

COMPLEX VIEW ON POISONING WITH NERVE AGENTS AND ORGANOPHOSPHATES

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Summary: OP/nerve agents are still considered as important chemicals acting on living organisms and widely used in human practice. Nerve agents are the most lethal chemical warfare agents. They are characterized according to their action as compounds influencing cholinergic nerve transmission via inhibition of AChE. The symptoms of intoxication comprise nicotinic, muscarinic and central symptoms, for some OP/nerve agents, a delayed neurotoxicity is observed. Cholinesterases (AChE and BuChE) are characterized as the main enzymes involved in the toxic effect of these compounds including their molecular forms. The activity of both enzymes (and molecular forms) is influenced by inhibitors and other factors such as pathological states. There are different methods for cholinesterase determination, however, the most frequent is the method based on the hydrolysis of thiocholine esters and following detection of free SH-group of the released thiocholine. The diagnosis of OP/nerve agents poisoning is based on anamnesis, the clinical status of the intoxicated organism and on cholinesterase determination in the blood. Some principles of prophylaxis against OP/nerve agents poisoning comprising the administration of reversible cholinesterase inhibitors such as pyridostigmine (alone or in combination with other drugs), scavengers such as preparations of cholinesterases, some therapeutic drugs and possible combinations are given. Basic principles of the treatment of nerve agents/OP poisoning are described. New drugs for the treatment are under experimental study based on new approaches to the mechanism of action.

Key words: *Organophosphates; Nerve agents; Acetylcholinesterase; Symptoms; Treatment; Diagnosis; Prophylaxis*

1. Introduction

Phosphorus plays a very important role in living organisms, e.g. in photosynthesis, metabolism, synthetic reactions, nucleic acids, coenzyme systems, transmission of signals etc. Organic phosphates are involved in energetic metabolism (ATP, phosphorylated saccharides) and influence the action of hormones or neuromediators (c-AMP, c-GMP). Chemically synthesized organic compounds of phosphorus show a broad variety of biological properties.

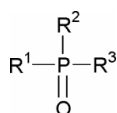
Organophosphorus inhibitors of cholinesterases (commonly called organophosphates, OP) are the most important chemicals in this group. They are anticholinesterase compounds. OP poisoning is commonly reported internationally (38,60,91). These compounds produce acute effect, which is characterized by influencing cholinergic nerve transmission. These compounds are used in industry, in veterinary or human medicine and, last but not least, these compounds are, unfortunately, usable (and used) for military purposes as chemical warfare agents and as poisons used by terrorists. Terrorists have deployed nerve agents in attacks on unprotected civilians, and terrorists have expressed interest of them (115). A Japanese religious

cult, Aum Shinrikyo, independently manufactured numerous chemical and biological agents. The first such attack with sarin occurred in Matsumoto in 1994 and Tokyo subway in 1995. Thousands of people were affected and dozens of people died (101,103,109,142,143). In Matsumoto (1994), 600 people were poisoned and hospitalized, and 7 died. The attack in Tokyo subway (1995) resulted in 5500 people seeking hospital evaluation and 12 deaths (101,104, 110). These compounds are commercially available, and are used in agriculture which leads to professional, suicidal or accidental intoxications. The mechanism of action, diagnosis and treatment of intoxications with OP and nerve agents is a very hot topic at present. Moreover, some principles of the effects, diagnosis and therapy are very similar for OP and highly toxic nerve agents and, therefore, the principles described in this article can be applied in general for both groups – OP and nerve agents.

2. Chemistry

OP and nerve agents are anticholinesterase compounds. A large variety of compounds with different physical chemical and biological properties including toxicity can be

observed in OP group. OP are liquids of different volatility, soluble or insoluble in water, organic solvents etc. and differ in their toxicity from practically non-toxic chemicals (malathion) to highly toxic agents such as VX and other nerve agents. Nerve agents are generally clear and often odourless liquids at room temperature (40). The components chemicals are available for benign chemical syntheses, and starting materials and methods for nerve agents synthesis are easily acquired (102). The most important group having a significant biological effect include compounds of the general formula



where R^{1-2} are hydrogen, alkyl (including cyclic), aryl and others, alkoxy, alkylthio and amino groups. R^3 is a dissociable group, e.g. halogens, cyano, alkylthio group, rest of inorganic or organic acid.

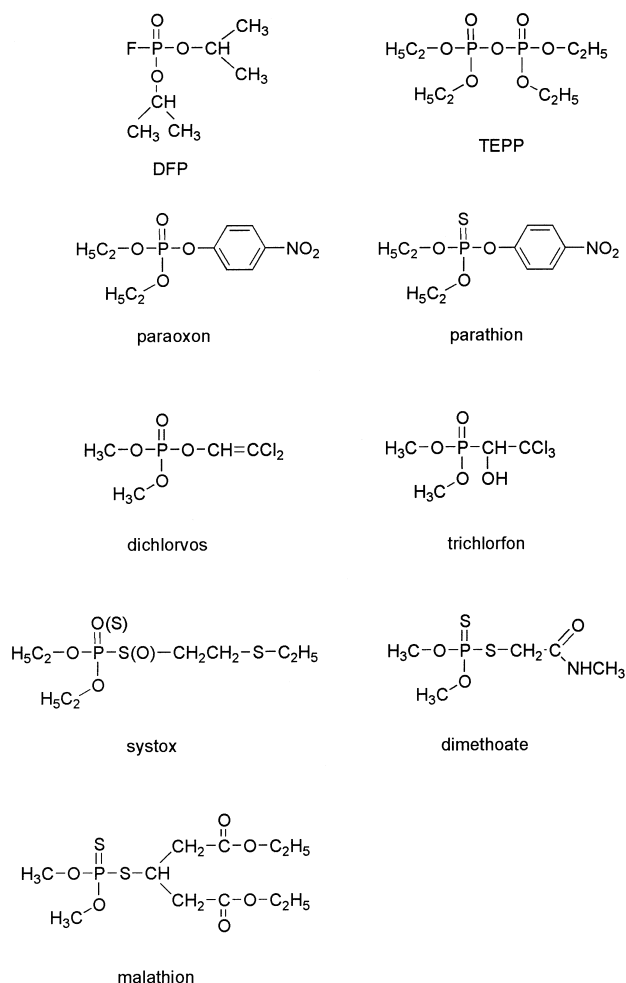


Fig. 1: Structural formulae of some OP.

Chemical formulae of some OP pesticides are shown in Fig. 1.

In the case of highly effective OP cholinesterase inhibitors (nerve agents), typical representatives are sarin, soman, tabun and cyclosarin (G compounds), VX and different V-compounds (V series); and GV compounds, combining in its molecule structure of both G and V compounds. The G series agents were synthesized in prewar Germany, while V series weapons (e.g. VX) were synthesized after World War II. The structural formulae of some highly toxic nerve agents are shown in Fig. 2.

3. Mechanism of action

The mechanism of action (toxicodynamics) of OP is based on irreversible acetylcholinesterase (AChE, EC 3.1.1.7) inhibition at the cholinergic synapses. The resulting accumulation of acetylcholine at the synaptic junctions overstimulates the cholinergic pathways and subsequently desensitizes the cholinergic receptor sites. OP/nerve agents

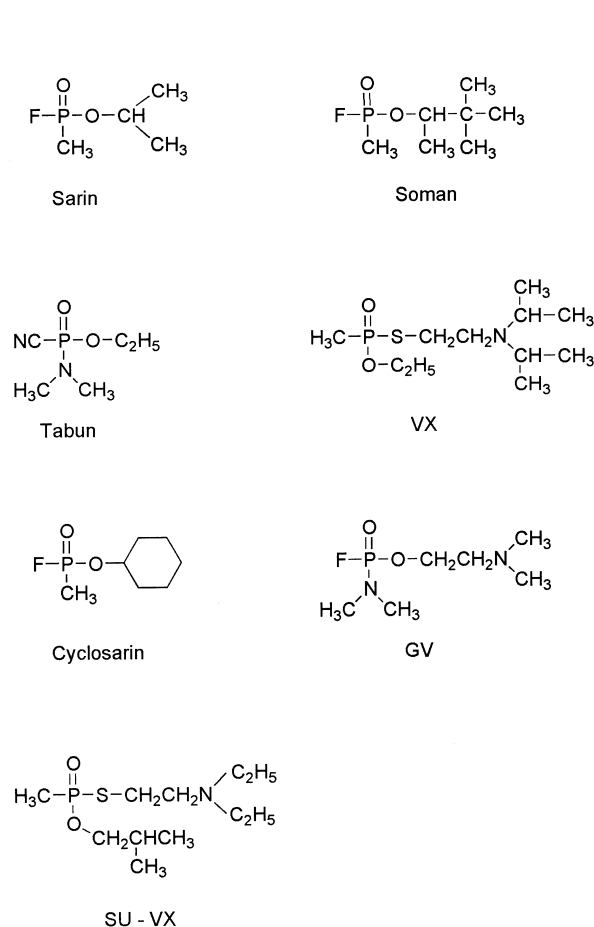


Fig. 2: Structural formulae of nerve agents.

also bind to cholinesterases in the erythrocytes and serum. Although pathophysiologically less important, activities of these enzymes serve as quantifiable proxies of exposure.

