Introduction

The threat of the use of chemical weapons not only in military conflicts but also in terrorist attacks is not excluded at present as it was clearly demonstrated in Tokyo (27) and Matsumoto (44) cities (sarin attack in the subway).

The very actual group of these chemicals are organophosphates (OP) including nerve agents. The most important nerve agents are represented by sarin (O-isopropyl methylphosphonofluoridate), soman (O-pinacolyl methylphosphonofluoridate) (these two compounds belong to so called G-compounds) and VX (O-ethyl S-2-diisopropylaminoethyl methyl phosphonothiolate) (V-compounds). Moreover, there are produced many organophosphorus compounds in the civilian facilities and used in industry, agriculture, medicine etc. Basic mechanism of action and antidotal treatment for these compounds are in principle the same and therefore some conclusions from this paper can be applied in the civilian medicine.

From the point of pharmacodynamics and therapeutic possibilities, soman represents the most serious poison: its toxicity is comparable with that of sarin and VX (7,8,15,16,35) but therapeutic efficacy of antidotal treatment with present and perspective drugs is not good enough (14,17,23,24,29). This is probably a reason for intensive research dealing with soman intoxication and its treatment.

Soman and sarin are quickly resorbed at all routes of administration including inhalation, percutaneous and oral administration (7) and inhibit cholinesterases (preferably acetylcholinesterase, AChE, EC 3.1.1.7) in the central and peripheral nervous system. Because of high lipophilicity of soman, it posses high affinity to brain AChE (1,8). Sarin is less lipophilic, however, its affinity to the brain AChE is also very high (8).

Soman and sarin are detoxified in the liver, plasma (21,37), according to some authors also in the lung (34) and therefore this part is excluded from the toxic effect. The losses of G-compounds in the organism are caused also by binding to nonspecific esterases and this part of soman and sarin is not able to cause toxic effect. It was assessed that only 1-3% from the dose administered of the both compounds inhibited AChE in the brain, i.e. 1-3% of the dose administered caused the basic toxic effect (7,22,26,34). Another factor (up to now not very elucidated) influencing soman and sarin poisoning is an existence of a depot in the organism from which the nerve agent can be released and causes new attack of intoxication. This depot was described for the skin, erythrocytes, muscles and lung (8,22). Bearing in mind very low portion of the dose administered causing
basic toxic effect (1-3%) , it is clear that releasing of a very small quantity of sarin and soman can influence significantly survival or death of intoxicated organism independently on the treatment.

On the other hand, V compounds are not detoxified in the organism (7). Probably this is a reason of 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 (42). The mechanism of action of VX is inhibition of AChE preferably in peripheral nervous system (8, 28). However, the inhibition of AChE in the brain parts was described to be selective and the most marked in the pontomedullar area of the brain (7,8).

The evidence supporting AChE as the primary site of the both OP and nerve agents action has been summarized by many authors (6,19,28,39). It includes the following observations: symptoms of OP poisoning are similar to those of the AChE inhibitor physostigmine; the in vivo LD50 for a variety of OP correlates well with the inhibition efficacy to AChE determined in vitro; and ChE reactivators (e.g. oximes), anticholinergics (e.g. atropine) and spontaneously reactivating AChE inhibitors (e.g. carbamates) can reduce OP toxicity, However, despite the model’s pragmatic success, a variety of data are inharmonious with AChE inhibition as the only important biochemical event in OP intoxication.

Thus, basic mechanism of action of nerve agents - similarly as for other OPs - is an intervention into cholinergic nerve transmission via irreversible inhibition of AChE and other hydrolases (6,28,39). Monitoring of cholinesterase changes - their development during the intoxication is at present the best reflection of a severity of OP poisoning as well as a reaction to antidotal therapy.

Both enzymes (AChE and butyrylcholinesterase, BuChE, EC 3.1.1.8) exist in multiple molecular forms (2-5,12,25,30,36,38). The activities of these forms are also influenced by many factors. The function of these forms is not known at present, however, their presence in the membrane structures at physiological conditions was demonstrated (5). There are a few data describing the changes of AChE molecular forms following intoxication with highly toxic OPs (25). Some experiments were performed with relatively less toxic OP (4,10,30).

Molecular forms of AChE showed different sensitivity to inhibitors in vitro (4,7,31) and in vivo (11,13,30,40,41). Following DFP (40) and highly toxic OPs (our results, and 25), the form with high molecular weight was the most sensitive. Intoxication with Parathion and Neguvon (less toxic OPs) caused medium inhibition of some forms of AChE (7,13,43). One can suppose that following determination of the whole AChE activity, a “mean” of activities of the forms could be determined: in case of death, some forms of AChE reached an unmeasurable level, some of them were unchanged: the whole AChE activity was about 10 % of controls. Thus, determination of AChE molecular forms can contribute to more precise diagnosis of OP intoxication.

