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Laboratory Diagnosis of Organophosphates/Nerve Agent Poisoning

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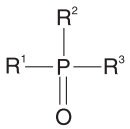
SUMMARY
OP/nerve agents are still considered as important chemicals acting on living organisms and widely used in human practice, either in positive or, unfortunately, in negative ways. They are characterized according to their acute action as compounds influencing cholinergic nerve transmission via inhibition of acetylcholinesterase (AChE). 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 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. For nerve agents intoxication, AChE in the red blood cell is more diagnostically important than BuChE activity in the plasma. This enzyme is a good diagnostic marker for intoxication with OP pesticides. Some other biochemical examinations are recommended usually not available in all clinical laboratories. These special examinations are important for prognosis of the intoxication, for effective treatment and for retrospective analysis of the agent used for exposure.
Key words: organophosphates, nerve agents, intoxication, cholinesterases, inhibition, laboratory diagnosis.

Introduction

Organophosphorus inhibitors of cholinesterases (commonly called organophosphates, OP) are used in industry as softening agents, hydraulic liquids, lubricant additives, plasticizers, antioxidants, and for antiflammable modifications. They are also used in veterinary or human medicine as drugs or chemicals for the study of nervous functions. They differ in their toxicity from practically non-toxic chemicals (malathion) to highly toxic agents such as sarin, soman, VX and other nerve agents known as the most important group of chemical warfare agents (1–9). Nerve agents were also used by terrorists in 1994 and 1995 in Japan (10, 11). Therefore, the mechanism of action, diagnosis and treatment of intoxications with OP and nerve agents is a very actual at present. 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.

OP includes a large variety of compounds with different physical chemical and biological properties including toxicity. OP are liquids of different volatility, soluble or insoluble in water, organic solvents etc. 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 (9).

Chemical formulae of some OP pesticides and nerve agents are shown in Fig. 1 and 2. Toxicity of chemicals is one of the basic characteristics for chemical compounds. Depending on the conditions of its determination, different types (acute, subchronic, chronic etc.) toxicity are differentiated. Acute toxicity is mostly characterized by LD50. The acute toxicities of different OP/nerve agents are shown in Table 1.