However, there is a variety of documented data showing that AChE inhibition is not the only important biochemical change during intoxication. These data have described many other changes accompanied with the development of intoxication that might contribute to OP toxicity. They have included changes of other enzymes, neurotransmitters, immune changes, anaphylactoid reaction, behavior etc. The evidence includes the data indicating that prophylactic/therapeutic drugs might also have multiple sites of action similar to those observed during intoxication (6,9,10,11,13, 23,32,62,88,93,132). The delayed neurotoxic effect is caused by a reason other than cholinesterase inhibition. The neurotoxic esterase has been described as the target site for this symptom, however, only some OP are neurotoxic in that sense (2,3,9,54,58,88,89). An interesting hypothesis was suggested by Cowan et al (32): acetylcholine acts as an agonist of autoacid release, and autoacids such as histamine can augment soman induced bronchial spasm. With respect to the demonstrable critical role of cholinergic crisis in OP/nerve agent toxicity, the precepts of neuroimmunology indicate that secondary adverse reactions encompassing anaphylactoid reactions may complicate OP toxicity.

The mechanism of AChE inhibition for the all OP and nerve agents is practically the same - it is its inhibition via phosphorylation or phosphonylation of the esteratic site of AChE. However, reactivation of inhibited AChE by oximes is different for different nerve agents: phosphorylated but reactivatable AChE is changed to a non-reactivable complex. The half-times for this reaction described as dealkylation (42) are different for various OP/nerve agents (9,10, 42,130) (see also Chapter 6).

There have been observed many kinds of specific and non-specific effects of OP using animal experiments. They

involve cholinesterase inhibition with subsequent changes of the neurotransmitters, changes in membrane permeability, and other metabolic imbalances. Markers of stress are also increased. In some OP, natriuretic affect was observed. Blood flow is decreased during OP/nerve agent intoxication (6,9,32,62,88,93,132) (Fig. 3).

Neuropathology can be observed in animals at subconvulsive doses (2). OP compounds can directly interact with numerous neurotransmitter receptors (e.g. acetylcholinergic, acetylcholine-muscarinic M2, GABA-A etc.), second messengers, and neuronal structural proteins (29,123, 137). Morphological changes following OP poisonings were demonstrated, too. Sližová et al. demonstrated changes in microvascularization of some rat organs (the most expressed in the liver) following soman administration in sublethal doses (124,125). Morphological changes in the brain and heart were observed by Tryphonas and Clement (133) following sublethal soman intoxication beginning 30 min after the injection of soman and progressing 7 day. Shih et al. (122) have demonstrated that soman-induced convulsions are associated with postexposure brain pathology. These findings lead to the hypothesis that central cholinergic mechanisms are primarily involved in eliciting convulsions following exposure to highly toxic OP such as soman and the subsequent recruitment of other excitatory neurotransmitter system. Loss of inhibitory control may be responsible for sustaining these convulsions and for producing the subsequent brain damage. The important role of glutamate and its transporters has been demonstrated during soman poisoning (62,84,135).

It has been described earlier that excitatory amino acids play an important role during OP poisoning. (126). On this basis, the good protective activity of adenosine receptor agonists has been demonstrated (135).

A scheme containing four basic actions (absorption, transport, metabolization and the toxic effect) was described

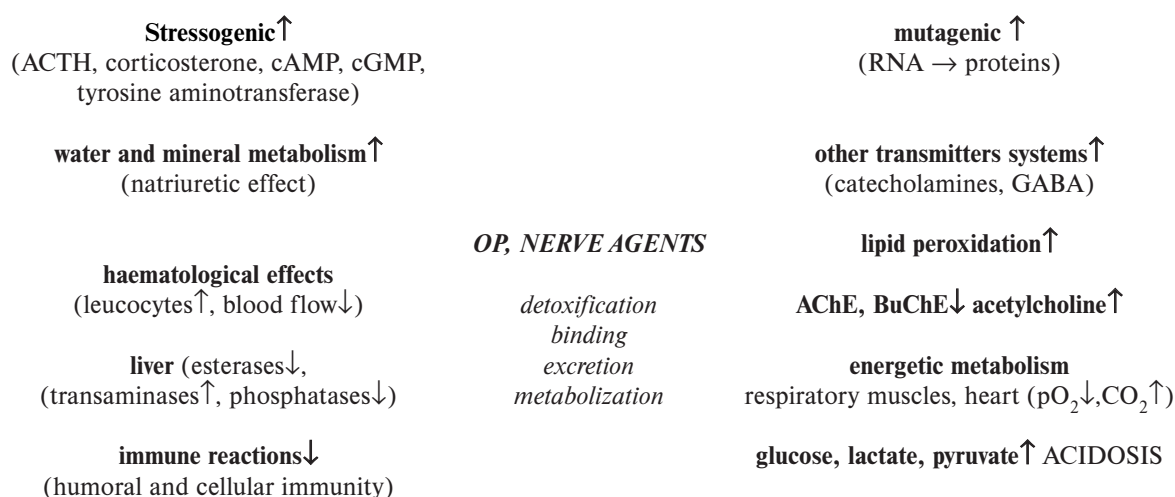


Fig. 3: Schematic representation of possible effects of OP/nerve agents (modified from cit. 9,10).

previously (9). The absorption is accomplished by penetration of OP through biological barriers into the blood representing the transport system. The losses originate either physically or biologically. This part of OP (reacting by this mechanism) is screened out from toxic action. The losses in the transport system originate from detoxification and non-specific binding to proteins and enzymes – esterases, AChE and butyrylcholinesterase (BuChE). Binding to plasma proteins is included, too. Inhibition of cholinesterases in the blood is practically the first target for OP according to the principle „first come, first served“ (24). The OP is carried out at the sites of metabolic and toxic effects. However, there are differences especially in the detoxification of highly toxic nerve agents: G-agents like sarin and soman are detoxified but compounds containing the P-S bond (V-agents) are not detoxified (9,13,14). The toxic effect site is a multi-compartmental system, minimally the central and peripheral nervous systems. In these places, OP reacts with cholinesterases – AChE and BuChE. Inhibition of cholinesterases is a trigger mechanism for the toxic action of OP. Important nerve agents, soman and sarin are rapidly absorbed at all routes of administration including inhalation, percutaneous and oral administration (9,13,14) and inhibit cholinesterases (preferably AChE) in the central and peripheral nervous system. Because of soman's high lipophilicity, it possesses a high affinity to the brain AChE (13,15). Sarin is less lipophilic, however, its affinity to the brain AChE is also very high (9,15).

Both groups of nerve agents (G- and V-compounds) are potent inhibitors of cholinesterases *in vitro* and *in vivo* (4,9,11). From the point of view of pharmacodynamics and therapeutic possibilities, soman represents the most serious poison. Its toxicity is comparable to that of sarin and VX (9,15,121) but the therapeutic efficacy of the antidotal treatment with current and perspective drugs is not good enough (9,13,17,63,93). This is probably a reason for intensive research dealing with soman intoxication and treatment.

In general, G compounds are detoxified in the liver, plasma (9) and, according to some authors, also in the lungs (120); and therefore this part is excluded from the toxic effect. The parent compounds can be monitored in the blood stream as well as metabolites which are excreted in urine (9,24,105–107). Binding to non-specific esterases also causes losses of G-compounds in the organism and this part of soman and sarin does not have a toxic effect. It was assessed that only 1–3% of the dose administered inhibited AChE in the brain, i.e. 1–3% of the dose administered caused the basic toxic effect (9,59,86,120). Another factor (up to now not very elucidated) influencing soman and sarin poisoning is the existence of a depot in the organism from where the nerve agent can be released and then causes a new attack of intoxication. This depot has been described for the skin, erythrocytes, muscles and lungs (9,59, 115). Bearing in mind the very low portion of the dose administered causing the basic toxic effect, it is clear that the release of a very small quantity of sarin and soman can sig-

nificantly influence the survival or death of the intoxicated organism independently of the treatment. V compounds are not detoxified in the organism (14). This is probably the reason for the higher toxicity of V compounds in comparison with G-compounds. The effect of V-compounds (especially VX) is prolonged in comparison with sarin and soman (134). The toxicokinetics of different nerve agents including stereoisomers have also been described (24). The mechanism of action for VX is inhibition of AChE preferably in the peripheral nervous system (9,93). However, inhibition of AChE in the brain parts was described as being selective and most marked in the pontomedullar area of the brain (9,14). Detoxification of OP with lower toxicity is also important. Moreover, for some OP especially those containing the P=S bond, oxidation giving rise to more toxic products is observed ($P=S \rightarrow P=O$). This reaction called „lethal synthesis“ is typical e.g. for malathion (oxidized to malaoxon) or parathion (oxidized to paraoxon). Oxo-derivatives (more toxic) are released into the transport system and can cause a new attack of intoxication. A similar reaction can be observed after releasing the OP from the depot, mostly from fat tissue (9,37,40). In place of the toxic effect (nervous system), the reaction with enzymes is important though some other direct interactions with receptors have been described and non-specific reactions (the stressogenic effect) have been also observed. Some OP can be mutagenic or carcinogenic (9,40). Depending on the target, acute, intermediate, chronic or delayed effects are manifested (9,88,93).

4. Toxicity

Depending on the conditions of its determination, different types of toxicity are differentiated. Acute toxicity is mostly characterized by LD50 (Table 1). Acute toxicities vary greatly among different species, they are dependent on many factors (sex, age, genetic disposition, body weight, diet, hormonal factors etc.). Especially in case of nerve agents, it can be of importance. These agents should be regarded as so-called “hit and run poisons” (24) and, therefore, e.g. time of the onset of convulsions (convulsive time, CT) or death (lethal time, LT) is very valuable information (46). The route of administration is also of great importance (9).

The modelling of OP poisoning has been extensively studied. These studies investigated, among other things, the symptomatologic assessment of OP-induced lethality in mice, expressing OP poisoning through mathematical equations, or evaluating a model for carbamate and OP-induced emesis in humans (9,53,95). Predicting toxicokinetic parameters in humans from the toxicokinetic data acquired from three small mammalian species was the aim of another study (8). Similarly, possible rat models for minimal brain dysfunction have been presented. Other studies correlating structure vs. activity of both OP and their antidotes have been presented (for a review, see 9). A very interesting

Tab 1.: Toxicities for rats (experimentally determined) and human (assessed) for different OP.