This approach could improve the therapy of OP and soman poisoning and simultaneously it could contribute to better understanding of cholinergic nerve transmission and thus to better insight to pharmacology and neuropharmacology in general.

Comparison of changes of AChE activity and its molecular forms following soman, sarin and VX poisoning is the aim of this study.

**Material and methods**

**Enzyme source**

Female Wistar rats (Velaz, Praha) weighing 170-200 g were used. The animals were killed by bleeding from the carotid artery, the brains were immediately removed, washed with saline and frozen. Then they were thawed and homogenized in an Ultra Turrax homogenizer (Janke and Kunkel, Germany) in distilled water and 0.5 % Triton X-100 to make a 10% (w/v) homogenate. The homogenate was centrifuged for 60 min at 105.000 g and 4°C (MSE, 50 T.C., England) and the supernatant was used as a source of soluble AChE.

**Disc electrophoresis**

Electrophoresis of the samples (20 µl) in 7.5% polyacrylamide gels was carried out for 60 min at 240 V and 40 mA as it was described previously (5).

**Determination of AChE activity**

AChE activity in the gel was demonstrated with acetylthiocholine iodide (Lachema Brno, Czech Republic) as substrate by the method described earlier (4,5). Colorimetric assay for AChE activity in the samples without electrophoretic separation was carried out according to Ellman et al. (18), with acetylthiocholine iodide as substrate and 5, 5-dithiobis-2-nitrobenzoic acid (Serva, Heidelberg, Germany) as chromogen. AChE activity was expressed as µmol of substrate hydrolysed per min per g wet wt. tissue or as percent of controls.

**Densitometric evaluation**

Following staining the gels were scanned on a Vitatron densitometer (Sci. Instr. Eefde, Holland) and the activity was expressed in arbitrary units or as percent of controls.

**Inhibition of AChE and its molecular forms by OPs in vivo**

Female rats (Velaz) weighing 180-200 g were divided into groups (n = 6). The animals in control group obtained saline, the animals in experimental groups were injected with sarin, soman and VX compound in a dose approximately 1 x LD50 (sarin - 0.2 mg/kg, soman - 0.12 mg/kg and VX - 0.05 mg/kg). The animals in both control and experimental groups were killed at different time intervals and supernatants from the pontomedullar area of the brain were prepared as described. The whole AChE activity in samples
removed 1, 3, 5 and 10 min (sarin, soman) and 15, 30 and 60 min (VX) after intoxication was determined. Samples removed 5 and 10 min (sarin, soman) and 30 and 60 min (VX) after intoxication were used for electrophoretic separation. The first interval chosen represents developed intoxication, the second one is practically the time of death.

The handling of experimental animals was under the supervision of the Ethics Committee of the Medical Faculty, Charles University, Hradec Králové.

**Statistical evaluation**

The results were calculated as the means with their 95% confidence limits. The differences between groups were evaluated by analysis of variance. Homogeneity of experimental groups was tested by Bartlett’s test. All calculations were done on a Hewlett Packard 9830.

**Results**

*Normal AChE activity and its molecular forms*

AChE activity in the brain homogenate was found to be 6 µmol of substrate hydrolysed per min per g wet wt. of tissue. AChE activity in the supernatant - i.e. activity of the soluble AChE - was approximately 95% of the activity in the homogenate.

Following electrophoretic separation of soluble rat brain AChE, the presence of four AChE molecular forms was demonstrated. They were designated by arabic numerals 1-4. Their percentage distribution was as follows: the forms with the highest electrophoretic mobility (forms 1,2) comprised about 1/5 of the whole activity and the forms with the lowest mobility (forms 3,4) contained the remaining ones, i.e. these forms represent the main part (about 80%) of the whole AChE activity (Table 1).

**Table 1:** Percentual distribution of the AChE forms according to densitometric evaluation

| form | 1    | 2    | 3    | 4    |
|------|------|------|------|------|
| %    | 8.0±2.4 | 10.1±2.8 | 33.9±3.5 | 48.0±3.1 |

**Inhibition of the total AChE activity**

The course of intoxication as well as a decrease (i.e. inhibition) of AChE activity was very fast for sarin and soman - within ten minutes the brain AChE activity reached the zero level (Fig. 1). On the other hand, following intoxication with VX, the time course of AChE inhibition was prolonged and reached to unmeasurable activity within one hour (Fig. 2). It was possible to calculate the half-lives of AChE inhibition using semilogarithmic transformation where the dependence of AChE activity changes vs. time gives the straight lines (Fig. 3). The half-life values were as follows: 24.1 ± 3.8 (VX), 3.1 ± 0.56 (sarin) and 2.2 ± 0.48 min (soman), respectively.

