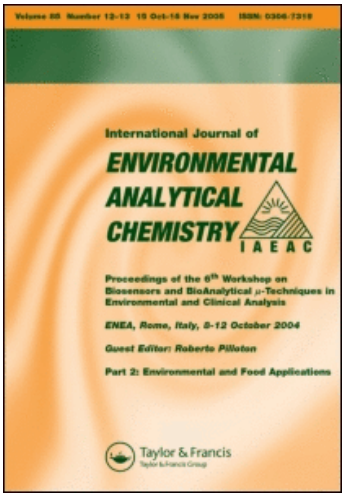

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The Chemistry of Reactivation at the Active Centres of Acetylcholinesterase and Receptor*

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Contrary to the overwhelming expectations in special papers and textbooks, the reactivation of the active centre of acetylcholinesterase, inhibited by organophosphorous compounds, with the help of so called "tailor-made-drugs", e.g. the oximes, never could be realized *in vivo*. However, the concept of the tailor-made drugs gave us a thorough insight in the function of synapses.

As the chemical background of the basic reactions of catalysis of the hydrolysis of acetylcholine, carbamates and organophosphates at the active centre is now investigated, the reason for the failure of the "tailor-made-drugs-concept" can be discussed. The rate limiting step in all naturally "reactivation-reactions" is the hydrolysis of the acetylated, carbamoylated and phosphorylated enzyme. Thus the acceleration of the hydrolysis of the phosphorylated enzyme proved to be the most promising way for reactivation *in vitro*. However, *in vivo* the oximes alone never proved to be successful. The only successful way in a very limited dose range is to combine the oximes with atropine and/or other anticholinergic drugs.

On the other hand, drugs without oxime groups, e.g. edrophonium, with obviously no capability to reactivate the enzyme blocked by organophosphates, behave almost

*Presented at the 2nd IAEAC Workshop on Chemistry and Fate of Organophosphorous Compounds, 10 November 1987, Barcelona, Spain.

like the oximes. Thus another mechanism concerning a direct action on the acetylcholine receptor has to be taken into account.

KEY WORDS: Organophosphate-poisoning, reactivation, sarin, soman, acetylcholinesterase.

INTRODUCTION

Acetylcholine (ACh) is attracted by the active site of acetylcholinesterase, classified by the IUB as acetylcholine acetyl hydrolase, EC 3.1.1.7 (AChE). The active site is composed of two subsites: the anionic site (AS) and the esteratic site (ES).[1,2] The kationic head of ACh is attracted by the AS and the molecule fits well in the enzyme cavity. Thus the carbonyle group of ACh comes close to the ES and the hydrolysis of ACh begins: in a first step the enzyme is acetylated and choline is liberated. The following step, the hydrolysis of the acetylated enzyme is the rate-limiting step.[3,4]

The chemical background of catalysis is not understood in all details, but the "charge relay-system",[5,6] which has been worked out for other hydrolytic enzymes, may be used as a working hypothesis. The entire catalytic process of acetylcholine breakdown is terminated within microseconds. The rate-limiting step also applies for hydrolysis of the carbamoylated and for the phosphorylated enzyme. The carbamoylation is terminated within hours but the phosphorylation is terminated after weeks.

Thus it is easily understood that phosphorylating agents may be seriously dangerous when applied to living creatures. Organophosphates are widely distributed, are used in synthesis, as drugs, insecticides and even as war gases. The overwhelming literature has been condensed in many books and journals. Without prejudice in respect of importance we only quote some of them, with the aim of giving a thorough review.[7–10]

Some pathways of organophosphate poisoning (OPP) are given in Figure 3.

Fortunately Tammelin-esters[11–15] are extremely labile. They are easily decomposed in the range of minutes.

As many of the problems concerning the therapy of OPP are unsolved, a worldwide research in this field is still justified. The oximes, originally synthesized as "tailor-made-drugs", failed

completely when they were used alone to counteract OPP *in vivo*. *In vitro* however, a beneficial effect can be shown, as demonstrated in Figure 4.

