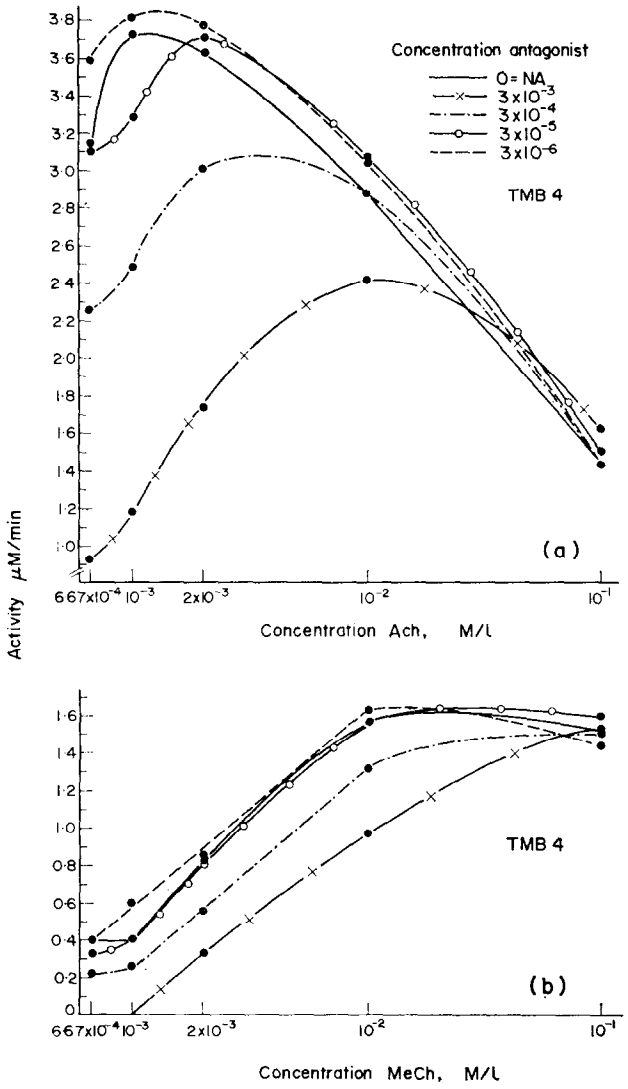


FIG. 1. Influence of various concentrations of TMB 4 on the activity of AChE toward the substrates acetylcholine (a), and acetyl-β-methylcholine (b). With the former the inhibitory effect is diminished with decreasing concentrations of the antagonist, and activation of the enzyme occurs whereas with the latter this is not the case.