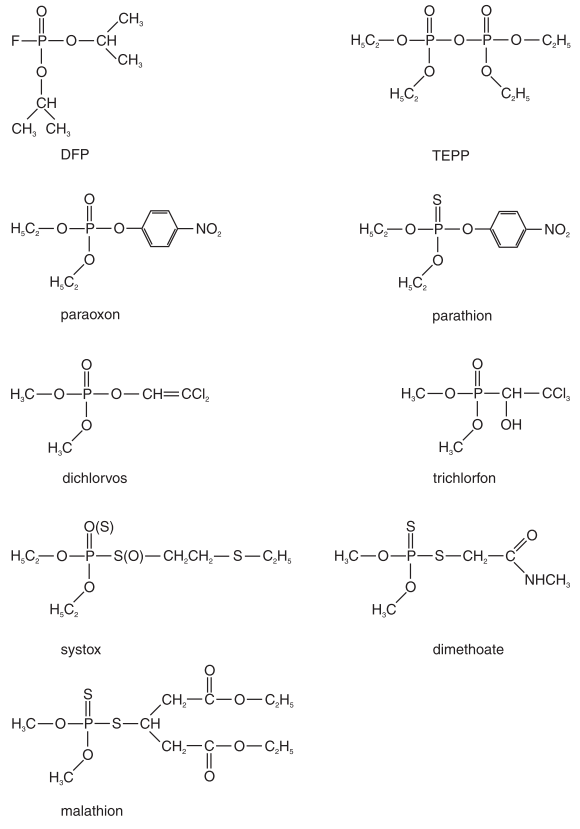


Fig. 1. Structural formulae of some OP

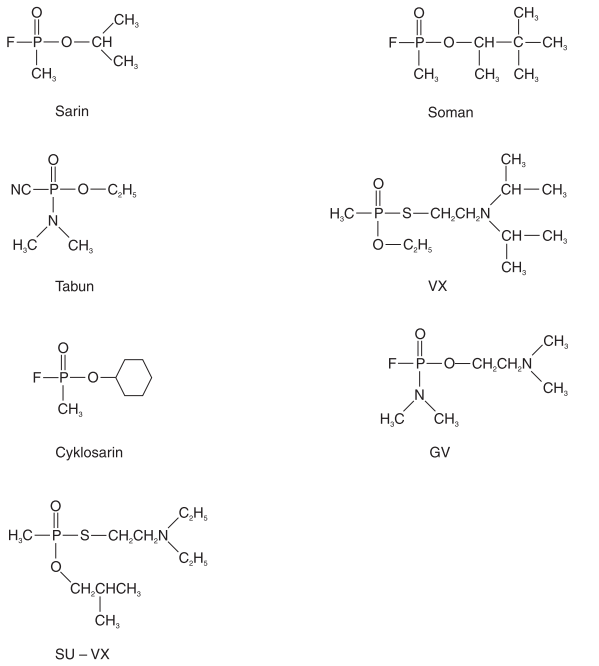


Fig. 2. Structural formulae of nerve agents

Table 1. Toxicities for rats (expwerimentally 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/70 kg)	[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	7–10 000	22	100–200	–
Diazinon	50–80 000	100–150	700–1200	–
Fosfamidon	10–15 000	27.5	100–180	–
Malathion	–	800–1200	grams	–

[a] experimental data from literature (1–8)

[b] assessed data from literature (1–4, 6)

[c] assessed data from literature (1)

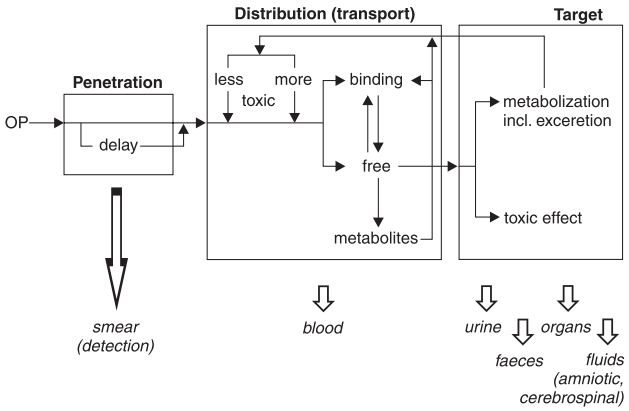
The toxidynamics of OP is known: it is based on irreversible acetylcholinesterase (AChE, EC 3.1.1.7) inhibition at the cholinergic synapses (1–6). The resulting accumulation of acetylcholine at the synaptic junctions overstimulates the cholinergic pathways and subsequently desensitizes the cholinergic receptor sites. This mechanism for the all OP and nerve agents is practically the same – the inhibition via phosphorylation or phosphonylation of the esteratic site of AChE. However, reactivation of inhibited AChE by oximes is possible but different for various nerve agents: phosphorylated but reactivatable AChE is changed to a non-reactivatable complex. The half times for this reaction described as dealkylation (aging) (12) are different for various OP/nerve agents (1–3, 6). Both the toxicodynamics and toxicokinetics of OP/nerve agents can be explained by their biochemical characteristics of interacting with cholinesterases and other hydrolases. Inhibition of cholinesterases in the blood is practically the first target for OP according to the principle "first come, first served" (8). 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 (1, 13, 14).

Thus, the basic trigger mechanism for nerve agents, similarly as for other OP, is an intervention into cholinergic nerve transmission via an inhibition of AChE and other hydrolases (1, 4, 6). Depending on the target, acute, intermediate, chronic or delayed effects are manifested (1, 4, 6, 15, 16). Monitoring the cholinesterase changes – their development during the intoxication is at present the best reflexion of the severity of OP poisoning as well as a reaction to antidotal therapy.