COMPOUND	TOXICITY (LD ₅₀)			
	^a i. m., rat (µg/kg)	^a p.o., rat (mg/kg)	^b p. o., human (mg/70kg)	^c i. m., human (µg/kg)
VX	12-16	0,08-0,09	5	20-25
sarin	200	0,7-0,9	8-12	-
soman	70	0,5-0,6	7-12	-
GV	17	0,19	8	20-25
DFP	800	1-13	20-80	40-50
TEPP	850	2-15	30-100	-
paraoxon	300-500	3	30-50	300-350
parathion	500-900	6-7	50-200	2800-3000
dichlorvos,DDVP	17 440	62	500-1000	150-200
trichlorfon	230 000	625	grams	-
systox	3110	9-14	20-100	4000
dimethoate	1000-2000	215-270	1-2 g	-
chlorfenvinfos	5000	15	40-100	-
dicrotofos	7-10000	22	100-200	-
diazinon	50-80000	100-150	700-1200	-
fosfamidon	10-15000	27,5	100-180	-
malathion	-	800-1200	grams	-

^a experimental data from literature (2,9,10,38,40,46,93)

^b assessed data from literature(9,14,93)

^c assessed data from literature (9) and Fig. 5

and perspective approach described by Maxwell et al. (95) using the multiple regression model for in vivo rate cholinesterase inhibition contained three independent variables (blood flow, carboxylesterase and cholinesterase) and this could account for 94% of the observed variation. A theoretical expression for the protection associated with stoichiometric and catalytic scavengers in a single compartment model of OP poisoning has been described (96,129). In our previous paper (9), we described the scheme of the multiple effects of OP including the influence on cholinesterases and other enzymes, detoxification, the possibility of metabolism etc. These studies were elaborated with the aim of extrapolating the data from animals to humans.

However, a simple correlation of toxicity and inhibition efficacy was not linear and statistically significant. A good correlation was achieved when the toxicity data was expressed as logarithm and the inhibition efficacy as a negative decadic logarithm of the I₅₀ value (pI₅₀). The value of pI₅₀ for human brain AChE interaction with OP allows us to extrapolate the corresponding toxicity data for humans (9,11,14). The equation of dependence pI₅₀ vs log LD₅₀ is following:

$$Y = 9.87 - 1.26x, p < 0.01, r_{xy} = -0.9489.$$

This extrapolation is possible for the highly toxic OP where the inhibition is the most important. This correlation is closer in case that the inhibition potency is expressed as the inhibition rate in vivo and correlated with the toxicity data (16).

These results dealing with the relationship between the inhibition efficacy and the toxicity of different OP showed a good correlation between these two parameters.

5. Symptoms of intoxication

Dominating signs of poisoning with OP and nerve agents are caused by hyperstimulation of the cholinergic nervous system due to an elevated level of acetylcholine caused by inhibition of AChE (acute cholinergic crisis). According to type and localization, peripheral and central muscarinic and nicotinic symptoms are observed. (Table 2). Peripheral muscarinic symptoms are observed in the exocrine glands - nasal mucosa (rhinorrhea), bronchial mucosa (bronchorrhea), sweat (sweating), lacrimal and salivary glands (lacrimation, salivation). An elevated level of acetylcholine in the smooth muscles causes miosis (iris), failure of accommodation (ciliary muscle), abdominal cramps, diarrhea (gastro-intestinal tract), micturition, increased frequency of urination (bladder) and bradycardia (heart). Peripheral nicotinic symptoms due to accumulation of acetylcholine include sympathomimetic effects, pallor, tachycardia, hypertension (autonomic ganglia) and muscular weakness, fasciculations and convulsions and later paralysis (skeletal muscles including diaphragm and intercostal muscles). Central (muscarinic and nicotinic) symptoms are not very specific and include giddiness, anxiety, restlessness, headache, tremor, confusion, failure to

Tab. 2: Main effects of OP/Nerve agents in various sites in the body.

Nerve agents (minutes)	CENTRAL giddiness, anxiety, restlessness, headache, tremor, confusion, failure to concentration, convulsions respiratory depression	OP (hours)
0-5 5-10 10-15	MUSCARINIC increased secretion – rhinorrhoea, salivation, lacrimation, bronchorrhea, sweating, miosis, failure of accommodation, abdominal cramp, diarrhoea, bradycardia, hypotension, involuntary micturition	0-1 1-3 3-8
20-60	NICOTINIC weakness, fasciculations, convulsions, generalized convulsions	2-8
30-60	DEATH failure of heart and ventilation functions	2-24

concentrate, convulsions, respiratory depression etc. These effects are called the cholinergic effects. OP/nerve agents have got many other effects that have an influence on various organs and systems. They are called non-specific (non-cholinergic) effects. These effects are usually registered later, after the manifestation of the cholinergic effects. Therefore, the OP/nerve agent poisoning can be divided into three phases (9,88,93): cholinergic phase characterized by cholinergic effects (also called acute cholinergic crisis), transitional phase characterized by mixed cholinergic and non-cholinergic effects and non-cholinergic phase characterized by the predominance of non-specific effects. The intermediate syndrome in OP poisoning is clinically characterized by weakness in the territory of cranial nerves, weakness of respiratory, neck and limb muscles, and depressed deep tendon reflexes. It occurs between the acute cholinergic crisis and the usual onset of OP-induced delayed neuropathy (3,58,59). Postexposure changes of neurological character have also been observed (26). It was recently demonstrated that low doses of nerve agents also caused long lasting changes in behavior and neuroexcitability in experimental animals (12,69). In the CNS, OP have both acute and long-term effects (12,61,69,98,115) The cholinergic system has widespread distribution in the CNS, and it plays primary roles in attention, arousal, and memory (115).

The time course of poisoning is dependent on the type of agent, the dose incorporated and the route of exposure. Symptoms appeared minutes after inhalation of nerve agents and minutes to hours after incorporation of OP pesticides. Death can be observed (without treatment) within minutes after nerve agent inhalation and within hours to days after OP pesticide exposure. (9,46,88,93 and others). In the sarin attack in Matsumoto, the critically ill were noted to have hyperglycaemia, hypokalaemia, and/or hypolipidaemia. An increased level of creatine phosphokinase was noted in more than 10% of victims. Neurologically, 22% had headache, 12% had malaise, 6% had dysesthesia and

some had EEG abnormalities. Low-grade fever and leucocytosis were noted in less than 10 % (97,108).

Following poisoning with some OP, the delayed neurotoxic effect called also OrganoPhosphate Induced Delayed Neurotoxicity (Neuropathy) (OPIDN) can be observed. It is characterized by sensoric and motoric disturbances of the peripheral nervous system (degeneration of axons and myeline and inhibition of so called „neurotoxic esterase“). OPIDN is manifested following OP exposure (some times it is not accompanied by acute syndromology) during days (weeks) after the exposure. After a latent period (1-4 weeks), cholinergic irritation can be observed in about 30% of patients (increased salivation, nose secretion, pharyngitis, laryngitis). Paralysis of the leg muscles follows these symptoms for 1-2 weeks, persisting 1-2 months without significant changes of sensitive innervation. Then denervation and atrophy of the leg muscles is observed. Partial restitution is possible, however, convalescence is too long, abnormal reflexes are observed for years. Tri-O-cresyl phosphate (TOCP) has been reported as the typical compound producing OPIDN (3,9,58,59). There has been a single case report of nerve agents exposure causing OP-induced neuropathy (57).

Sarin-exposed victims, when compared with unexposed victims at 1 and 3 years after the Matsumoto attack, had significantly different complaints. The severity of their fatigue, asthenia, shoulder stiffness, insomnia, slight fever, narrowing of the visual field, blurred vision, and asthenopia was positively associated with the grades of exposure (104). Indications were obtained for long-term effects of low-level inhalation exposure of rats to sarin. Some changes in behavioural characteristics, such as decrease in activity and mobility, persisted for 3 to 12 months (12,69). Thus, nerve agent poisoning is associated with long-term CNS changes both in experimental animals and in humans appearing especially following exposure to low concentrations of nerve agents. The data available for nerve agents (especially sarin) do not support a hypothesis on carcinogenic, mutagenic and teratogenic properties of nerve agents (9,88,93).

6. Cholinesterases

Cholinesterases belong to the group of hydrolases splitting the ester bond, i.e. the esterase subgroup catalyzing the hydrolysis of esters to alcohol and acid. Cholinesterases hydrolyze choline esters more rapidly than other esters and are sensitive to OP and eserine.

According to the affinity to natural substrates – choline esters – cholinesterases are divided into AChE and BuChE. AChE, specific or true cholinesterase, the “e” type of cholinesterase (EC 3.1.1.7) with a higher affinity to acetylcholine than to butyrylcholine, and splitting acetyl-beta methylcholine. It is inhibited by an excess of substrate. High AChE activity was observed in erythrocytes, the brain, the electric organ of *Electrophorus Electricus* and the neuromuscular junction. However, AChE activity was observed in many tissues including plants, e.g. onion (55). AChE is composed from subunits. BuChE, pseudocholinesterase, non-specific cholinesterase, the “s”-type of cholinesterase (EC 3.1.1.8) is present in the plasma (serum), pancreas and liver (where it is synthesized). It is ubiquitous enzyme present not only in some human and animal tissues but also in many plants, microorganisms etc. BuChE does not hydrolyze acetyl-beta-methylcholine and has a higher affinity to butyryl- and propionyl choline in comparison with acetylcholine. Substrate inhibition was not observed. There exist BuChE isoenzymes that are genetically determined. Depending on the genetic material, some individuals have a very low or no BuChE activity (1,9,27,139).