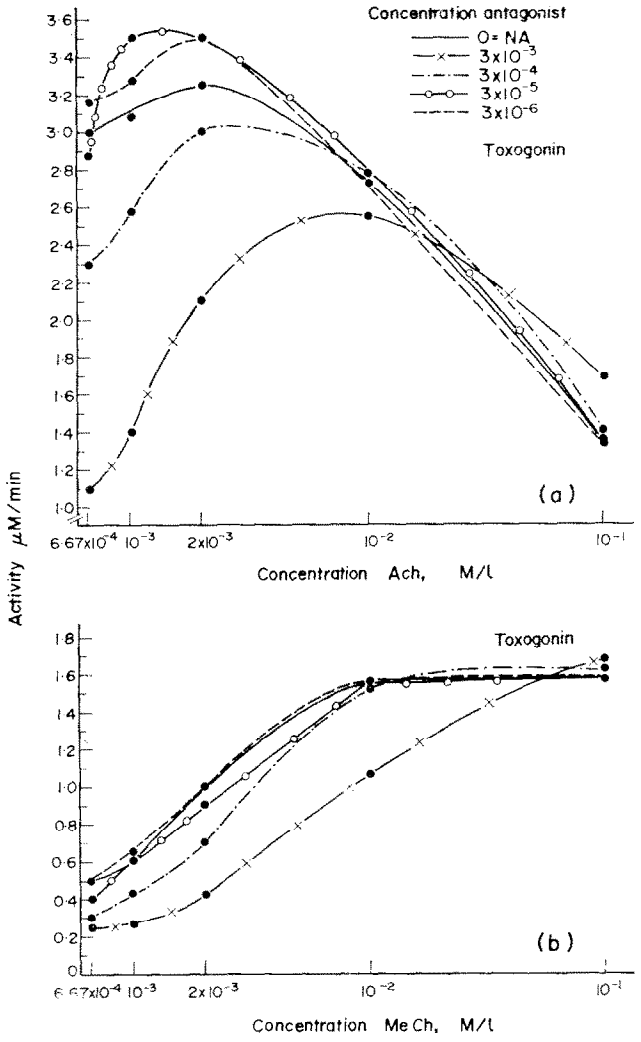


FIG. 2. Influence of various concentrations of toxogonin on the activity of AChE toward the substrates acetylcholine (a), and acetyl-β-methylcholine (b). The inhibition of the enzyme is decreased with MeCh, although the enzymatic activity is not potentiated. The action of this antagonist is similar to that of TMB 4.

Increasing concentrations of the competitive inhibitor carbamoylcholine result in a decrease of the activity of AChE (see Fig. 4a). At concentrations of 3.10^{-4}–3.10^{-6} M carbamoylcholine, the rate of inhibition of the enzyme depends only on the concentration of the inhibitor. However at a 3.10^{-3} M inhibitor concentration a protective effect of the substrate is observed which increases with increasing substrate concentrations. At this inhibitor concentration toxogonin produces no reactivation of the inhibited enzyme (see Fig. 4b). For all other carbamoylcholine concentrations studied

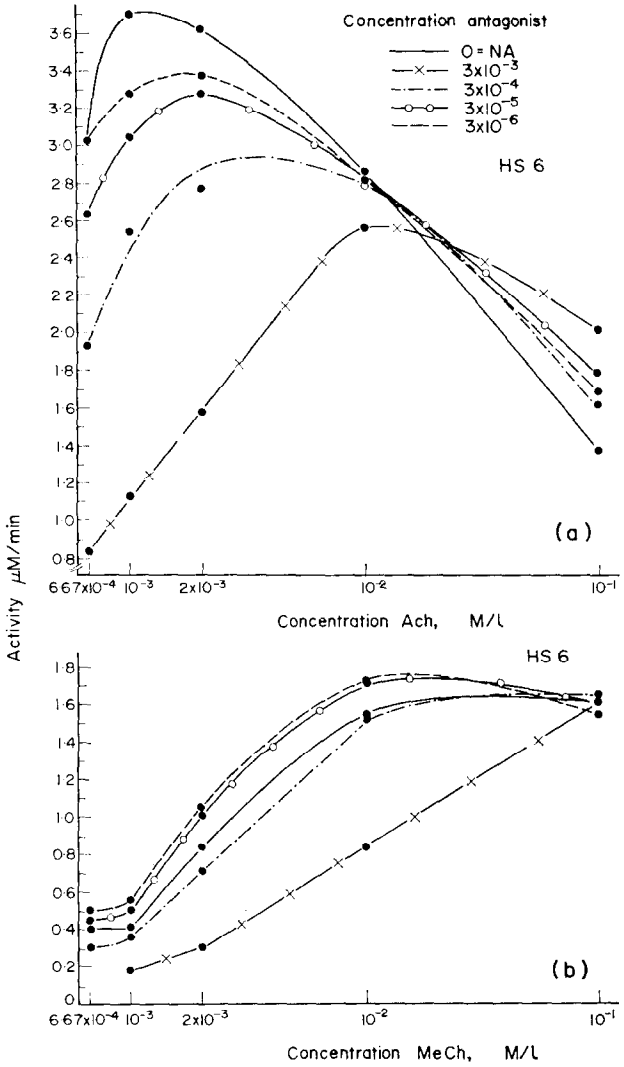
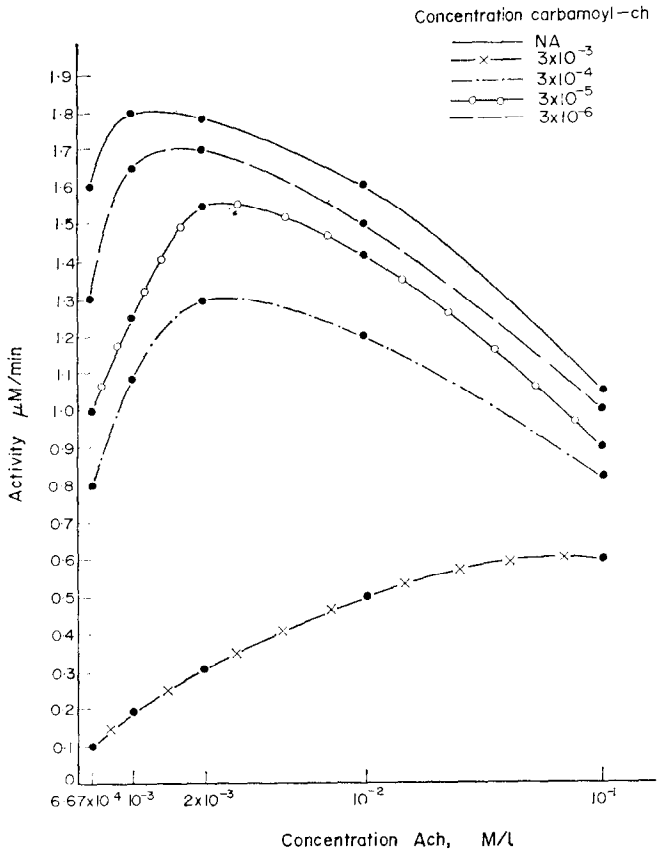


