cofactor of tyrosine hydroxylase. However, since all the three stereoisomers of trihydroxypropyltetrahydropterin have similar characters as cofactor, it may be necessary to compare the properties as cofactor with the stereochemical isomers of tetrahydrobiopterin.

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SHORT COMMUNICATION

The influence of some acetylcholine-receptor activating and blocking agents on the esteratic site of acetylcholinesterase

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The sulfonylation of acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) by methanesulfonyl fluoride is greatly accelerated by tetraethylammonium[1]. Tetraethylammonium changes the reactivity of the esteratic site of the enzyme, probably by changing the structure of the site[2]. On the other hand, the structure of tetraethylammonium is similar to that of the quaternary ammonium part of acetylcholine, which is the natural activator of acetylcholine-receptor; the receptor is supposed to undergo a structural change under the influence of an activator. Thus, the supposed change in the structure of the esteratic site of acetylcholinesterase under the influence of tetraethylammonium points to a similarity between the active site of the enzyme and that of the receptor. In order to obtain more data concerning this similarity the present investigation on the influence of some receptor activating and blocking agents on the esteratic site of acetylcholinesterase was undertaken. Experiments on the methanesulfonylation of acetylcholinesterase from the electric organ of the fish *Electrophorus electricus* in the presence of some typical activating and blocking agents of the acetylcholine-receptor from the same organ were performed and compared with those performed in the absence of these agents.

Methods and materials

The methanesulfonylation of acetylcholinesterase follows the scheme[3, 4]

$$EH + I \xrightarrow{k_a} E' + HF$$

with EH, the esteratic site of the enzyme; I, the inhibitor methanesulfonyl fluoride; E', the sulfonylated esteratic site; HF, hydrogen fluoride; and k_a, the second order rate constant for sulfonylation. The effect of an agent on sulfonylation is reflected in k_a and the corresponding thermodynamic quantities ΔH^+, ΔS^+, and ΔG^+. From these data conclusions can be drawn about the influence of the agent on the structure of the esteratic site. For our purpose typical activating and blocking agents of the acetylcholine-receptor from the electric organ of *E. electricus* were chosen: decamethonium, carbamycholine, D-tubocurarine, and gallamine.

The rate of the methanesulfonylation of acetylcholinesterase was studied in the presence of each of these agents at 5° and 25°, pH 8·4, ionic strength 0·2. The concentration of decamethonium ranged from 2 to 200 μM, of carbamylcholine from 10 to 500 μM, of D-tubocurarine from 2 to 200 μM, and of gallamine from 10 μM to 10 mM. The second order rate constant for sulfonylation in the presence of a given agent at a given concentration was determined as previously described[2]. From the dependence of the second order rate constant on the concentration of the agent, the maximum value of this constant was extrapolated and used for the calculation of the thermodynamic quantities for activation; this calculation was done as already described[2].

The enzyme preparation was acetylcholinesterase from the electric organ of *E. electricus*, Worthington, ECHP 1 JA, 1·079 units/mg. Methanesulfonyl fluoride, for chemical purposes, was obtained from Eastman Organic Chemicals; decamethonim iodide, pure, from Koch-Light Lab.; carbamylcholine chloride, for investigation use, from Mann Research Lab.; and gallamine, Lot No. 1570, from Specia.

Results and discussion

The results are summarized in Table 1. It can be seen that decamethonium accelerates the methanesulfonylation of acetylcholinesterase at 25° by about 28 times. This is in agreement with Suszkiw's result[5], considering the differences in methods and experimental conditions.

Table 1. Effect of some pharmacologically active agents on the methanesulfonylation of acetylcholinesterase

Methanesulfonylation in the presence of	k_a, 25° (l.mole^{-1}.sec^{-1})	k_a, 5° (l.mole^{-1}.sec^{-1})	$\Delta G^{\neq}$ 25° (kcal.mole^{-1})	$\Delta H^{\neq}$ 25° (kcal.mole^{-1})	$\Delta S^{\neq}$ 25° (cal.mole^{-1} × deg^{-1})
Decamethonium	88	20	14·7	11·8	−9·6
Carbamylcholine	4·2	0·85	16·6	12·2	−14·6
D-tubocurarine	2·8	0·61	16·9	12·1	−16·1
Gallamine	2·8	0·60	16·9	12·1	−16·1
Tetraethylammonium†	85	15	14·7	13·9	−2·7
Without agent†	3·1	0·68	16·8	12·1	−15·8

*From M. R. Pavlič, *Biochim. biophys. Acta* **327**, 393 (1973).