A qualitative difference between the BuChE of suxamethonium sensitive individuals and that of other patients were demonstrated by Whittaker (139). These people with genetically diminished BuChE activity may be at higher risk when exposed to pesticides or suxamethonium (1,87). The plasma of individuals with normal BuChE activity hydrolyzes succinylcholine or bind a part of OP pesticide and, therefore, the real dose of these compounds penetrating to the target sites is diminished. In case of absence of BuChE, the dose administered is not decreased and, therefore, relative overdosage is occurred.

AChE and BuChE differ in their enzymatic properties and physiological function (9,94,112). AChE splits neurotransmitter acetylcholine at the cholinergic synapses. It was also observed in erythrocytes but its function here is not yet known in detail similarly as the function of BuChE activity in plasma, though there is evidence that BuChE plays an important role in cholinergic neurotransmission and could be involved in other nervous system functions, in neurological diseases and in non-specific detoxification processes (112).

A more detailed knowledge of cholinesterases occurred with the description of the molecular structure of AChE (127). Like other serine hydrolases, AChE contains a catalytic triad so-called the esteratic site (Ser₂₀₀-His₄₄₀-Glu₃₂₇) at the bottom of a deep and narrow cavity, known as the „aromatic gorge“. In addition to the catalytic center sub-

sites, AChE possesses one or more additional binding sites for acetylcholine and other quaternary ligands. Such peripheral anionic binding sites are at the lip of this gorge. In BuChE, Trp₂₇₉, an important component of the peripheral binding site in AChE is missing. This site is believed to be responsible for substrate inhibition, which is one of the features that distinguishes AChE from BuChE. Hypothetic scheme of the active surface of AChE is given in Fig. 4.

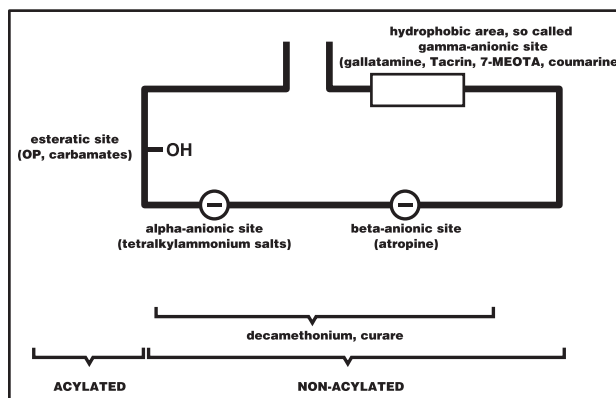


Fig. 4: Schematic structure of the active surface of AChE indicating different binding sites for various ligands.

Determination of cholinesterase activity is based on many principles. In general, an enzyme is added to the buffered mixture and the enzymatic reaction is initiated by adding the substrate. Different parts of the reaction mixture are determined (continually or discontinually), i.e. unhydrolyzed substrate or reaction products, both directly or indirectly. The conditions must be chosen very carefully because of different factors influencing the activity.

According to the procedure and laboratory instrumentation, the most common methods of cholinesterase determination are as follows:

Electrometrical, titrimetric, manometric, colorimetric detection of the unhydrolyzed substrate, measurement by the change of pH using an indicator, spectrophotometric, fluorimetric, radiometric, calorimetric, polarographic, enzymatic, and others e.g. near infrared spectroscopy. These methods are also suitable for the detection of cholinesterase inhibitors using biosensors or immunochemical assay for detection of chemical warfare agents. More detailed review dealing with the methods of cholinesterase determination including literature sources was given previously (9,11).

A very sensitive and commonly used method for cholinesterase determination was described by Ellman et al. (39), based on hydrolysis of the thiocholine substrates acetyl- and butyrylthiocholine or others. After enzymatic hydrolysis, the relevant acid and thiocholine are released and thiocholine by its SH-group is detected using 5,5'-dithio-bis-2 nitrobenzoic acid forming 5-mercapto-2-nitrobenzoate

anion determined spectrophotometrically at 412 nm. Sometimes this method is used with specific inhibitors and there are many modifications described in the literature. This method is in good correlation with other methods. It is sufficiently specific and sensitive and it is used for different purposes in many laboratories around the world. Expression of the activity varies greatly, usually as μmoles of substrate hydrolyzed per min (time) per ml of material examined (e.g. plasma, serum) or per mg of weight tissue (wet, dry, mg of nitrogen etc.). From these values, the expression of the activity in Units can be derived (it is the quantity of enzyme catalyzing μmol of substrate per min at standard conditions). In the clinical laboratory, the activity can be also expressed as catal per litre, i.e. 1 mol of substrate hydrolyzed per sec per litre or kg (cat/l, kg) which is hydrolysis of 1 mol of substrate hydrolyzed per sec per l or kg ($\text{mol} \cdot \text{sec}^{-1} \cdot \text{l}^{-1}$ or kg^{-1}). There are many publications dealing with the review and modifications of cholinesterase determination. One of the last methodical works improving the Ellman's method (39), including a description of the methods, is a paper published by Worek et al (140).

Inhibition of enzymes can basically be divided into reversible or irreversible. OP are apparently irreversible inhibitors of cholinesterases (AChE and BuChE) and the reaction can be expressed by a simple scheme (Fig. 5).

The principle of this reaction is phosphorylation (phosphorylation) of the serine group on the catalytic triade (active center) of AChE. The rate of spontaneous dephosphorylation is very low and it can be omitted in most cases. However, it can be improved/increased using cholinesterase reactivators (oximes) able to reactivate OP/nerve agent-inhibited AChE. These compounds with an ionized oxime group will break the bond between AChE and OP and restore enzyme activity (reactivation) by the nucleophilic attack on phosphorylated or phosphonylated serine at the active center of the AChE molecule and liberate the free enzyme (9,63,80,81). This fact is limiting factor for the therapy with reactivators. The rest of the OP/nerve agent forms a complex with the reactivator (more toxic but less stable) hydrolyzing practically immediately and not having high importance for the course of intoxication. OP inhibits AChE via phosphorylation of the esteratic site. The efficacy of oxime reactivation is dependent on both oxime and the conjugated phosphonate structure (90). Simultaneously, the microenvironment of the gorge plays a significant role in determining the selectivity of the substrate and inhibitors for cholinesterases. Depending on the structure of the inhibitor, inhibited AChE is dealkylated (aged) and the complex formed is resistant to the reactivation effect ($\text{EP1} \rightarrow \text{EP2}$). The molecular mechanism is explained by

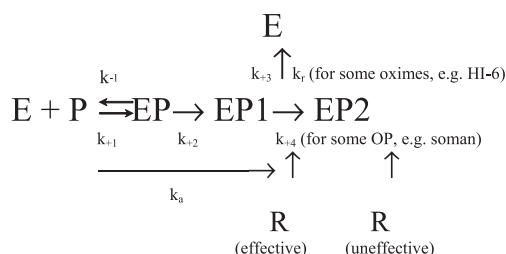


Fig. 5: Simplified representation of reaction of OP inhibitor (P) with AChE (E). The enzyme reacts with inhibitor forming intermediate complex (EP). This reaction is reversible characterized by relevant rate constants of the first order kinetics (k_{+1}, k_{-1}). Intermediate complex is changed with relevant rate constant k_{+2} to phosphorylated (phosphonylated) stable enzyme (EP1). The reaction from the beginning to formation of the stable complex ($\text{E} + \text{P} \rightarrow \text{EP1}$) is characterized by bimolecular rate constant k_a . The rate of spontaneous dephosphorylation (dephosphorylation) characterized by constant k_{+3} is very slow and can be omitted. However, the complex EP1 can be reversed by cholinesterase reactivators forming free enzyme (E). Therefore, the complex EP1 is reactivatable. The rate constant of reactivation (k_r) is very important for the treatment. For some OP/nerve agents, the change of EP1 to EP2 is occurred. This reaction called "aging" or dealkylation leads to forming of unreactivable complex (EP2) is characterized by k_{+4} rate constant. This reaction is very fast for soman-inhibited AChE. The products formed during these processes are not shown for simplicity.

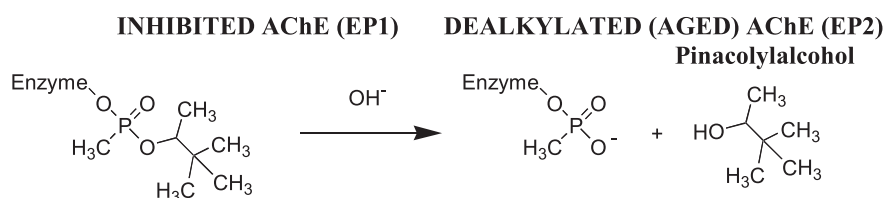


Fig. 6: Schematic representation of aging (dealkylation) of soman inhibited AChE (Enzyme). The enzyme is dealkylated and remains unreactivable and relevant alcohol (pinacolylalcohol) is released.

the splitting of the complex forming the alcohol and unreactivable enzyme (Fig. 6). This reaction, called aging or dealkylation is very fast for soman-inhibited AChE (the half-life is about 10 min) and it is less expressed for sarin (the half-life is about 10 hours), for VX-inhibited AChE this reaction was not observed within 24 hours (9,40,42,130). This is one of the reasons for difficult therapeutic interventions of soman intoxication (9,13,18,64,65). Peripheral site ligands may have selective effects on AChE phosphorylation. The importance of the orientation not only of the OP molecule but also the reactivator has been described by Luo et al. (90).