FIG. 3. Influence of various concentrations of HS 6 on the activity of AChE toward the substrates acetylcholine (a), and acetyl-β-methylcholine. The inhibitory effect which appears in presence of ACh occurs with MeCh only at high antagonist concentrations. At lower doses the enzyme is activated.

here, bell-shaped concentration–response curves are observed for the inhibition of enzyme (see Fig. 5a) as well as for the reactivation by toxogonin (see Fig. 4b upper curves, Figs. 5a and 5b). The upper curve of Fig. 4b shows that a reactivation of the inhibited enzyme to the level of normal activity occurs only at substrate concentrations between 3.10^{-1} and 1.10^{-1}. From Fig. 5 it can be seen that, at carbamoylcholine concentrations of 3.10^{-5} and 3.10^{-6}, toxogonin not only reactivates the inhibited enzyme but in fact increases the enzymatic activity beyond the normal level. Furthermore the family of dose–response curves of the reactivated enzyme shows a shift of the maximum substrate decomposition rate towards higher substrate concentrations as

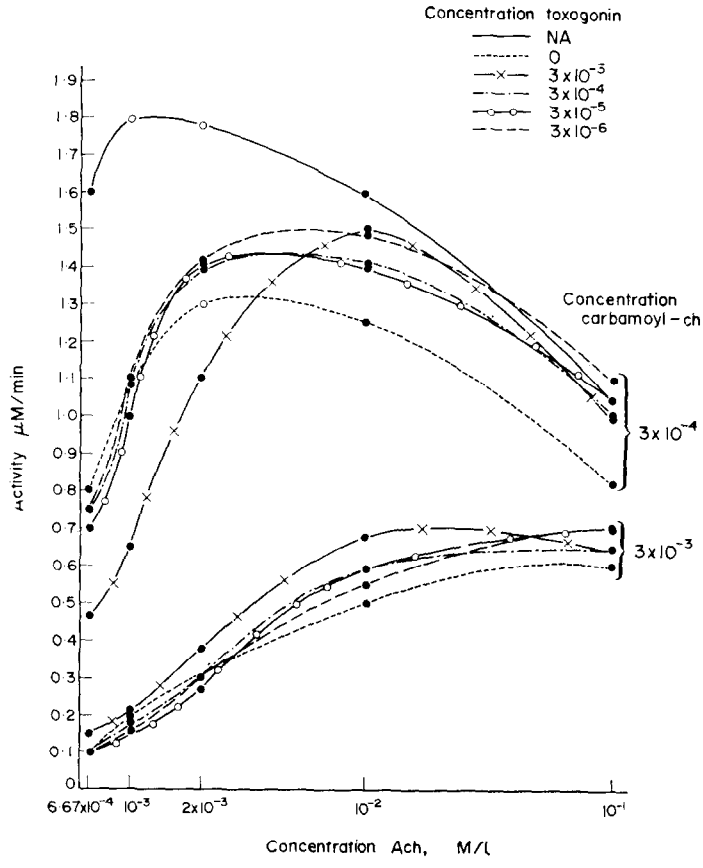


4 (a)

toxogonin concentration is increased. At low substrate concentrations, however, the enzymatic activity is decreased by 3.10^{-3} M toxogonin. Here an additional inhibitory effect of toxogonin occurs; but from 1.10^{-2}–1.10^{-1} M substrate a decomposition rate higher than that of the normal activity in this range is shown. The maximum is shifted considerably to a substrate concentration of 2.10^{-2} M ACh.