Detoxification of OP with lower toxicity is also important. These compounds can be hydrolysed (alkaline or acid hydrolysis), alkylated, splitted by alkali or enzymes and conjugated. Moreover, for some OP especially those containing the P = S bond, oxidation giving rise to more toxic products is observed (P = S → 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 (1, 2, 17). 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. 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. A delayed neurotoxic effect is caused by inhibition of a quite different enzyme from cholinesterases – neurotoxic esterase (16).

From toxidynamic point of view, OP is penetrated into organism depending on the route of administration. Except i. v. injection administered directly into the blood stream (transport system), OP is penetrating into the transport system (and it is more or less delayed), and it is distributed (transported) into the sites of metabolic and toxic effect. Metabolites (less or more toxic than the parent compound) are released into the blood stream and distributed to the target sites. During this process, some losses are occurred and some reactions are observed. OP can be metabolized, can be bound to pro-

teins, enzymes, etc. (1, 2, 7, 8). Thus, there are some possibilities for biological sampling as it is shown in Fig. 3. Possible biological samples (obtained pre or post mortem) can be analyzed in different ways as it is demonstrated in Fig. 4. However, the fluids and organs are of low importance for the detection of poisoning in human (it is more important for laboratory diagnosis of other diseases). In experimental studies on animals, the organ cholinesterases are frequently used not only for diagnostic purposes but, especially, for the studies dealing with mechanism of action and the effects of antidotal therapy.



Important steps for toxicodynamic processes
material for laboratory diagnosis, post or ante mortem

Fig. 3. Possible materials for laboratory diagnosis of OP/nerve agent poisoning

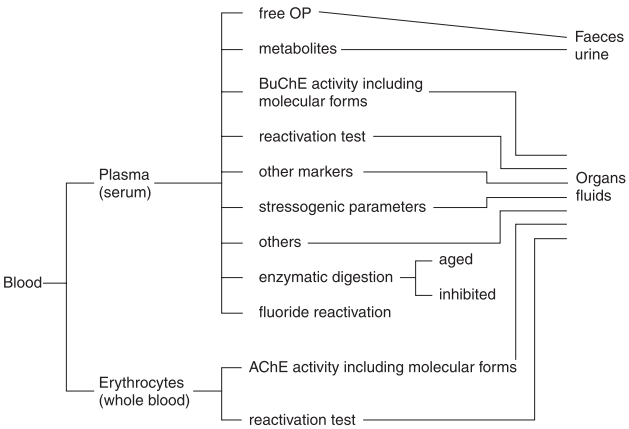


Fig. 4. Possible materials and methods for laboratory diagnosis of OP/nerve agent poisoning

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. An excess of substrate inhibits it. 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 (18). AChE is composed from subunits and can be separated into the different molecular forms. 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 synthetized). 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. The people with genetically diminished BuChE activity may be at higher risk when exposed to pesticides or suxamethonium. 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 over dosage is occurred (1, 2, 4, 19–21).

AChE and BuChE differ not only in their enzymatic properties but in physiological function, too (1, 22, 23). AChE splits neuromediator 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, in non-specific detoxification processes and lipid metabolism (22).

Determination of cholinesterase activity

Determination of cholinesterase activity is based on many principles. In general, an enzyme is incubated in 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 (1, 24–26). The conditions must be chosen very carefully because of different factors influencing the activity (13, 27).

According to the procedure and laboratory instrumentation, the most common methods of cholinesterase determination were described (1).

Electrometrical (e. g. 28), titration (e. g. 29), manometric (e. g. 30), colorimetric detection of the unhydrolyzed substrate (e. g. 31), measurement by the change of pH using an indicator (32), spectrophotometric (e. g. 33–35), fluorimetric (e. g. 36, 37), radiometric (e. g. 38), calorimetric (39), polarographic (40), enzymatic (41, 42) and others e. g. near infrared spectroscopy (43). These methods are also suitable for the detection of cholinesterase inhibitors using biosensors (44–46) or immunochemical assay for detection of chemical warfare agents (47).

A very sensitive and commonly used method for cholinesterase determination was described by Ellman et al. (48), based on hydrolysis of the thiocholine substra-

tes 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'dithiobis-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. One of the methodical works improving the Ellman's method, including a description of the methods, is a paper published by Worek et al. (35).