PAM, the most important fragment of "Tammelin-ester-hydrolysis", can reenter the circuit, thus reactivating the blocked active site in an autocatalytic way. This is the reason why the oximes are acting beneficially *in vitro*. However, it has to be taken into account, that the oximes have to be applied in a concentration which is already considerably high. We discussed this problem earlier.[16] One of the most potent oximes, obidoxim, only could reactivate about 38% of the blocked enzyme after 18 hrs in a concentration which never would be tolerated in humans.[17] The reason why only 38% are reactivated, may be due to the blocking activity of the oxime itself, which will be attracted by the open active sites, not already occupied by the organophosphate.

In vivo however, a continuous concentration of the oximes cannot be maintained in the tissues, as the oximes are readily excreted. Further it has been demonstrated that the oximes cannot penetrate the blood-brain-barrier. On the other hand a slight beneficial effect *in vivo* could be demonstrated, but only when the oximes were combined with parasympatholytic drugs, e.g. atropine. However, it has to be clearly pointed out, that the mixture has to be injected in animal experiments, prior to organophosphate poisoning, when a beneficial effect in lethally poisoned animals should be demonstrated. On the other hand, drugs with similar structures to the oximes, but without their property to reactivate phosphorylated AChE may have a beneficial effect, when used in combination with parasympatholytic drugs, e.g. SAD-128.[18,19] A recent review was given at the occasion of a "symposium on prophylaxis and treatment of organophosphate poisoning", headed by Koelle.

In animal experiments and in humans, accidentally poisoned by organophosphates, contrary to the suboptimal dose of oximes a beneficial effect could be observed. It is unlikely that the low concentration of oximes might have had any useful effect on reactivation. Therefore a direct action on the cholinergic receptor has to be taken into consideration. It is well known that carbamates do have a direct action on the cholinergic receptor. The receptor may be occupied, or even better be desensitized by the interaction with adequate drugs. It has long been known that carbamoylation