†k_a is the second order rate constant for methanesulfonylation at the indicated temperature and pH 8·4. In the case of the first four agents it was obtained by extrapolation towards infinite concentration of the agent; in the case of tetraethylammonium it was obtained at 50 mM concentration of tetraethylammonium.

According to Table 1, acceleration in the presence of decamethonium is due to an increase in the activation entropy. This indicates a change in the structure of the esteratic site of acetylcholinesterase under the influence of decamethonium.

As seen from the table, methanesulfonylation is accelerated also by carbamylcholine. The difference in the activation quantities for methanesulfonylation in the presence and absence of carbamylcholine, is small and lies within the limits of experimental error. However, the difference is qualitativily equal to that in the case of decamethonium; this indicates small structural changes under the influence of carbamylcholine.

Methanesulfonylation is slightly inhibited by the blocking agents D-tubocurarine and gallamine. The degree of inhibition is not in good agreement with that reported by Suszkiw[5]; his data, however, concern membrane-bound enzyme. The corresponding changes in the activation quantities are small. It seems probable that the effect of both blocking agents is due to nothing but a partial competition: the approach of the methanesulfonyl fluoride molecule to the esteratic site is hindered by a D-tubocurarine or gallamine molecule bound near the esteratic site; the hindering, however, is not great, since the methanesulfonyl fluoride molecule is small.

At this point it must be remembered that tetraethylammonium accelerates the methanesulfonylation of acetylcholinesterase and does so by increasing the activation entropy which more than compensates a higher activation enthalpy in the presence of tetraethylammonium[2]. Tetraethylammonium is a week 'inhibitor' of the acetylcholine-receptor from *E. electricus* [6]. This is in accordance with the fact that tetraethylammonium is known to be mainly a blocking agent for acetylcholine-receptor. However, this action does not seem to be simple. From the work of Koketsu[7] it can be concluded that this drug would block neuromuscular transmission only at a relatively high concentration: at a relatively low concentration the action of tetraethylammonium seems complex. This complex situation is clearly shown in the paper of Paton[8] according to which the action of tetraethylammonium on the receptor seems to be a double one: at ganglionic synapses the binding of tetraethylammonium to the receptor results in either an excitation or a block. Thus, tetraethylammonium could be, in some experimental conditions, an activating agent.

The present results together with those previously published[2, 5, 9] indicate that the tested acetylcholine-receptor activating agents do and receptor blocking agents do not, accelerate the methanesulfonylation of acetylcholinesterase and change the structure of the esteratic site of the enzyme. The change in the activation entropy in the presence of each tested activator is positive in each case; this seems to indicate that these activating agents change the structure in a specific way. The exact nature of this change is difficult to conceive. It may be, for example, a conformational change, a change in hydration, or both, something like a conformational perturbation of a receptor under the influence of a drug[10]. The tested blocking agents, on the other hand, do not seem to change the structure at all. It should be noted here that tetraethylammonium behaves in respect to the influence on the enzyme like the tested activators except in that the positive change in the activation entropy is accompanied with an increase of the activation enthalpy.

The effect of a receptor activating agent on the receptor is supposed to involve structural changes in the receptor, whereas the effect of a blocking agent seems to be connected mainly with a competition between the blocking agent and the activator. Thus, it seems that the effect of the tested acetylcholine-receptor activating and blocking agents on acetylcholinesterase parallels the effects of these agents on the acetylcholine-receptor. The somewhat special position of tetraethylammonium as a blocking agent might be connected with its special position as an accelerator. Of course, this parallelism might be a coincidence. More substances should be tested with respect to their possible effect on the structure of acetylcholinesterase and more direct evidence of structural changes should be obtained. Nevertheless, the parallelism points to similarities between acetylcholinesterase and acetylcholine-receptor which might be important in connection with the view that acetylcholinesterase and acetylcholine-receptor are closely related or identical[cf. 10–14].