The reversibility of inhibition is very important for carbamates. They react with AChE in the same manner forming a carbamylated (inactive) enzyme preventing phosphorylation of the carbamylated portion of the enzyme; however, spontaneous decarbamylation occurs very quickly and the released enzyme serves as a normal enzyme source, provided that no inhibitory concentration of an AChE inhibitor is present anymore. Therefore, reversible inhibitors (mostly carbamates) are used as prophylactics against OP/nerve agents intoxications (9,18,45,66,68,70).

The influencing of BuChE activity by gamma-irradiation, stress, gravidity, some neurological and psychiatric disorders, hormones and medical drugs has been demonstrated (9,11,27,33,112,139). The elevation of BuChE activity is not so frequent; an increase in children with nephritic syndrome has been observed (9,139); an elevated ratio of BuChE/LDL cholesterol indicates an increase in the risk of cardiovascular diseases. The involvement of BuChE with the fat (cholesterol) metabolism has been suggested (9). The relationship between BuChE activity and experimentally induced diabetes mellitus in rats was also mentioned (for a review, see also 9,11,112).

Determination of AChE activity is not so widely used in clinical laboratories. A decrease in red blood cell AChE activity in pernicious anaemia has been demonstrated; diminished erythrocyte AChE activity is typical for paroxysmal nocturnal haemoglobinemia and ABO incompatibility (9,11). AChE activity in the erythrocyte membrane can be considered as an indicator of erythrocyte membrane integrity. Increased AChE activity in rectal biopsy is of great diagnostic significance in Hirschsprung's disease, especially in the presence of its atypical molecular form (9,19). There are other papers demonstrating increased AChE activity in the amniotic fluid during pathologic development of the neural tube (25). AChE activity in the erythrocytes and cerebrospinal fluid is also diminished in some endogenous depressions and other psychiatric disorders; however, the results presented are not quite clear at present (for a review see, e.g. 9,11,112).

On the other hand, influencing of the cholinergic nervous system is one of the most important pathological changes in Alzheimer's disease. A lack of the cholinergic mediator, acetylcholine, was observed. This has led to attempts to correct cholinergic deficiency at various levels of

cholinergic functioning: inhibitors of cholinesterases like physostigmine were used; however, physostigmine was not found to be an ideal drug for clinical use because of its short half-life, side effects etc. (44). Clinical study results showed perspective results with the acridine inhibitor of cholinesterase - Tacrin (1,2,3,4-tetrahydro-9-aminoacridine); its 7-methoxyderivative (7-MEOTA) was described as a compound of low toxicity and with good therapeutic effect in experimental intoxication with anticholinergics (44,112). Biochemical studies dealing with its effect on cholinesterases *in vivo* showed that 7-MEOTA inhibited BuChE in the liver and AChE in brain parts with the highest sensitivity in the frontal cortex (9,11,44,112).

Metal cations are an interesting group of compounds modifying cholinesterase activity. Their effect was studied on relatively simple cholinesterase models. It was demonstrated previously that Hg ions especially diminish AChE activity in low concentrations (14).

There are other drugs influencing AChE activity, however, without basic knowledge on their mechanism of action. It was demonstrated recently that cyclosporine A diminished AChE activity in selected parts of the rat brain after administration *in vivo* (56). Similar studies were demonstrated with Daunorubicin in rabbits: cholinesterases in different parts of the heart were decreased in animals treated with this drug (47-50).

The factors influencing cholinesterases are not limited to chemicals only. AChE activity is connected with cholinergic activity in the brain. Using determination of AChE, combined with defined lesions of the different parts of the brain, it was possible to demonstrate cholinergic projections in the central nervous system (9,14).

AChE shows a polymorphism of quaternary structures, of similar catalytic activity but differing in their properties. Catalytic subunits, which may vary in glycosylation can oligomerise into dimers or tetramers, giving rise to the globular (G) forms: G1, G2 and G4. These forms can further be divided depending on their amphiphilicity. Attachment of a collagen-like tail to one, two or three catalytic tetramers gives the A4, A8 and A12 asymmetric forms, which bind to basal lamina. Tetramers are formed by electrostatic and hydrophobic interaction between two disulphide-bonded dimers (9,94,112).

Multiple molecular forms (AChE and BuChE) are also influenced by many factors (9,11). The function of these forms is not known at present. There are only scarce data describing the changes of AChE molecular forms following intoxication with highly toxic OP (15). Some experiments were performed with relatively less toxic OP (21). From the group of highly toxic OP compounds, sarin, soman, and VX were found to be the most effective (15).

Molecular forms of AChE showed different sensitivity to inhibitors *in vitro* and *in vivo* (9,11,15,112). Intoxication with Paraoxon (less toxic OP) caused medium inhibition of some forms of AChE (21). Using thermal denaturation, it was demonstrated that they are not artifacts formed during

homogenization or other treatment of the brain tissue (14). The overall data show that the catalytic activity of AChE molecular forms is different and that their inhibition by various inhibitors may be heterogeneous. This heterogeneity was demonstrated for AChE phosphorylating inhibitors as well as for inhibitors with different binding sites for the enzyme.

7. Diagnosis

A chemical exposure should enter the differential diagnosis in all mass casualty events manifesting with respiratory and neurologic symptoms. Carbon monoxide, hydrogen sulfide, certain metal exposures, and xylene can cause an acute convulsions. Only two classes of chemical warfare agents can cause the acute onset of respiratory symptoms and neurologic dysfunction: nerve agents and cyanides. Cyanide victims present without miosis and usually without cyanosis. Although seizures may be present, neuromuscular symptoms are absent (9,115). The effect of OP/nerve agents is characterized by their interference with cholinergic nerve transmission via inhibition of AChE and BuChE. The cholinergic crisis (accumulation of neurotransmitter acetylcholine) is accompanied by other changes – disturbed membrane permeability, the stressogenic effect, inhibition of enzymes other than cholinesterases, changes in the cyclic nucleotide levels, oxygen saturation etc. There are also morphologic and immunologic changes observed soon after the intoxication. Diagnosis of OP poisoning is based on clinical signs and cholinesterase determination using the most suitable material for laboratory diagnostics – the blood. However, there are other changes in the biochemical parameters during the intoxication/exposure to OP depending on the type of such compound (mutagenicity, delayed neurotoxicity etc.).

Cholinesterase activity is fundamentally important for the diagnosis of intoxication with cholinesterase inhibitors, including OP and carbamates (9,93,88). On the other hand, the activity depends on many other factors and, therefore, cholinesterase determination is of diagnostic importance in different pathological states, i.e. not only intoxications (9, 11,92). The activity of these enzymes (AChE and BuChE) is influenced by sex, age, nutrition, hormonal factors, irradiation etc. (11,27,139). The variation of BuChE activity is greater than that of AChE (11,139) and it is genetically determined (139). There is a wide variation in the population for AChE erythrocyte levels by ethnic groups, age, and reproductive status. Red blood cell AChE levels are lower in infants than adults (33). In pregnancy, erythrocyte AChE activity can be elevated and serum BuChE levels can be reduced compared with non pregnant controls. Measurements of both cholinesterases is still useful for conforming the diagnosis, for monitoring recovery, or for forensic study (9,115).

Clinical monitoring of intoxication and determination of cholinesterases in the blood are basic methods for the diagnosis and differential diagnosis of the intoxication with OP/nerve agents (9,23,115). It is necessary to examine the

whole picture of intoxication, i.e. not only biochemical examinations but clinical signs allowing more precise assessment the prognosis of the intoxication. As for clinical biochemistry, it is necessary to have biological samples, mostly blood and urine. The OP/nerve agent in the urine can be detected, however, their degradation is fast and therefore the time where detection in the urine is possible short. The detection of metabolites is also possible but limited for such OP metabolizing to the specific products e.g. para-nitrophenol in the paraoxon poisoning (107). For the diagnosis, the direct determinations of the toxic agent (OP or nerve agent) in the circulating system is also possible. However, the parent compound will circulate intact for a short period of time and detection will not be possible for more than approximately hours after exposure. Metabolites circulate for a longer time period and are mostly excreted in urine. A metabolite of sarin (O-isopropyl methylphosphonic acid) could be traced in urine and plasma from victims after the Tokyo subway sarin terroristic attack (105,106). For some OP pesticides (parathion, paraoxon), detection of p-nitrophenol in urine is an indicator of exposure (9). However, the retrospectivity of these methods is limited. The detection using an immunoassay of nerve agents is now in progress. The antibodies against soman may have the appropriate specificity and affinity for immunodiagnosis of soman exposure (85).

Therefore, the blood remains to be the main source of biological material for biochemical examination.

For occupational medicine purposes, the determination of cholinesterases in the blood of workers with OP is obligatory. A decrease of the activity below 70% of normal values is an indicator that the worker should not come into contact OP. However, the normal values varied within the laboratories depending on the method of determination. For practical purposes (individual and interindividual variation), determination of individual normal activity was recommended (this approach is more better than that of calculating the decrease from an average value) as well as separate determination of both cholinesterases, the red blood cell AChE and plasma BuChE. The erythrocyte AChE activity seems to be more useful for diagnostic purposes than BuChE activity in the plasma. In clinical biochemistry, BuChE determination in the plasma or serum is more frequently used than that of AChE in the red blood cells.

There are many other factors influencing BuChE activity and the diagnostic importance of diminished BuChE activity is important for the following states – except hereditary decrease of the activity and poisoning with OP/nerve agents and carbamates – congenital deficiency, liver damage, acute infection, chronic malnutrition, metastasis (especially liver), myocardial infarction, dermatomyositis, intoxication with carbon disulphide or mercury and obstructive jaundice (7,9,20,23,60,92).