DISCUSSION

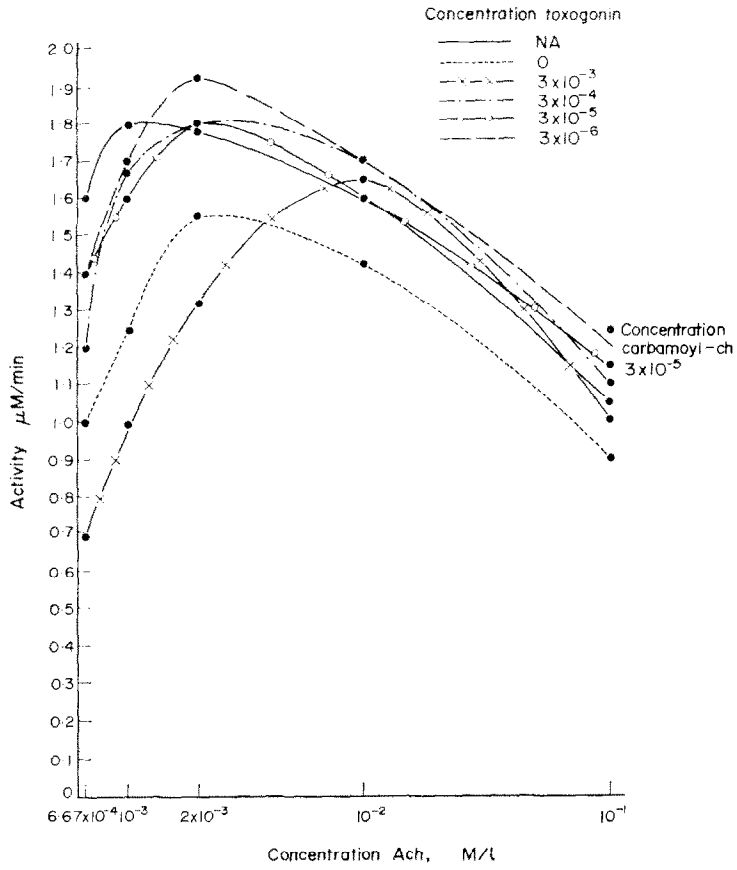
The mechanism of action of the three bis-pyridinium compounds on AChE is different for the substrates ACh and MeCh. TMB 4 and toxogonin, which are able to inhibit or to activate the enzyme (depending on the substrate concentration) in the case of ACh, exert only an inhibitory or nearly no effect with MeCh as substrate. At ACh concentrations of $6{\cdot}67.10^{-4}$–2.10^{-1} M, HS 6 acts as an inhibitor. When MeCh is chosen as substrate the activity of the enzyme is potentiated at concentrations of 3.10^{-4}–3.10^{-6} M antagonist. This indicates that the mechanism of action of the bis-pyridinium compounds with MeCh as substrate is different from the mechanism involved with ACh as substrate. In a previous paper[2] it was suggested that the mechanism of action of TMB 4 and toxogonin with ACh as substrate on the enzyme AChE is a



4 (b)

FIG. 4. (a) Influence of different concentrations of carbamoylcholine on the activity of AChE. (b) Influence of toxogonin on the carbamoylcholine-inhibited enzyme. At 3.10^{-3} M carbamoylcholine the enzyme is not significantly reactivatable. At 3.10^{-4} M inhibitor the reactivation rate depends not only on the concentration of the reactivator, but also on that of the substrate acetylcholine. The curve for 0 toxogonin concentration in (b) is the same curve appearing in (a) (second curve from the bottom).