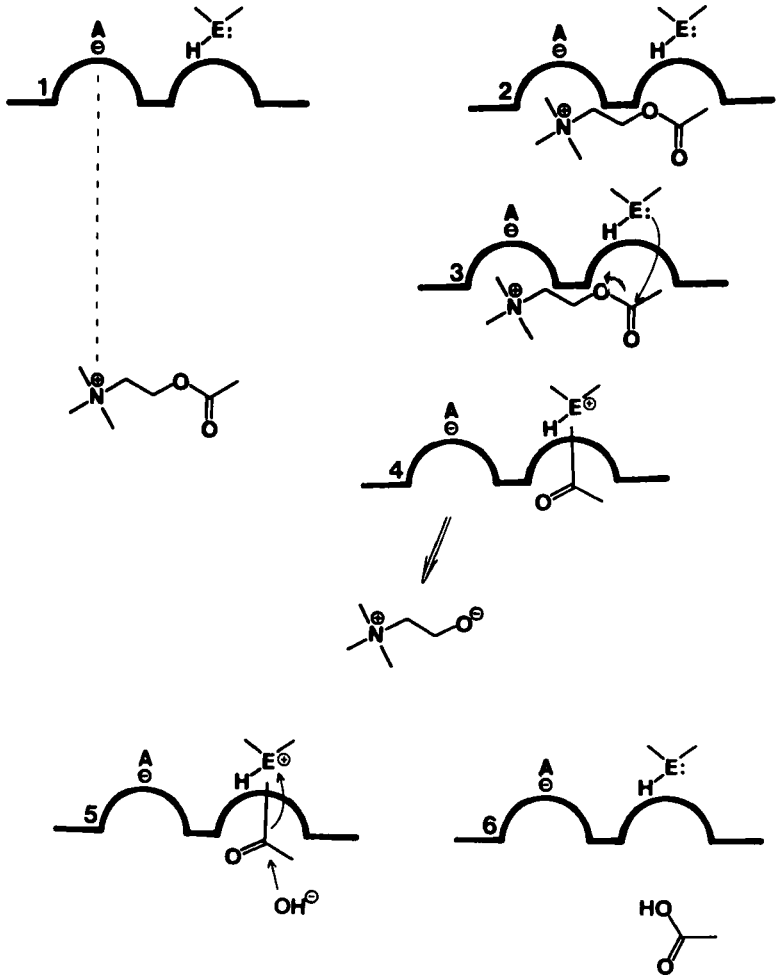

Figure 1 Catalytic process of ACh-hydrolysis. ACh is attracted by ionic forces from the anionic site (1). When placed in the appropriate position (2) the nucleophile group of the esteratic site binds to the carbonyle group of ACh (3), and the choline-moiety is leaving the active site (4). The hydrolysis of the acetylated enzyme (5) is the rate limiting step, and afterwards the active site is again ready to catalyze the hydrolysis of another ACh-molecule (6).

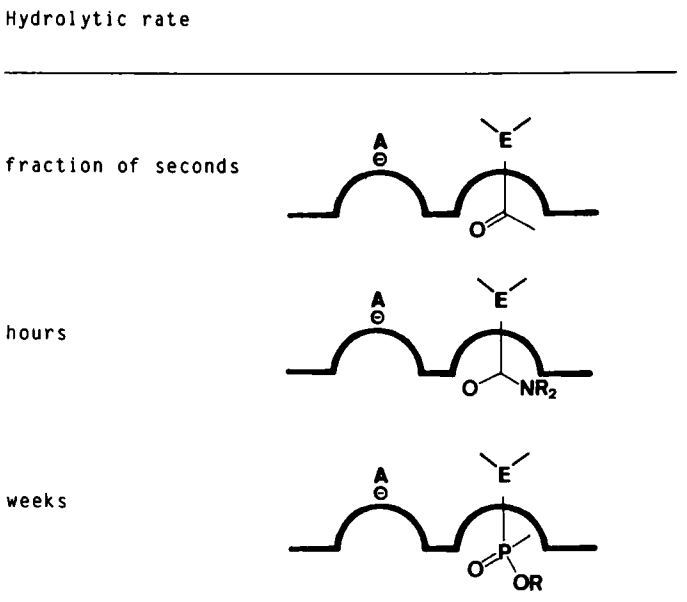

Figure 2 Rate of hydrolysis of the acetylated, carbamoylated and phosphorylated AChE.

protects the enzyme from phosphorylation.[20,21] Drugs like neostigmin or pyridostigmin may be used for this purpose. The hydrolytic product of neostigmin, however, is a product very similar to edrophonium, with the only difference that one ethyl-group on the cationic head of edrophonium is replaced by a methyl-group. Figure 5 illustrates this point.

For our purpose to analyze the beneficial reaction pathways, the group of edrophonium and similar compounds proved to be useful for two reasons:

1) Edrophonium has no capability to reactivate.

2) Edrophonium has no capability to carbamoylate.

To our surprise edrophonium *in vivo* showed a beneficial effect, when applied together with a parasympatholytic drug for therapy of OPP. This effect was similar to the effect of the oximes.