![Fig. 1: Changes of AChE activities following intoxication with soman (S1) and sarin (S2). The results are means only, for errors see Table 2.](image1)

![Fig. 2: Changes of AChE activities following intoxication with VX (S1). The results are means only, for errors see Table 2.](image2)

**Inhibition of AChE molecular forms**

The changes of AChE molecular forms were studied at time of developed intoxication and at time very near to death. These changes are summarized in Table 2. It is clear that the most sensitive was the form 4 having the lowest electrophoretic mobility. Its activity is reaching the zero at the time of death. On the other hand, the forms with highest electrophoretic mobility (forms 1 and 2) were relatively resistant to the inhibitory effect of OPs used (Table 2).

**Table 2:** Changes of total AChE activity and its molecular forms following sarin, soman and VX poisoning

| time | Total AChE | form 1 | form 2 | form 3 | form 4 |
|------|------------|-------|--------|--------|--------|
| min  | % control  | % control | % control | % control | % control |
| 5    | 39.0±1.4   | 93.3±6.8 | 35.5±4.5 | 70.0±4.9 | 9.2±2.5 |
| 10   | 10.0±1.8   | 87.7±4.6 | 18.7±5.4 | 14.0±3.2 | 3.0±1.8 |
| SORIN |            |        |        |        |        |
| SORIN | 5          | 25.0±3.5 | 95.5±5.4 | 31.2±4.0 | 44.0±2.6 | 8.2±3.9 |
| SORIN | 10         | 5.2±1.1  | 89.2±7.6 | 13.0±5.1 | 9.3±0.6 | 1.5±1.2 |
| VX    |            |        |        |        |        |
| 30    | 40.2±4.1   | 94.3±5.8 | 70.0±4.9 | 62.2±4.1 | 13.2±4.9 |
| 60    | 29.7±2.4   | 94.3±5.3 | 64.8±5.0 | 39.8±3.4 | 5.3±2.2 |
For this reason, a distribution profile of different forms was drastically changed and the main part of the whole activity was represented by forms 1 and 2 as it is demonstrated in Fig. 4. However, this change was relative only because the calculation of percentage distribution was made using the values decreased.

Fig. 3: Changes of AChE activities following intoxication with sarin and soman. Semilogarithmic transformation.

![Graph showing changes in AChE activities](image)

Fig. 4: Changes in relative distribution of the AChE forms following sarin, soman and VX intoxication.

![Graph showing relative distribution of AChE forms](image)

When the values are expressed as the activity for each form as 100%, it is possible to compare real values of AChE activity determined for the unseparated enzyme and calculated (as weighted means) values of different molecular forms (Table 3). It is demonstrated that real and calculated values are in very good agreement differing from 0.1 (minimum) to 6.1 (maximum) %. It means that really determined AChE activity is corresponding to distribution of AChE molecular forms and therefore, the whole activity is a „mean” of the activities of AChE molecular forms.

Table 3: AChE activity and its molecular forms following sarin, soman and VX poisoning: real and calculated values

| Time (min) | Total AChE | Form 1 | Form 2 | Form 3 | Form 4 |
|-----------|------------|--------|--------|--------|--------|
|           | REAL       | CALC   | % cont | % total| % cont | % total| % cont | % total| % cont | % total|
| 5         | 39.0       | 39.1   | 91.3   | 7.3    | 35.5   | 3.6    | 70.0   | 23.8   | 9.2    | 4.4    |
| 10        | 10.0       | 15.1   | 87.7   | 7.0    | 18.7   | 1.9    | 14.0   | 4.8    | 3.0    | 1.4    |
| 5         | 25.0       | 29.6   | 95.5   | 7.6    | 31.2   | 3.1    | 44.0   | 15.0   | 8.2    | 3.9    |
| 10        | 5.2        | 11.3   | 80.3   | 6.4    | 13.0   | 1.3    | 9.3    | 3.2    | 1.5    | 0.7    |
| 30        | 40.2       | 41.9   | 94.3   | 7.5    | 70.0   | 7.0    | 62.2   | 21.1   | 13.2   | 6.3    |
| 60        | 29.7       | 30.0   | 94.3   | 7.5    | 64.8   | 6.5    | 39.8   | 13.5   | 5.3    | 2.5    |

Discussion

It appears from our results that inhibition of the brain AChE by G compounds (sarin and soman) is very fast reaching to 50% activity within minutes. For VX, there is a delay and decrease of AChE activity was observed after more than twenty minutes. The half-lives are very dependent on the dose of the agent administered, on the species and other factors and therefore it is difficult to compare our results. In general, inhibition of AChE in vivo is faster for G-compounds in comparison with V-compounds (1,8,25).