In clinical biochemistry, BuChE determination in the plasma or serum is more frequently used than that of AChE in the red blood cells. Except for intoxication with OP or carbamates, a BuChE decrease indicates either a diminishment of the enzyme synthesis or a decrease in the number of production cells in the liver (49). A special case of diminished BuChE activity is the hereditary affected presence of atypical variants of BuChE mentioned previously (1, 2, 4, 19–21).

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 (1, 14, 50). The elevation of BuChE activity is not so frequent; an increase in children with nephritic syndrome has been observed (1, 20); an elevated ratio of BuChE/LDL cholesterol indicates an increase in the risk of cardiovascular diseases (1, 51). The involvement of BuChE with the fat (cholesterol) metabolism has been suggested by van Lith et al. (52, 53). The relationship between BuChE activity and experimentally induced diabetes mellitus in rats was also mentioned (54). The influencing of BuChE activity by gamma-irradiation, stress, gravidity, some neurological and psychiatric disorders, hormones and medical drugs has been demonstrated (1, 13, 14, 19).

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 was observed in paroxysmal nocturnal haemoglobinaemia and ABO incompatibility (55). 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 (1,55–57). There are other papers demonstrating increased AChE activity in the amniotic fluid during pathologic development of the neural tube (58). 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. 1, 13, 55, 56). Inhibition of cholinesterases in different organs including brain and its parts was demonstrated following effect of OP and other cholinesterase inhibitors *in vitro* and *in vivo*.

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 assymetric forms, which bind to basal lamina. Tetramers are formed by electrostatic and hydrophobic interaction between two disulphide–bonded dimers (23).

Multiple molecular forms (AChE and BuChE) are also influenced by many factors (1, 44). 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 and some experiments were performed with relatively less toxic OP (59–61). From the group of highly toxic OP compounds, sarin, soman, and VX were found to be the most effective (61).

Diagnosis of OP/nerve agent poisoning

Monitoring of signs 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. However, 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 parathion and paraoxon poisoning (62). Therefore, the blood remains to be the main source of biological material for biochemical examination. Serum biochemical and haematological parameters were examined in the rhesus monkeys following acute poisoning with cyclosarin. Significant increase in creatine kinase, lactate dehydrogenase, transaminases (AST, ALT) and potassium ion associated with damage to striated muscle and metabolic acidosis occurred in the treated group (atropine and oxime) two days after the exposure. Total protein, albumin, red blood cell count, haemoglobine concentration and haematocrit were decreased in the treated group at 7 days (63). These other methods are not very specific (1, 63).

In general, AChE activity in the red blood cell can be considered to be more important for diagnosis with the nerve agents than the plasma BuChE activity. The plasma BuChE activity is in some cases a good marker for diagnosis of OP pesticides poisoning. It is necessary to exclude a diminishing of BuChE activity caused by other reasons. In all cases, the simple cho-

linesterase determination gives us information about the decrease of the enzyme activity without specification of the factor causing this phenomenon. A more detailed specification is possible using special methods not available in all clinical laboratories.

The determination of cholinesterases in the blood of workers with OP is obligatory for occupational medicine purposes. 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 norm 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 activity determined in the whole human blood corresponds to about 10% of BuChE and 90% of AChE but the ration is different for different species. The erythrocyte AChE activity seems to be more useful for these purposes than BuChE activity in the plasma (1, 64).

It is necessary to check vital functions (cardiac, ventilation) and other clinical signs and according to the symptoms to apply different biochemical examinations and treatment.

In the circulating system, the direct determinations of the toxic agent (OP or nerve agent) are also possible. However, the detection of the parent compound 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 and plasma from victims after the Tokyo subway sarin terroristic attack (65–67). For some OP pesticides (parathion, paraoxon), detection of p-nitrophenol in urine is an indicator of exposure (1, 62). However, the retrospective validity 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 (1, 47, 68).