mixed competitive–noncompetitive one, whereas HS 6 is a competitive inhibitor. The mixed competitive–noncompetitive mechanism is attributed to a secondary binding site at the enzyme. The existence of a secondary binding site on AChE was previously confirmed by Kato *et al.*[3–5] The occupation of this secondary binding site by ACh is possibly one of the reasons for the so-called substrate inhibition at high substrate concentrations. An inhibitory effect at the active centre of the enzyme is induced by this occupation. This effect would be additional to the well-known competition[6] of the substrate molecules involving two-point attachment at the active site. Probably the affinity of TMB 4 and toxogonin for the secondary binding site is higher than that of ACh. If these compounds are bound an activation or prevented inhibition of the enzyme results (see Figs. 1a and 2a), provided that no competition at the active site takes place at the same time.



5 (a)

The substrate MeCh may also be bound at the secondary binding site. However, in this case no substrate inhibition results (see Fig. 1b and 2b); the inhibitory influence on the active site is lacking. The affinity of MeCh with the secondary binding site may be even higher than that of TMB 4 and toxogonin. When this binding site is occupied by MeCh, the potentiating activity of the bis-pyridinium compounds cannot occur. Therefore the enzymatic activity remains unchanged in presence of TMB 4 or toxogonin if MeCh is chosen as substrate.

The affinity of the antagonist HS 6 is lower than that of ACh but higher than that of MeCh. Thus the substrate decomposition rate decreases with ACh, and increases with MeCh. Purdie has described[7] a similar reaction of AChE from bovine red cells inactivated by *N,N*-dimethyl-2-phenylaziridinium. After initial inhibition of the enzyme with this competitive inhibitor, a later reaction resulted in a product with an activity greater or less than that of AChE, depending on the substrate.

Carbamoylcholine was studied, because, being a competitive inhibitor,[8] it reacts with the same site of the enzyme as do substrates and organophosphates.[9,10] It forms a moderately stable carbamylated complex with the enzyme. Berry and Davies[11] confirmed that carbamylation of AChE by physostigmin has a protective effect even

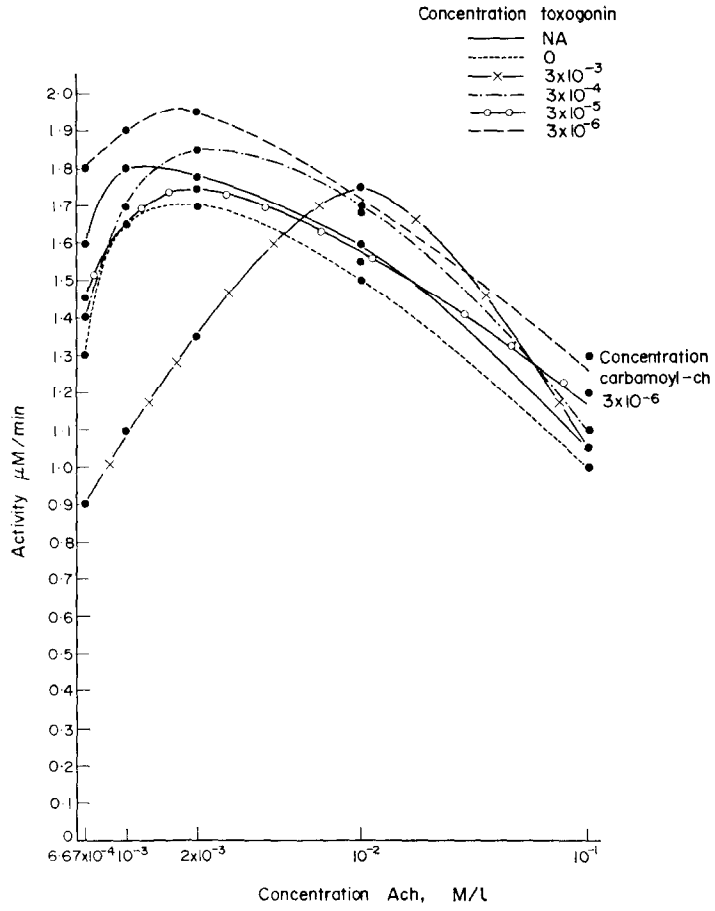


FIG. 5. Influence of different concentrations of toxogonin on AChE which has been partially inactivated by 3.10^{-5} M (a), and 3.10^{-6} M (b) carbamoylcholine. The inhibited enzyme is not only reactivated by the antagonist; the enzymatic activity is potentiated beyond the level of the normal activity. The maxima are shifted to higher substrate concentrations with increasing toxogonin concentration.

