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THE FIRST MINI-CHEMICAL AND BIOLOGICAL MEDICAL TREATMENT SYMPOSIUM (THE m-CB MTS, PMMA I)

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1. SOME FACTORS INFLUENCING THE EFFECTIVENESS OF TREATMENT OF SOMAN POISONING

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Treatment of soman intoxication represents very serious problem especially dealing with type and time of therapeutic intervention. One of the factors limiting the therapeutic effectiveness is rapid dealkylation of soman inhibited cholinesterases (acetylcholinesterase, AChE, EC 3.1.1.7 and butyrylcholinesterase, BuChE, EC 3.1.1.8). However, there exist other factors: following the inhibition (cholinergic crisis), there are observed changes of non-cholinergic parameters like corticosterone, tyrosinamino-transferase and others (stressogenic effects). Therefore influencing of the both types of changes would led to more effective antidotal therapy. Regarding to the time course of these changes (rapid start of cholinergic symptoms and delayed long lasting the non-cholinergic changes), we tried to improve the treatment of soman poisoning using repeated administration of different drugs in different time intervals after the intoxication. The doses of all drugs used for the treatment were in the range of human doses. Improvement of therapeutic efficacy was achieved when repeated doses and types of antidotes were administered in interval 2-8 h after the intoxication. Repeated administration of reactivators was not effective. It appears from these results that treatment of soman poisoning may be improved by careful choice of antidotes administration and their timing.

2. DESIGN AND CONSTRUCTION OF BUTYRYLCHOLINESTERASE MUTANTS THAT HAVE ORGANOPHOSPHORUS ACID ANHYDRIDE HYDROLASE ACTIVITY

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Our goal is to design, express and characterize mutants of cholinesterases that resist or hydrolyze the organophosphorous (OP) nerve agents. Our initial studies have been concentrated on human serum butyrylcholinesterase (BuChE; EC 3.1.1.8) because of its relatively open active site region. By computer-aided molecular modelling based upon the crystal structure of acetylcholinesterase, several residues were selected for site-specific replacement with histidine. We reasoned that introducing an appropriately positioned imidazole group could promote general base catalysis to hydrolyze the phosphylated active site serine. The approach was oligonucleotide-directed mutagenesis in M13mp19 and subsequent stable expression in both CHO and human 293 cells by using a cytomegalovirus promoter and the geneticin drug resistance gene. One of the histidine mutants, G117H, was found to retain butyrylthiocholine (BuSCh), acetylthiocholine and benzoyl-choline (Bz) activity at pH 7.4 with a K_m^2 = 0.23+0.017 mM for BuSCh. Wild type, recombinant BuChE had a K_m^2=0.20+0.016 mM for BuSCh. Using BuSCh to measure activity, we found that the inhibition rates for the BuChE G117H mutant were markedly decreased for soman, sarin, tabun, DFP, echothiophate (EcSH) and VX. For soman, sarin and DFP inhibition, wild type BuChE has k_i values in the range of 20,000 M^{-1} sec^{-1}; for VX the k_i is 30,400 M^{-1} sec^{-1}. However, for G117H the k_i was 3.0 for soman, 2.7 for sarin and 8.7 for VX. GB and VX-inhibited G117H undergo (relatively) rapid spontaneous reactivating with k_{max} values of 6.8 x 10^{-5} and 16.3 x 10^{-5} sec^{-1}, respectively. This reactivation constitutes hydrolysis of these nerve agents and represents a rate enhancement of 100- and 2000- fold for GB and VX, respectively, at pH 6.0. Comparison of these kinetic data with those of G117K indicates that the observed characteristics of G117H are not due to the presence of a positive charge at the 117 position. Insertion of a second mutation, E197Q, results in an enzyme that also catalyzes the hydrolysis of soman.