There are other biological materials available for special purposes (not for the diagnosis of OP poisoning) – amniotic fluid, cerebrospinal fluid, and biptic materials. From

these samples, tissue obtained by the rectal biopsy is used most frequently (diagnosis of Hirschsprung's disease). An elevated AChE activity in the rectal tissue/homogenate (detected histochemically/biochemically) is one of the good diagnostic markers indicating a need for surgical treatment of Hirschsprung disease and a criterion for diagnosis and management of obstipation (11,74). The presence of an unusual AChE band after the electrophoretic separation supports the diagnosis (11,19). The same (either AChE elevation or the presence of a new electrophoretic AChE form) in the amniotic fluid can be applied for the diagnosis of malformation of the neural tube development during pregnancy (25). AChE activity in the cerebrospinal fluid is also changed in some pathological states, however, the diagnostic validity is not so high and can be considered as a complementary examination (9,11,76,113).

Recently, a method was developed which is based on reactivation of phosphorylated cholinesterase and carboxylesterase (CaE) by fluoride ions (114). Based on this method for retrospective detection of exposure to OP, the exposure of victims of the Tokyo incident to an OP, probably sarin, could be established from analysis of their blood samples (41,114). A novel and general procedure for diagnosis of exposure to OP, which surpasses the limitations of the fluoride reactivation method was described (136). It is based on the rapid isolation of BuChE from the plasma by the affinity chromatography, digestion with pepsin followed by liquid chromatography with the mass spectrometric analysis of phosphorylated nonapeptides resulting after the digestion of inhibited BuChE with pepsin. The method can be applied for the detection of exposures to various OP pesticides and nerve agents including soman (105–107). The development of the new specific methods mentioned (fluoride reactivation, tandem MS analysis of enzymatic digests of BuChE) are of high importance for more precise diagnosis of OP/nerve agents poisoning. An extensive review of Noort et al. (105) dealing with biomonitoring of exposure to chemical warfare agents (not only nerve agents) can be strongly recommended.

As was mentioned previously, a decrease in cholinesterase activity is the factor indicating (after the exclusion of other factors) an exposure to OP/nerve agents or other cholinesterase inhibitors. This simple determination does not allow us to make some decisions dealing with the antidotal therapy (especially the repeated administration of reactivators) and then have low prognostic validity. Therefore a new test of the reactivation has been described (14). The principle of the reactivation test is double determination of the enzyme, the first without and the second one with the presence of a reactivator in the sample. The choice of reactivator is dependent on the availability of the oxime, however, in principle it is necessary to have in these parallel samples the same concentrations of the reagents. Using this method, *in vitro* reactivation of the whole human blood *in vitro* inhibited by various nerve agents (VX, sarin, soman) was determined. This reactivation test was used for deter-

mination of the reactivatability in rats and dogs intoxicated with the same nerve agents (sarin, soman, GV and VX) (10). From these results, differential diagnosis can be derived – in the case of low reactivation (0–10%), soman as the toxic agent is the most probable. A middle reactivation of about 50% indicates sarin intoxication and a high reactivation is typical for VX (9,10,14). The delayed neurotoxic effect can be monitored by the determination of neurotoxic esterase. The determination of this enzyme in the lymphocytes soon after injection of neurotoxicants (15–30 min) permits an assessment the progress of delayed neurotoxicity (72). *In vitro* techniques for the assessment of neurotoxicity have been elaborated, too (9,58).

An interesting and new approach was described by Gopalakrishnakone (52). The human brain cell lines were exposed to various concentrations of soman for a period of one and two day. A total of 115 and 224 genes involved in signal transduction, metabolism, cell growth, development, apoptosis and immune response were either up- or down-regulated, respectively. This approach needs to be elaborated in more detail.

Determination of AChE or BuChE molecular forms can be interesting and useful for improvement of the diagnosis of OP poisoning. It was demonstrated that these forms are inhibited in different manners – some of the forms are resistant (a low molecular weight), some of them are very sensitive (a high molecular weight). When the total AChE activity is determined, the value obtained is a „mean“ of the activities of these forms (15). From practical point of view in the clinical laboratory, it is necessary to monitor basic physiological functions, cholinesterases and other biochemical parameters not only for diagnostic purposes but also preferably for the regulation of treatment.

8. Prophylaxis

The decontamination can be considered as a prophylactic measure, however, it is not medical protection (prophylaxis). The prophylaxis will be focused on protection of AChE against the inhibition using reversible cholinesterase inhibitors. The diminishing the level of OP using enzymes hydrolyzing these agents or enzymes binding the agents (to specific proteins or to antibodies) and thus reducing the OP level (and inhibition of cholinesterases) in the organism can be described as detoxification. Another approach to prophylaxis is based on using present antidotes. It can be characterized as a treatment “in advance”. The problem with this approach is how to achieve sufficient levels of antidotes for a relatively long time. Combinations of these approaches are also possible. Unlike other OP, the treatment of soman poisoning is very difficult and unsatisfactory. This is the reason for intensive studies using pre-treatment/prophylaxis allowing survival and increasing the resistance of the organism exposed to soman and tabun.

Keeping AChE intact is a basic requirement for effective prophylaxis, i.e. to change the enzyme in a way that will

make it resistant to OP. This can be achieved by using reversible inhibitors, which are able to inhibit AChE reversibly and after spontaneous recovery of the activity, normal AChE serves as a source of the active enzyme. Moreover, AChE inhibited by carbamates is resistant to OP/nerve agent inhibition (9,18).

The ability of some carbamates to protect an organism poisoned with OP has been known for many years. Physostigmine and neostigmine have been used to protect animals against DFP. The number of OP studied for protection was enlarged, as well as the number of carbamates studied. These studies were performed both *in vitro* and *in vivo*. The results are very dependent on experimental conditions; nevertheless, the protective effect of physostigmine, aminostigmine, pyridostigmine and others against AChE inhibition caused by different OP (mostly soman) has been demonstrated (9,46,93,121,131). There have been numerous studies demonstrating the effectiveness of carbamate pre-treatment/prophylaxis against intoxication with OP. From the results published (and unpublished) it appeared that pyridostigmine was the most promising prophylactic drug especially against soman poisoning (9,17,18,64–70,93). On the basis of these results, pyridostigmine was introduced into some armies as a prophylactic against nerve agents. Its prophylactic effect (like the effects of other carbamates) is limited by its dose. With a higher dose, a higher efficacy was observed, but the side effects were more expressed, too. This problem can be solved by the adding of pyridostigmine antagonizing drugs – anticholinergics. Many anticholinergics have been tested to protect the organism against intoxication with soman (and other nerve agents) and, on the basis of this research, the prophylactic combination of pyridostigmine with trihexyphenidyle and benactyzine (9, 17,18,45,68,70) was introduced into the Czech Army as PANPAL. The presence of these two anticholinergics allowed us to increase the pyridostigmine dose and to increase its prophylactic efficacy. This combination (including follow-up therapy) is not limited to soman, sarin and VX poisoning but its high efficacy against tabun, GV and cyclosarin intoxications was observed (9,18). The prophylactic antidote combination called PANPAL has no side effects as it has been demonstrated on volunteers: no statistically different changes in the actual psychic state as well as no negative changes in the dysfunction time were observed (45).

Other carbamates also have a good prophylactic efficacy, especially physostigmine (due to its central effect on the contrary to pyridostigmine). Human study with transdermal physostigmine suggests a serious interest in the prophylactic use of this drug (9,73,99). Mobam and decarboxyfurane were also experimentally considered as potential candidates for prophylaxis. Among other inhibitors, aminophenols and OP were tested but their effects were lower in comparison with pyridostigmine (18,93).

Structurally different inhibitors from the carbamate and OP groups were also studied. From these compounds (preferably binding to the AChE anionic site), tacrine, 7-MEO-

TA and huperzine A were considered and experimentally studied with respect to prophylaxis *in vitro* and *in vivo* (9,43,83,111). The most interesting results were obtained with huperzine A. It is an inhibitor of the rat brain AChE (83,111). Very similar results were obtained with enzymes from other sources. Huperzine A was tested as a potential candidate against OP for its long-lasting efficacy and relatively low toxicity. However, the results obtained do not support replacement of pyridostigmine by these drugs (for a review, see 18).

Detoxification principle can be used in two different ways: administration of enzymes splitting the OP or specific enzymes which bind the OP (cholinesterases). OP is bound to the exogenously administered enzyme and thus the OP level in the organism is decreased (it acts as “scavenger”). Enzymes hydrolysing OP are under research (30). On the other hand, many studies have been made with cholinesterases as scavengers. BuChE and AChE were observed to be very effective in protection against OP intoxication (18,31, 34,35,100,117,118). The administration of enzymes as scavengers seems to be very promising: the enzyme is acting at the very beginning of the toxic action, without interaction with the target tissues and without side effects (34,35,117). All of these features are of great interest and they are yielding practical results – isolation of the enzyme, examination for lack of and auto immune response, stability, and establishment of pharmacokinetic and pharmacodynamic properties (117). Moreover, BuChE pretreatment also showed protective effects on AChE inhibition in the brain parts following low level sarin inhalation exposure (118). Given our increasing knowledge in bioengineering and biotechnology, the connection between these two enzymes will be possible with the aim of obtaining a modified enzyme splitting OP and simultaneously reacting with AChE as a scavenger (30). Antibodies against OP are in the stage of research and they are more focused on the detection of OP (85).