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Enzymic hydrolysis of malaoxon by mouse liver homogenates

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In a previous communication [1] we reported that malathion (*S*-(1,2-dicarbethoxyethyl) *O,O*-dimethyl phosphorodithioate) is hydrolysed by two types of esterases: malathion B-esterase which is predominently microsomal and acts at a pH between 7·4 and 7·6, and malathion A-esterase which is predominently in the cell-sap, acts at a pH of 8·8 and requires SH group activators such as 2-mercaptoethanol or reduced glutathione. The malathion esterase activity was determined by a newly developed colorimetric procedure [2].

Malaoxon (*S*-(1,2-dicarbethoxyethyl) *O,O*-dimethyl phosphorothioate) is the toxic oxygen analogue of malathion. It is thus an anti-cholinesterase (anti-ChE) agent and a carboxylesterase inhibitor. It has two carboxyethyl ester groups, and by analogy with malathion, it is considered that detoxification is effected by enzymatic hydrolysis of one of these ester groups by the non-specific B-esterases [3, 4]. There is, however, no definite evidence to show that malaoxon is, in fact, hydrolysed by the same enzymes which are also inhibited by it. The only work in which a carboxylesterase is implicated in the hydrolysis of malaoxon is that of Murphy and DuBois [5] who used a preparation of malaoxon obtained by the oxidation of malathion by bromine water. The enzymatic hydrolysis of their preparation was shown to be inhibited by EPNO (*O*-ethyl *O*-*p*-nitrophenyl phenylphosphonate, the oxygen analogue of EPN) which is a powerful B-esterase inhibitor. This is considered as evidence that malaoxon is hydrolysed by a B-type of esterase, and a dual role is assigned to malaoxon as an inhibitor as well as a substrate for such esterases [3, 4].

Recently we developed a method [6] for the determination of malaoxon in the presence of malathion. Using the method we analysed malaoxon preparations obtained by the procedure of Murphy and DuBois [5]. The results in Table 1 show that the amount of malaoxon in these preparations is low (about 7%) the remainder being malathion. To obtain higher yields more bromine water has to be used. Also, the technique used by Murphy and DuBois [5] and by Cohen and Murphy [7], to measure malaoxon esterase activity was a bio-assay in which the un-hydrolysed malaoxon was determined by its anti-ChE activity. This procedure, understandably, will not distinguish between a carboxylesterase of the B-type and a phosphotriesterase which belongs to the A-type according to the classification of Aldridge [8]. The question is thus open whether malaoxon is both an inhibitor and a substrate for non-specific carboxylesterases. In this connection it would be of interest to study which of the two malathion esterases [1] acts upon malaoxon.

The malaoxon used in our studies was obtained from American Cyanamid Co., Princeton, N.J. As determined by our procedure [6] it was quite pure, containing not more than 0·6% malathion. Malaoxon suspensions of

Table 1. Conversion of malathion to malaoxon by bromine water

Expt. No.	Vol. in ml of saturated bromine water (approx 0·1 M) added to 10 μmoles of malathion	Per cent conversion to malaoxon
1	0·024	7·3
2	0·20	22·4
3	0·40	30·2
4	0·60	60·4
5	0·80	76·2
6	1·00	100·0

Expt. 1 was performed according to Murphy and DuBois [5]. 5 ml of 1×10^{-3}M aqueous suspension of malathion was treated for 1 hr with 2 ml water and 3 ml of dilute bromine water (0·1 ml of saturated bromine water diluted to 25 ml). The volume of bromine water given in table is the equivalent in terms of saturated bromine water. In other experiments 1 ml (10 μmoles) of malathion in propylene glycol was treated with saturated bromine water as indicated. After a few minutes the mixture was diluted to 10 ml with water containing 0·1% Triton X-100, extracted with 10 ml cyclohexane and the solvent layer separated and clarified by anhydrous sodium sulphate. Aliquots of the solvent phase were leached with alkaline hydroxylamine to remove malaoxon and the residual malathion was assayed as described in Ref. 6. The malaoxon content was obtained by difference. The values are averages of 3 experiments.