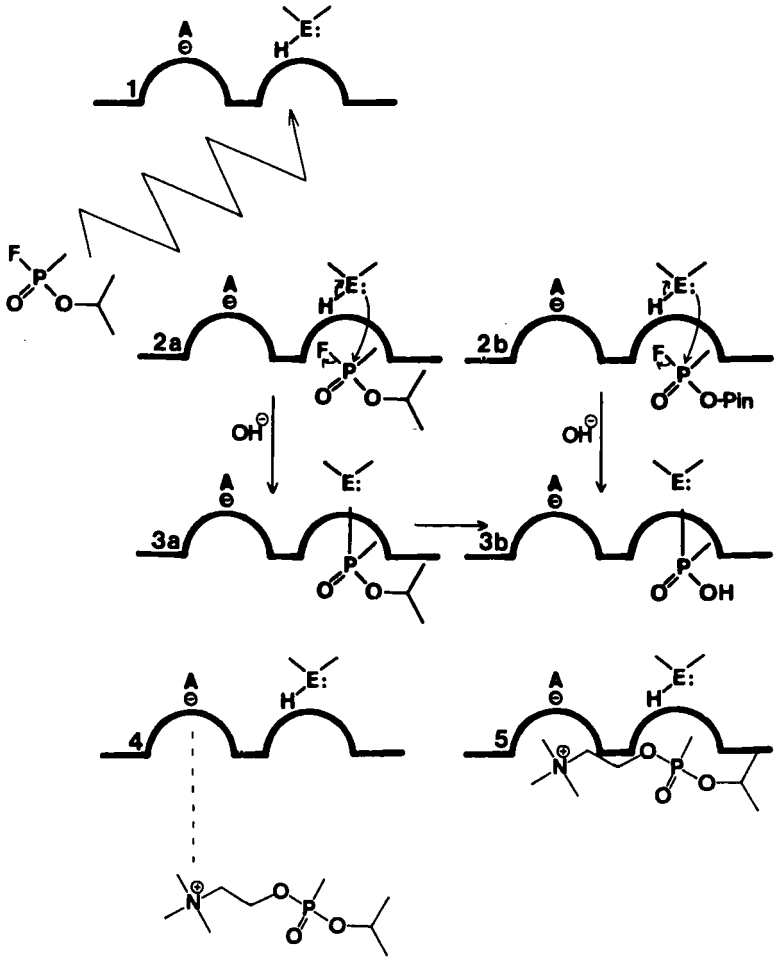

Figure 3 Binding of organophosphates to the active site. Contrary to ACh which is attracted by ionic forces to the anionic site, the organophosphates (1), e.g. sarin (2a) or soman (2b), have to approach the active site by diffusion. The intermediate product from sarin (3a) is able to be reactivated for a short time, the intermediate of soman (3b) however, which is identical to the end-product of sarin-binding, does not react with the conventional oximes. Similar to ACh the so-called Tammelin-Esters (4) are also attracted by ionic forces, and bound to the active site (5) thus outperforming the native organophosphates by a factor of about 1000.