3. MECHANISMS OF DISTURBANCES IN XENOBIOTIC DETOXIFICATION AFTER ALKYLATION OF LIVER MICROSOMES

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We studied the effects of aldehydic products of lipid peroxidation, malondialdehyde (MDA) and 4-hydroxynonenal (HNE), on the structure and function of cytochrome P-450 (CYP). Either MDA and HNE decreased activities of NADPD-oxidase, NADPH-cytochrome P-45- reductase and microsomal oxidases (aniline hydroxylase, ethylmorphine- N-demethylase, ECOD, EROD, PROD). MDA (15-30 (M) similarly to p-chlormercuribenzoate decreased the CYP content by 50% and lowered microviscosity of lipid surrounding of the spinabel OTMB bound to SH-groups of membrane

proteins. OTMB was effectively reduced by $K_3Fe(CN)_6$ in microsomes pre-incubated with MDA (20 (M), but not in native microsomes. MDA increased the content of eximers of the fluorescent probe, pyrene, bound bilayer ((f=470 nM), after stimulation of tryptophan residues (ex=280nM)). HNE and aliphatic aldehydes, valeric and caprylic, but not the corresponding aliphatic acids (10 (M) decreased the CYP content by 90%, 80% and 65%, respectively. HNE (10 (M) increased microviscosity of the OTMB lipid environment. The further increase of HNE concentration did not affect this parameter. Both MDA and HNE absorbance at 420 nm, which indicated inactivation of CYP by changes in hydrophobicity surrounding. We suggest that HNE and aliphatic aldehydes at low concentrations can enter into hydrophobic environment of CYP, binding to its SH-groups, which led to inactivation of CYP. At the same time, the modification of membrane surface layer and subsequent decrease of hydrophobicity CYP environment preceded the binding of MDA to the SH-group of CYP to develop its inactivating effect.

4. THE IMPORTANCE OF REACTIVATION OF PHOSPHORYLATED ACETYLCHOLINESTERASE IN VITRO FOR THE EVALUATION OF THE EFFICACY OF OXIMES AGAINST NERVE AGENTS

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In vitro reactivation is an important part of experimental methods concerning the development of new acetylcholinesterase reactivators from their synthesis to their practical use. It makes the explanation of their biological efficacy based on their physical and chemical properties possible. In vitro reactivation study can be also used for the qualified choice of reactivators for further examination.

In vitro testing of acetylcholinesterase reactivators involves a standard collection of experimental procedures. The reactivating efficacy of oximes in rat brain homogenate is evaluated following pre-incubation of homogenate with organophosphate. The enzyme activity is measured by potentiostatic method. The data about initial rate of enzyme reaction with substrate make the calculation of the dependence of percentage of reactivation on enzyme concentration, dissociation constant of enzyme - inhibitor - reactivator complex and pseudo-monomolecular as well as bimolecular rate constant possible. In the case of organophosphate with rapid aging of reaction with acetylcholinesterase, e.g. soman, the procedure must be modified. The reaction of enzyme, substrate, and inhibitor is investigated at the same time.

In this study, new acetylcholinesterase reactivator, transbutene analogue of HI-6, designated BI-6/1-(2-hydroxyiminomethyl-pyridi-nium)-4-(4-carbamoylpyridinium)-2-butene diibromide/, synthesized in our Department of Toxicology, was tested. In vitro reactivating efficacy of BI-6 against nerve agents (soman, sarin, GF agent) was compared with other oximes (pralidoxime, obidoxime, methoxime, HI-6, HLö-7). BI-6 is almost as efficacious as H oximes. In addition, obidoxime as well as pralidoxime are very poor reactivators of nerve agent inhibited acetylcholinesterase. Obidoxime even intensifies soman induced inhibition of acetylcholinesterase. In the case of GF agent, methoxime seems to be as efficacious as H oximes.

5. INHIBITION OF CHE WITH THE OP COMPOUNDS - CORRELATION WITH PHYSIOLOGICAL, MORPHOLOGICAL, AND BIOCHEMICAL CHANGES

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At present, besides inhibition of cholinesterase (ChE) and stabilization of acetylcholine there are recognized also many other mechanisms in toxic action of OP compounds. The main purpose of the present work is to follow the relationship between ChE inhibition in vitro by OP compounds, and the its use in the case of study of new drugs. Further, the ChE inhibition in vivo is connected with a variety of biochemical, physiological, histochemical and ultramorphological changes, after intoxication with OP and consequent therapy. This serve basis for critical evaluation of the ChE activity (inhibition and/or reactivation) for different purposes in the toxicological practice.