The methods for determination of the blood cholinesterases inhibition (AChE and BuChE) do not allow identification of the OP and do not provide reliable evidence for exposure at inhibition levels less than 10–20%, especially in cases of exposure to low-level concentrations to OP (67, 69). Moreover, they are less suitable for retrospective detection of exposure due to *de novo* synthesis of enzymes. Recently, a method was developed which is based on reactivation of phosphylated cholinesterase and carboxylesterase (CaE) by fluoride ions. Treatment of the inhibited enzyme with fluoride ions can inverse the inhibition reaction yielding a restored enzyme and a phosphofluoridate which is subsequently isolated and quantified by gas chromatography and phosphorus-specific or mass spectrometric detection (70–72). This method allows partial identification of the OP whereas the lifetime of the phosphylated esterase (and consequently the retrospectivity of the method) is only limited by spontaneous reactivation, in vivo sequestration and aging. Fluoride induced reactivation of OP-inhibited AChE is a reliable and retrospective method to establish OP-exposure (71–73). It is limited to compounds that regenerated with fluoride ions. A novel and general procedure for diagnosis of exposure to OP, which surpasses the limitations of the fluoride reactivation method was described (74). 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 phosphylated 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. This approach is very valuable and represents a new field for the improvement of diagnosis with nerve agents and OP. A comprehensive review of the methods for retrospective detection of exposure to toxic scheduled chemicals has been published by Noort et al. (66, 67).

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. The concentration of the reactivator (usually trimedoxime, but other oximes such obidoxime, pralidoxime or HI-6 are also possible) must not be higher than the oxime concentration which causes the hydrolysis of the substrate (acetyl- or butyrylthiocholine), i. e. the oxime concentration in the sample for reactivation test is lower than 10–3 M because these higher concentrations of oximes cause artificial hydrolysis of the substrate. Using this method (reactivation test), in vitro reactivation of the whole human blood in vitro inhibited by various nerve agents (VX, sarin, soman) was determined and compared with results *in vivo* on dogs. The reactivations in vitro and in vivo were in good correlation (1, 3, 14).

Following intoxication with sarin, soman, and VX, the highest sensitivity for the high molecular AChE form was observed (61). Determination of the whole AChE activity is partly misleading because of the different distribution of AChE molecular forms in the sample. Following determination of the whole activity, a “mean” activity containing the activities of the forms is determined. Therefore, in studies requiring high sensitivity (e. g. the studies of antidotal action), AChE molecular forms would be of choice for more detailed information about the functional stage of AChE – an important marker of cholinergic nerve transmission.

A new and interesting approach was described by Gopalakrishnakone (75). 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.

Monitoring of the delayed neurotoxic effect can be realized 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 (76). *In vitro* techniques for the assessment of neurotoxicity have been elaborated by Harry et al. (77).

Stressogenic markers are also influenced during the OP/nerve agent intoxication. However, the changes in the cyclic nucleotides are interesting but not very valid for blood. They were determined during animal experiments with toxic OP and are of more interest in connection with the nervous system. Esterases and AP, generally hydrolases, are sensitive but the inhibition potency of different OP is very variable: for nerve agents these hydrolases are not of great interest, and, for some OP insecticides like malathion they are sometimes more sensitive than cholinesterases. There are observed changes in other markers especially those connected with stressogenic reaction (corticosterone). This increased level of corticosterone was observed in inhalation intoxication of guinea pigs in soman intoxication at low concentrations, too (78). 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. (67) dealing with biomonitoring of exposure to chemical warfare agents (not only nerve agents) can be strongly recommended. 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.

Conclusions

It appears from these results that the mechanism of action of OP/nerve agents needs to be elaborated in a more detailed way including not only cholinergic but the other neurotransmitter systems. The study on binding of different ligands to the molecules of AChE and BuChE including the molecular forms and receptors with the aim of eluciding cholinergic nerve transmission requires further study It is necessary to study the relationship between cholinesterases and their functions including the changes in OP/nerve agents intoxication and pathological states. The gene expression profile after OP/nerve agent intoxication which is important for the development of mechanism-based therapies should be considered.

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