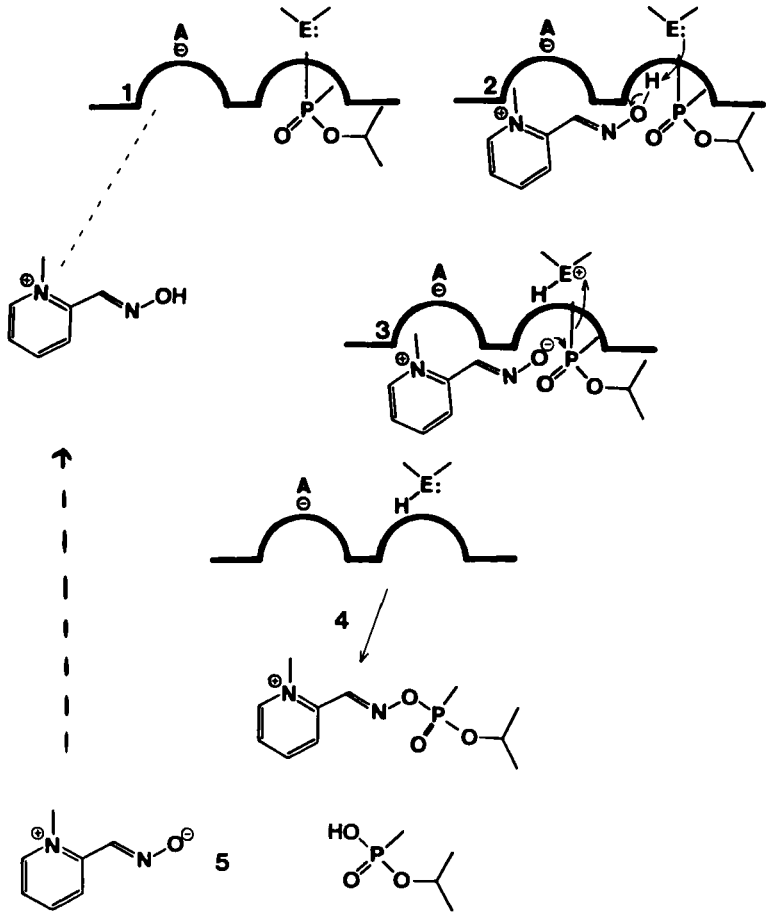

Figure 4 The action of "tailor-made-drugs". In analogy to ACh an oxime e.g. pralidoxime (PAM) is attracted to the active site by ionic forces (1), and as it easily ionized (2) it is able to bind to the phosphor (3), thus forming a so called "Tammelin-ester" (4) which hydrolyzes within minutes. The corresponding fragments are PAM (5), and a harmless phosphate.

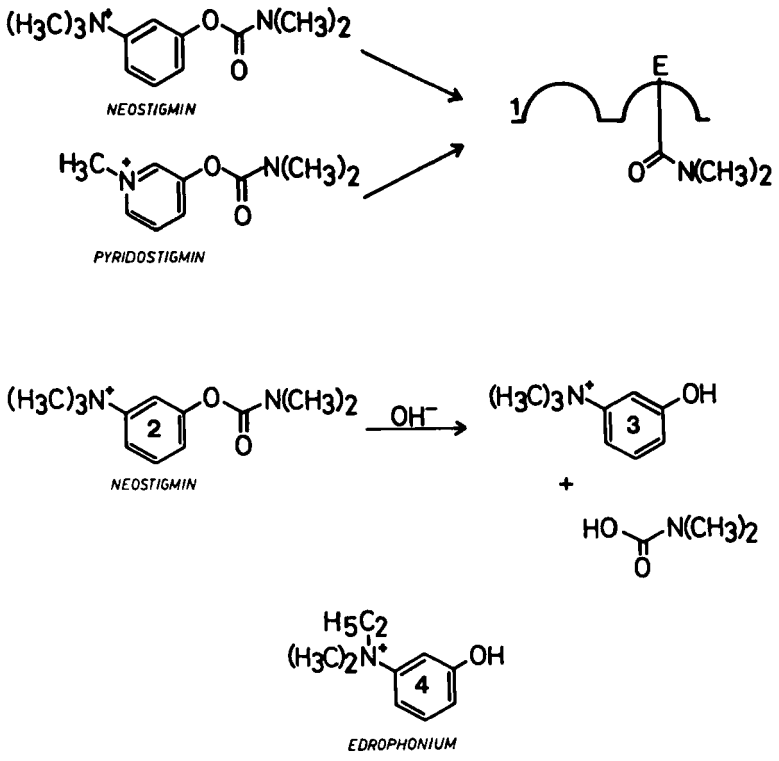

Figure 5 Action of carbamates with the active site (1) of AChE. The hydrolysis of neostigmin (2) yields 3-hydroxy-trimethyl-anilinium ion (3), which is very similar to edrophonium (4).

EXPERIMENTAL

Syntheses

All solvents and compounds for our syntheses were of analytical grade. Edrophonium was a gift from F. Hoffmann-La Roche and the other compounds, including the organophosphates, were synthesized in our laboratory. N,N-diethyl-1,3-diamino propane was purchased from Fluka AG, CH 9470 Buchs, Switzerland. The syntheses of the other compounds will be published elsewhere.

Animal experiments

We used mice (ICRZ, Charles River) and rats (SIVZ 50 S. Ivanovas, Kisleg, Germany) of both sexes from the Institut für Zuchthygiene der Universität Zürich. They were fed NAFAG laboratory diet (NAFAG, Nähr und Futtermittel AG, CH 9202 Gossau, Switzerland) and water *ad libitum*.