against the very strong inhibition by soman. Figure 4b, 5a and 5b show that AChE inhibited by carbamoylcholine in a concentration from 3.10^{-4} to 3.10^{-6} M can be reactivated by toxogonin. The maximal position of the dose–response curve is the same as for that of the inhibited enzyme, except for 3.10^{-3} M toxogonin; these maxima are shifted to substrate concentrations which are higher than that for the non-inhibited enzyme.

According to van Rossum[6] the mechanism of reactivation of carbamoylcholine-deactivated enzyme by toxogonin involves a competition of the two compounds at the active site. Our results show that, with decreasing carbamoylcholine concentration, the reactivation even leads to a substrate decomposition rate higher than the normal

activity of the enzyme. A similar potentiation of AChE by quaternary nitrogen compounds has been reported by Roufogalis and Thomas.[12,13]

At a concentration of 3.10^{-3} M, toxogonin reacts with carbamoylcholine-deactivated enzyme in another way as compared with lower concentrations of toxogonin. The maximum of the substrate decomposition rate is shifted to a substrate concentration which is 10-fold that of the normal activity, thus indicating a noncompetitive mechanism.[6] This is probably due to a competition between toxogonin and substrate at a secondary binding site of the enzyme. Occupation of this binding site by toxogonin partially prevents the substrate inhibition and leads to a higher enzymatic activity than the normal substrate decomposition rate in the absence of the antagonist. It must be considered that the inhibition of AChE by carbamoylcholine cannot be strictly compared with inactivation by organophosphates because the former is a reversible inhibition and the latter an irreversible inhibition of AChE. Furthermore Hastings *et al.*[14] pointed out that bovine erythrocyte AChE exists in multiple forms which may be inhibited at significantly different rates, depending on the inhibitor. *In vitro* at relatively higher substrate concentrations, the noncompetitive reaction at a secondary binding site of the enzyme may also play a role in determining enzymatic activity.

Acknowledgements—The author is gratefully indebted to Mr. A. Schrichten and Mr. E. Schmidt for technical assistance in these investigations, and to Dr. J. C. Thompson for help in the preparation of the manuscript.

REFERENCES

1. H. KUHNEN, *Europ. J. Pharmac.* **9,** 41 (1970).
2. H. KUHNEN, *Toxic. appl. Pharmac.* **20,** 97 (1971).
3. G. KATO, J. YUNG and M. IHNAT, *Biochem. biophys. Res. Commun.* **40,** 15 (1970).
4. G. KATO, J. YUNG and M. IHNAT, *Molec. Pharmac.* **6,** 588 (1970).
5. G. KATO and J. YUNG, *Molec. Pharmac.* **7,** 33 (1971).
6. J. M. VAN ROSSUM, in: *Molecular Pharmacology* (Ed. E. J. ARIENS) Vol. 2, p. 233. Academic Press, New York, London (1964).
7. J. E. PURDIE, *Biochim. biophys. Acta* **185,** 122 (1969).
8. K. B. AUGUSTINSON, *Ark. Kemi.* **6,** 331 (1953).
9. I. B. WILSON, M. A. HATCH and S. GINSBURG, *J. biol. Chem.* **235,** 2312 (1960).
10. I. B. WILSON, M. A. HARRISON and S. GINSBURG, *J. biol. Chem.* **236,** 1498 (1961).
11. W. K. BERRY and D. R. DAVIES, *Biochem. Pharmac.* **19,** 927 (1970).
12. B. D. ROUFOGALIS and J. THOMAS, *J. Pharm. Pharmac.* **20,** 135 (1968).
13. B. D. ROUFOGALIS and J. THOMAS, *J. Pharm. Pharmac.* **22,** 649 (1970).
14. F. L. HASTINGS, A. R. MAIN and F. IVERSON, *J. Agr. Food Chem.* **18,** 497 (1970).