The idea on use of standard antidotes as prophylactics is very simple – to achieve sufficient level of antidotes in the blood vessel before intoxication. Standard antidotes were studied in this respect i.e. anticholinergics, reactivators, anticonvulsants and others (18,28,77,93,97). The problem with their use is the timing and duration and achievement of sufficient levels of these antidotes after administration. However, the prophylactic efficacy is good as it has been demonstrated in treatment studies – administration of the antidotes mostly takes place very shortly (minutes) after the intoxication. The prolongation of the duration of the antidote effects by achievement of their sufficient level in the blood by oral administration is not possible (especially reactivators) and therefore it is excluded. It was a reason for searching for other routes of administration. Transdermal administration of one of the most effective reactivators (HI-6) was shown to be the most realistic approach (9,18, 36). The final result was the new prophylactic transdermal antidote TRANSANT containing the reactivator HI-6. This preparation was clinically tested (including dermal sensi-

Tab. 3: Drugs used in the prophylaxis against OP poisonin (relatively perspective drugs are in bold).

Principle	Drug group	Drug	Duration	Equipment of the army	Efficacy	Comment
Protection of cholinesterase inhibition	carbamates	Pyridostigmine, Aminostigmine, Physostigmine Syntostigmine, Eptastigmine, Mobam Decarbofuran, Heptylphosostigmine Huperzine A Tacrine, Methoxytacrine	8 hours	PYRIDOSTIGMINE BROMIDE	+++	Dose limited, side effects Alone is not very effective, following antidotal treatment enhances its effect
	others	organophosphates aminophenols				
Simulation of treatment	anticholinergics	Biperidene, Scopolamine, Benactyzine Atropine, Apropfen, Hyoscin Adiphenine, Caramiphen Pentamethonium, Mecamylamine Trihexyphenidyle				
	reactivators	HI-6 PAM, Obidoxime, Trimedoxime Methoxime	8 hours	TRANSANT (HI-6, transdermal administration)	+	Alone is not effective
Detoxification	others	Suramine Benzodiazepines, Tubocurarine Mementine, Procyclidine Nimodipin, Clonidine				Very perspective
	cholinesterases enzymes hydrolyzing OP monoclonal antibodies against OP	Butyrylcholinesterase, Mutants Acetylcholinesterase Triesterase Paraoxonase	24 hours?? 8 hours	FOURCOMBINATION? PANPAL (pyridostigmine, trihexyphenidyle, benactyzine) PANPAL+ TRANSANT	++++? ++++ +++++	No sufficient information Efficacy is increased with following antidotal treatment In combination, the best prophylactic efficacy
Combinations						

vity) without any harmful effects and field testing was also successful and TRANSANT was introduced into the Czech Army. The prophylactic efficacy of other drugs was studied. As anticonvulsant drugs, benzodiazepines (diazepam, midazolam, alprazolam, triazolam, clonazepam) were studied, but isolated prophylactic administration has not had very good effects (4,9,18,93).

There are other drugs tested as prophylactics. Calcium antagonists (nimodipine), neuromuscular blockers (tubocurarine), adamantanes (memantine), and the opiate antagonist meptazinol were also tested with different results but they were not very useful for practical use (18). On the other hand, a positive prophylactic effect has been demonstrated with procyclidine (antimuscarinic, antinicotinic and the anti-NMDA receptor drug) (9,18). Special importance can be focused on suramine (a protease inhibitor). Administration of this compound prior to soman intoxication (and followed by administration of atropine) showed good prophylactic effect (18,32). However, all these studies are experimental ones and they have not reached the practical output stage. The combinations of various drugs as prophylactics can be of very different character. They can be used simultaneously (a combination of different drugs) or as pre-treatment and following treatment with different antidotes. Administration of pyridostigmine (or other inhibitors) prior to intoxication and treatment with different drugs is a typical example (4,9,17,18,45,66–68). There are other combinations such as the administration of triesterase, procyclidine, clonidine, sustained release of physostigmine and scopolamine (9,18,73,99). The results are very dependent on experimental conditions but this approach – administration of different drugs – has yielded some good results though up to now they have been on an experimental level. Only three prophylactics have been introduced into the armies – PANPAL composed of pyridostigmine, benactyzine and trihexyphenidyle, TRANSANT (HI-6) and pyridostigmine bromide, respectively. There are some indications on fourcombination introduced in one east army but without further specification cannot be considered as serious (Table 3).

It appears from these results that simple prophylaxis (without postexposure treatment) against OP/nerve agents is not sufficient enough. Therefore, pyridostigmine has importance as a prophylactic drug especially when it is connected with postexposure antidotal treatment. For further development, it is necessary to search for new prophylactic drugs and new routes of administration. In this connection, preparations of cholinesterases are of special importance for the development of more effective prophylactics.

9. Treatment

The general priorities after OP/nerve agent intoxication are: evacuate the toxic zone, decontaminate, resuscitate victims while initiating antidotal treatment, and secure definitive treatment. The pillars of complex therapy for any nerve

agent/OP casualties include: intensive respiratory care, antidotal therapy, treatment of complications and monitoring, and care for long-term sequelae. Based on our knowledge of the mechanism of action, two therapeutic principles for antidotal treatment are used. The main drugs are anticholinergics that antagonize the effects of accumulated acetylcholine at the cholinergic synapses (also called symptomatic antidotes) and cholinesterase reactivators (oximes) to reactivate inhibited AChE (causal antidotes). Their effects are synergistic. Central nervous antidepressants such as benzodiazepines are also used to treat convulsions (anticonvulsants). Due to the high toxicity of OP/nerve agents, first aid is important for the future fate of the intoxicated organism. It consists of interrupting contacts with the poison (evacuation, protective mask), administration of antidotes if possible, and decontamination. Support of vital functions (the heart, artificial respiration) is necessary. Though administration of the above-mentioned antidotes is recommended, successful therapy of moderate OP/nerve agent and carbamate poisoning has been described using atropinization and the treatment of acidosis with sodium bicarbonate only (22,109).

Atropine is the anticholinergic drug most frequently used for the treatment of human poisoning. This muscarinic cholinergic antagonist acts by blocking the overstimulating effects of acetylcholine at the muscarinic sites and has little effect at the nicotinic sites. It does not readily cross the blood-brain barrier but it has central ameliorative effects. Atropine reverses central apnea, relieves bronchoconstriction, and dries secretion, i.e. it reverses the peripheral muscarinic symptoms (e.g. secretion etc.) and arrests the early phase of convulsions when given within minutes of exposure (98). Atropine will not improve neuromuscular function, particularly diaphragmatic function. Ketamine (an antagonist of glutamatergic NMDA receptor and bronchodilator) should be used with the caution in nerve agent poisoning. In animal experiments, central apnea quickly ensued when NMDA receptor antagonists were administered before atropine (18). Atropine pre-treatment prevented this effect (9,18). In experiments on animals, the good therapeutic efficacy of benactyzine and biperidene was observed especially against soman poisoning due to its better central effect in comparison with atropine (66). Though some doubts exist about administration of very high doses of atropine, the treatment of human casualties and experimental intoxications are clear (18,38,71,96,115): in severe poisoning both the animal and human data show that very high doses of atropine are life saving and well tolerated. Animals exposed to 2xLD50 soman were capable of tolerating 0.5–3.0 mg/kg, i.v. atropine: this equates to 35–210 mg in a 70 kg human. These doses agree with Iranian casualty data. Animal studies show lower doses of atropine (0.1–0.2 mg/kg). Therefore higher doses of diazepam or more efficient anticonvulsant are required. Lower atropine doses increase the risk of lethality from poor cardiorespiratory response and long period of unconsciousness and possibly

seizure activity, and, therefore, increase the potential for neurological damage. The further course of OP intoxication is negatively influenced by a low dosage of atropine (9,23,115). High doses of atropine return consciousness more rapidly and support cardiorespiratory efforts. Aggressive atropinization and prolonged administration of the oxime improved the fate of OP-intoxicated patients (9,23,115). Other anticholinergics may be even more efficacious. The centrally acting anticholinergics (benactyzine, biperidene) can be very useful in the therapy and reduce the necessary amounts of benzodiazepine anticonvulsants (9,51,75). Nerve agents have a long-term effect on the behavior of experimental animals lasting months after intoxication with low doses of nerve agents and were eliminated with pharmacological pretreatment followed by antidotal treatment (9,12,69,77). However, adding diazepam into the therapeutic mixture improved the survival of tabun-intoxicated mice when combined with atropine and methoxime (119). The anticonvulsant action of some anticholinergics in soman poisoning was demonstrated (28).

The current standard treatment with reactivators includes different types of oximes with a similar basic structure differing by the number of pyridinium rings and by the position of the oxime group in the pyridinium ring. Oximes hydrolytically cleave the OP/nerve agent from AChE, restoring enzymatic function. From the common oximes, mono- and bisquaternary pyridinium oximes are frequently used such as pralidoxime, obidoxime, trimedoxime, methoxime

and HI-6 (Fig. 7). Because of some doubts about the use of oximes in the treatment of OP poisoning, Eddleston et al. (38) published a systematic review of clinical trials dealing with oxime therapy in acute OP poisoning. His generalized statement that pralidoxime should not be used in OP poisoning was not supported by the published results. The use of the reactivators is supported by the observations of OP-poisoning (9,37,128,138,141).

The effectiveness of antidotal treatment is dependent on the reactivatability of AChE by the reactivator used (9,23,63). Generally, the conventional oximes (pralidoxime or obidoxime) have been considered to be sufficiently effective against VX, sarin and cyclosarin, and rather ineffective against soman (9,63,75).

The differences in the oxime efficacy against various nerve agents are mainly due to the various aging rates at which inhibited AChE is converted to a form that can no longer be reactivated by oximes (9,40,42,63). The reactivation of VX, sarin, or GF-inhibited AChE is still possible hours after the intoxication while soman-inhibited AChE becomes unreactivable within minutes and, therefore, renders the treatment of soman poisoning much more difficult (9,10,63).