All animals which did not receive any treatment, thus serving as controls, were centrally anesthetized.

We feel deeply obliged to the recommendations of the Swiss Academy of Medical Sciences to save the life of animals, whenever possible. For this reason we kept the number of animals at the lowest level.

RESULTS

Edrophonium combined with benactyzine, when applied prior to OPP showed a beneficial effect (Table 1).

This effect resembles the effect of PAM and other oximes. Additionally the combination of edrophonium and benactyzine showed a beneficial effect, even when applied in case of soman poisoning (Table 2).

The reason why only 4 of the 6 originally used animals survived may not be explained yet.

The synthesis of oxime-like substances without an oxime-group was stimulated by the fact that SAD had a beneficial effect to counteract OPP. For this reason we synthesized 1,3-*bis* (4-hydroxymethyl-pyridonium) propane, R8103, 1,3-*bis* (piperidinium-4-carbonic acid ethyl-ester) propane, R8107, 1,3-*bis* (3-hydroxyphenyl-dimethyl-ammonium) propane, R8120, and last but not least N,N-diethyl-1,3-diamino propane (NNP).

The chemical structures are shown on Figure 6, which allowed us to propose a structure activity relationship (SAR).

We did not undertake toxicology studies with R8103, R8107, and R8120, but used doses which approach the optimum doses of oximes by the molecular level (Table 3).

NNP seems to be less toxic than all other substances used before, but on the other hand only shows a beneficial effect when used at a much higher dose-level (Table 4).

Table 1 Therapy of sarin-poisoning in rats with edrophonium and benzactyzine. Edrophonium and benactyzine were given in mg/kg. 1 mg/kg of sarin was applied by s.c. injection at the neck 5 min. after prohylactic treatment. Edrophonium in doses above 17.5 mg/kg were not tolerated

Edrophonium	*Benactyzine*	*Lethality*	*Time to death (min.)*
—	—	6/6	2.28 ± 0.09
4.5	10	2/6	9.39 ± 5.49
17.5	10	0/6	—
17.5	—	6/6	5.27 ± 1.49

Table 2 Therapy of sarin- and soman-poisoning in mice with edrophonium and benactyzine. Edrophonium and benactyzine were given in mg/kg. 0.5 mg/kg of sarin or soman were applied by s.c. injection at the neck 5 min. after prophylactic treatment. Edrophonium in doses above 17.5 mg/kg was not tolerated

OP	*Edrophonium*	*Benactyzine*	*Lethality*	*Time to death (min.)*
sarin	17.5	10	2/6	27
sarin	15	15	1/6	13
sarin	—	—	4/4	10
soman	15	15	2/6	10:30
soman	—	—	4/4	7

Table 3 Therapy of sarin-poisoning in rats with R8103, R8107, R8120 and benactyzine. All compounds were given in mg/kg. 1 mg/kg of sarin was applied by s.c. injection at the neck 5 min. after prophylactic treatment

Substance	*Benactyzine*	*Lethality*	*Time to death*
—	—	3/3	3:04 min.
R8103 84	—	1/3	1 day
R8103 42	10	0/3	—
R8103 20	10	0/3	—
R8107 103	—	3/3	1 day
R8103 51	—	1/3	1 day
R8107 22.5	10	0/3	—
R8120 48	10	1/3	13:54 min.

Table 4 Therapy of sarin-poisoning in mice with N,N-diethylamino-1,3 propyldiamine (NNP) and benactyzine. NNP and benactyzine were given in mg/kg. 1 mg/kg of sarin was applied by s.c. injection at the neck 5 min. after prophylactic treatment. NNP was tolerated in doses up to 500 mg/kg, but animals dosed from 250 mg/kg and higher showed moderate sedation

NNP	*Benactyzine (B) Atropine (A)*	*Lethality*	*Time to death*
10	(B) 10	6/6	25 min.
—	—	6/6	9 min.
20	(B) 10	2/6	2–3 days
20	(A) 10	1/6	3 days
100	(B) 10	1/6	1 day
200	(B) 10	0/6	—
400	(B) 10	6/6	2 days