From our results describing multiple molecular forms of AChE can be concluded that AChE in the brain exists in molecular forms. These forms were observed also by other authors (3,5,12,13,31,40,43). These forms are different for various species. However, the electrophoretic mobility of AChE components from the rat, rabbit, mouse and human brains suggested that there are generally two types of AChE forms having high and low molecular weight. One BuChE and two AChE bands in the rat hippocampus after electrophoresis in polyacrylamide gel were observed (41). The distinction between the two AChE forms is difficult without electrophoresis. They differ in electrophoretic mobility and they can be well differentiated by electrophoresis.

Subcellular localization of AChE suggested that in nerve ending particles and microsomal fractions 2 - 4 AChE forms are present, in the mitochondrial fraction only one was detected (4). The microsomal form absent in the mitochondrial fraction is the most sensitive to OPs in vivo. From previous studies is known that high molecular form of AChE has the lowest K_m value (2) and the highest decrease of this component after deafferentation was also observed (3). These results suggested that this form of AChE would be very important for normal cholinergic nerve transmission. It arises the question on existence of the forms under physiological conditions. Using thermal dena-
turation, it was demonstrated that they are not artifacts formed during homogenization or other treatment of the brain tissue (5). These findings were also described by other authors (2,5,23,25,33,41,43). The overall data show that catalytic activity of AChE molecular forms is different and 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.

The results with another type of inhibitor - 7-methoxytacrine (7-MEOTA) fit well with our previous findings indicating a greater sensitivity of slowly migrating molecular forms separated by polyacrylamide gel electrophoresis (10). In fact, it has been demonstrated recently that slowly migrating forms of cortical AChE correspond to G4 forms separated by sedimentation analysis (43). On the other hand, recent data indicate an almost equal sensitivity of G4 and G1 forms of both soluble and membrane-bound whole brain AChE to this type of inhibitor (31). It is not excluded that the reversible inhibitors such as 7-MEOTA modify its interaction with the active site resulting in a preferential inhibition of G4 forms. It is of interest that the introduction of a heptyl group into physostigmine modified its interaction with the AChE molecular forms, heptylphysostigmine showed a stronger inhibition for G4 than for G4 forms while in the case of the parent compound similar inhibition of the 2 forms was observed (31).

The data of 7-MEOTA are different from those obtained for DFP and paraoxon showing similar IC_{50} values for G4 and G1 forms (43). These findings have been confirmed for membrane-bound AChE (31). This is not surprising since the interaction of OP compounds (and physostigmine) with the active site of enzymatic molecule is different from that for 7-MEOTA-type compounds. In fact, OP compounds inhibit AChE by phosphorylating the esteratic serine in the catalytic site. On the other hand, acridine derivatives bind to the hydrophobic area close to the active site of AChE simultaneously affecting its catalytic center via an allosteric mechanism (20,32,38).

As regards the data on AChE molecular forms, they confirm previous findings indicating a more pronounced sensitivity of G4 forms, as compared to G1 forms, in brain of rats injected with paraoxon (43). Somewhat lower inhibitory effects of the same dose of paraoxon (0.25 mg/kg s.c.) as well as a somewhat lower contribution of G4 forms to total AChE activity in untreated rats were observed in another experiments (11) in comparison with those reported by Volpe et al. (43) and may depend on regional differences (cerebral cortex and whole brain).

In the case of brain AChE, as has been pointed out (43), G4 and G1 forms represent distinct pools in the cell, the former being mainly associated with membranes, with its catalytic site exposed to the extracellular space, and the latter confined to the intracellular compartment.

It appears from our results that following intoxication with nerve agents studied the highest sensitivity for high molecular AChE form was observed. Determination of the whole AChE activity is partly misrepresenting because of different distribution of AChE molecular forms in the sample. Following determination of the whole activity, a "mean" activity containing activities of the forms is determined. It can be concluded that in studies requiring high sensitivity (e.g. the studies of anticholinergic action), AChE molecular forms could be of choice for more detailed information on functional stage of AChE - important marker of cholinergic nerve transmission.

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94

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Doc. MUDr. Jiří Baigar, DrSc.,
Military Medical Academy,
500 01 Hradec Králové,
Czech Republic.