This fact led to the synthesis of a series of bisquaternary oximes, designated as „H-oximes“, that in combination with anticholinergic drugs have been relatively successful in antagonizing soman intoxication (9,63,121,122). Among the H-series oximes, HI-6 has been the best studied and, there-

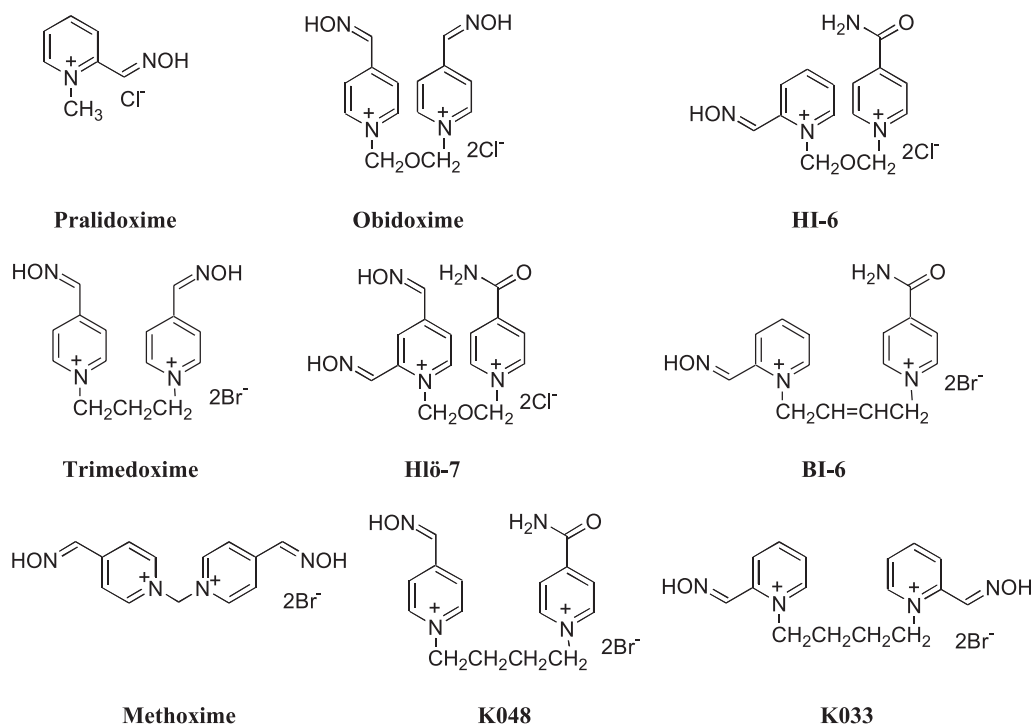


Fig. 7: Structural formulae of some reactivators.

fore, seems to be the most promising oxime against soman poisoning (9,63). Worek et al. (141), based on experimental testing of the reactivation potency of obidoxime, pralidoxime, HI-6 and HLö-7 in human erythrocyte AChE inhibited by nerve agents, suggested that HLö 7 may serve as a reactivator in nerve agent poisoning at doses therapeutically relevant in humans.

If we compare the AChE reactivatability of different oximes and various nerve agents, i.e. the dependence of the percentage of reactivation vs. concentration of the oxime (9,63), basically two different types of the curves can be obtained: the first depending on the oxime concentration shows an increase with a maximum followed by a decreased part of the curve. The second type is a sigmoid curve reaching to the maximum but the decrease cannot be demonstrated because of a too high concentration of the oxime (very probably it will be the same, i.e. containing a decreasing part). Obidoxime and pralidoxime are effective against cyclosarin and sarin-inhibited AChE at concentrations reaching to 10^{-3} – 10^{-2} M (Fig. 8). Therefore the effectivity of the oxime in a human can be influenced by the concentration in the target organs, i.e. when administered parenterally, in the dose range of 470–2280 $\mu\text{mol/kg}$, the concentration in the brain can be about 10^{-4} – 10^{-5} M (9,63). These concentrations are able to reactivate sufficiently inhibited AChE in

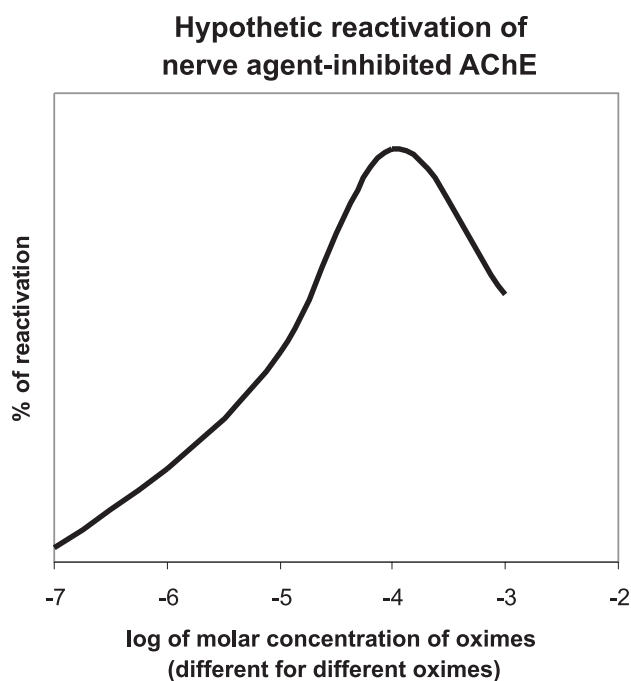


Fig. 8: Hypothetic reactivation of AChE inhibited by nerve agents. In this case, concentration 10^{-4} M is the maximum available in human, concentration 10^{-6} (in this case) is the minimum necessary for “life saving” AChE reactivation (10–20% reactivation). However, different reactivators differ in these concentrations and this is limiting factor for antidotal therapy.

the brain especially in the ponto-medullar area (the increase by 10–20%): the minimal level of AChE activity in the pontomedullar area necessary for the survival of nerve agents-intoxicated animals was assessed to be about 5 % (9,14).

The crucial question dealing with the reactivator’s effect on the central nervous system was discussed in the past. Because of their quaternary structure, at intact BBB, the penetration of the reactivators is slow. In order to reach an effective concentration of the reactivator in CNS, its extremely high plasma concentration is necessary. On the other hand, the central reactivation effect exists (9,13,14). It is known from other results that the inhibition and reactivation of AChE in the brain is selective for different OP and following administration of the reactivators to nerve agent-intoxicated animals, reactivation of AChE in different brain parts was demonstrated (9,13). The ability of oximes to penetrate the blood brain barrier was confirmed by Sakurada et al. (116).

There were and are some attempts to synthesize new reactivators with the aim of making them universal or more effective especially against soman or tabun inhibited AChE either in the past (for a review, see 9,63) or presently (78–82). A number of alternative oximes have been shown to be significantly more effective and have a broader spectrum of action than the pralidoxime and several of these may be as or more effective than HI-6 (80–82) (Fig. 8). However, the results obtained up to now are not of interest for introducing them into medical practice. It can be concluded that currently available oximes (pralidoxime, methoxime, obidoxime) are sufficient for therapy of poisonings with OP but they are not very effective against nerve agent (especially soman) poisoning. The H-oximes (HI-6, HLö-7, in some cases methoxime) appear to be very promising antidotes against nerve agents including soman. However, there is no universal oxime suitable for antidotal treatment of poisoning with all OP/nerve agents.

Seizures should be prevented and treated when they occur. Against convulsions caused by OP/nerve agents, anticonvulsants were studied empirically. These studies were not carried out only for the treatment of seizures. The control of seizures is strongly associated with protection against lethality and brain pathology (122). Different results were obtained using different anticonvulsants such as barbiturates, hydantoins, local anesthetics, calcium channel blockers, sometimes perspective, however, benzodiazepines were chosen as the most effective (9,93). However, anticonvulsant actions also have some anticholinergics (atropine, scopolamine, biperidene, trihexyphenidyle, procyclidine) (4,9,75). The GABA uptake inhibitor tiagabine, the glutamate receptor antagonists (e.g. memantine, antinicotinic mecamlamine, the $\alpha(2)$ -adrenergic agonist clonidine) were not very effective (5,9,121,122). Benzodiazepines were effective against soman-induced seizures with the strong synergistic effects when combined with centrally active anticholinergic drugs. Different benzodiazepines were tested (avizafon, clonazepam, diazepam, lorazepam, loraze-

pam, midazolam,) and the most pronounced antiseizure activity of diazepam and midazolam was demonstrated. Midazolam may be the most effective anticonvulsant after nerve agents exposure, but, despite its efficacy, it has not yet been approved as a drug for OP-induced seizures. It is notable for its water solubility, short-term onset of action, short half-life, and lack of active metabolites (115). Diazepam has been recommended for standard treatment therapy of convulsions caused by OP/nerve agents, however, midazolam has very similar or better effects (9,97,115,122) and these results have led to a study with nasal administration of this drug (51). All these studies were performed experimentally on animals pretreated with pyridostigmine and treated with atropine and a reactivator (pralidoxime, trimedoxime, HI-6) to eliminate the lethal effects. A complex therapy including all necessary biochemical examination is necessary to prevent complications and chronic health disturbance.

10. Conclusions

- Though the mechanism of action of OP/nerve agents is extensively studied, it needs to be elaborated in more detailed way especially in connection with an influence of non cholinergic transmitter systems.
- It is necessary to study the modelling of OP/nerve agents intoxication and the effect of antidotes and prophylactics including all factors involved.
- The relationship between cholinesterases and their functions needs further study for both enzymes (AChE and BuChE).
- Obtaining more detailed information regarding the long-term effects of OP/nerve agents at low doses (concentrations) is necessary.
- There is a need to search new drugs for prophylaxis and treatment. Cholinesterase preparations are of special interest.
- The gene expression profile after OP/nerve agents intoxication needs to be considered in more detailed way.

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