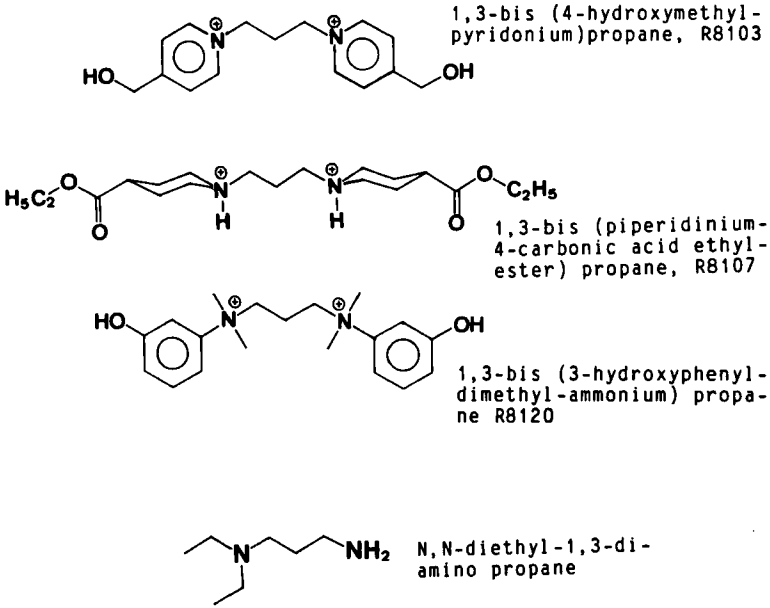

Figure 6 Structure-activity relationship (SAR). Some compounds with beneficial effects for the treatment of organophosphate poisoning.

DISCUSSION

The only antidotes suggested in our days to counteract OPP poisoning are the oximes combined with parasympatholytic drugs. Prominent oximes are based on the structure of pralidoxim, obidoxim, and TMB-4. As the oximes alone are useless *in vivo*, they have to be combined with parasympatholytic drugs. For therapy in humans atropin and similar compounds are recommended. Benactyzine is preferred in animal experiments.

In vitro however, the oximes proved to be effective. Stoichiometrically blocked AChE (sarin), could be reactivated to a certain degree.[17]

Thus the beneficial effect of the oximes combined with the parasympatholytic drugs *in vivo*, cannot be contributed fully to the reactivating property. On the other hand drugs with no capability to reactivate (SAD 128, edrophonium and similar compounds) had a beneficial effect similar to the oximes. The carbamates do have a direct effect at the cholinergic receptor. They can interfere with its activity and/or desensitize the receptor's active site.

We tried to find out a common mechanism for the action on the receptor, and found that structures related to the central part of obidoxim or TMB4 do have almost the same beneficial effect. We used N,N-diethylamino-1,3-propyldiamin because it fits this concept, and because of its ability to penetrate the blood-brain-barrier.

CONCLUSIONS

We have to be aware that the lethal effect of the organophosphates is not a direct effect. Death is caused by the numerous direct actions of ACh on the peripheral, and even more important, on the central nervous system. As the quaternary oximes only can act peripherally, because they cannot penetrate the blood-brain-barrier, we can conclude that the central actions of ACh play a very important role. Now we can understand why conventional oximes never will be successful *in vivo*, when they are applied as single drugs. They have to be combined or even better, preceded by parasympatholytic drugs, e.g. atropine or benactyzine, which easily penetrate the blood-brain-barrier. It has been clearly demonstrated that substances with

no capability to reactivate the blocked AChE showed beneficial therapeutic effects in the treatment of OPP. From this point of view it can be concluded that these substances do have a direct action on the cholinergic receptors. It is not our aim to suggest the substances discussed in this paper to be used as therapeutic agents, but they have been proven to be useful tools. It is our aim to stimulate basic research in the field of muscarinic and nicotinic receptors.

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