6. SEVERITY SCORE MODELLING OF OP INTOXICATION AS A TOOL FOR EVALUATION OF EFFECTIVENESS OF PROPHYLACTICS AND THERAPEUTICS

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Most in vivo methods reported in the literature for assessing nerve agents antidotal efficacy of drug combinations have utilized as analytical endpoints either percentage of survival following a single agent challenge dose or protective ratios (LD_{50} of the agent with treatment versus LD_{50}

without treatment). While these methods are themselves useful for determining response to pre- and/or post-challenge therapy, only limited information could be derived how much each component of the treatment would contribute to an observed effect. Response surface methodology (RSM) allows both characterization of the interactions occurring between the compounds of a combination tested, and prediction of results due to combinations not performed, thus identification of optimal pre/post-treatment combination. To assess the efficacy of combination consisting of cholinesterase reactivator, cholinolytic(s) and anti-convulsants as a pre- or post-treatment for OP-challenged rats, both survival and severity scores, a measure for incapacitation experienced, were evaluated following different doses OP. The method of maximum likelihood was used for the estimation of model parameters, derived from the experimental data.

7. PREPARATION AND CHARACTERIZATION OF BIOSCAVENGERS FOR POSSIBLE USE AGAINST ORGANOPHOSPHATE TOXICITY

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Enzymes (AChE, BChE and OP hydrolases) have been demonstrated to be effective pretreatment drugs against organophosphate (OP) toxicity in experimental animals. The efficacy of AChE as a bioscavenger of OP was amplified by combining enzyme pretreatment with oxime reactivation. To further improve the efficacy, mutants were developed that are more easily reactivated (essentially non-aging) than wild-type enzymes. Also, the wild-type enzymes that catalyzed the hydrolysis of various OPs and ChE mutants with similar ability were tested as bioscavengers. Some native enzyme bioscavengers, and all recombinant ChEs produced by biotechnology, wild type or mutants have very short biological half life, limiting their usefulness as stable, long-lasting efficient bioscavengers. This may be due to (a) incomplete glycosylation, (b) incomplete capping of some of the oligosaccharides and (c) the lack of subunit assembly of recombinant enzymes. With the proper understanding of the relationship between glycosylation and circulatory properties of ChEs, subunit assembly, and the production facilities of biotechnology, it is possible that in the very near future one or more bioscavenger enzymes, having efficient catalytic activity, biological half life and stability can be generated in sufficient quantities for human use as pre- or post treatment drugs for OP toxicity. Practically, these enzyme bioscavengers can be used now for *in vitro* sequestration of OPs.

8. CHOICE OF THE METHOD OF DETERMINATION OF THE BLOOD CHOLINESTERASE ACTIVITY IN PERSONS EXPOSED TO ORGANOPHOSPHORUS WARFARE AGENTS

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The catalytic activity of cholinesterase is exhibited by its ability to hydrolyze the substrate, therefore, the activity of the enzyme can be measured by the rate of change of the substrate content. Differences in the values of the catalytic activity of the enzymes when measured by various methods, reflect special features of the effect of those factors on which the activity of the enzyme depends under the experimental conditions, hence various methods may yield various results. We have chosen three most widely used methods of measuring the catalytic activity of cholinesterases, i.e., the method of Hestrin with a modification, the one of Ellman with a modification and the method of potentiometric titration for experimental approbation. A comparative experimental assessment of the three methods has been performed using the blood of healthy and sick people. A comparative evaluation of the method of Hestrin and that of potentiometric titration has been performed. We have established that the values of the activity of cholinesterase measured by the method of potentiometry may be higher than those measured by the method of Hestrin. It has been established as a result of the studies conducted that the methods of Hestrin and Ellman to measure the activity of cholinesterases of the blood of healthy and sick people at a sampling of 60 individuals give practically identical results and are equally appropriate for the measurement of the activity of the enzymes; however, we showed earlier that dilution of the inactivated enzyme could substantially lower the degree of its inactivation at the expense of the restoration of the catalytic activity, especially in those cases when the interaction of the enzyme with the inhibitor occurs in the presence of the substrate. As most marked this effect is observed for bifunctional inhibitors, such as toxic agents of the Vx type. In this connection, for the measurement of the catalytic activity under the conditions of a possible contact with organophosphorus warfare agents, it has been proposed that the method of Hestrin should be used.

9. PERSPECTIVES IN NERVE AGENT MEDICAL COUNTERMEASURES

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Protection or effective therapeutic strategies against the organophosphorus nerve agents has been a difficult problem for the world's defence research community since the end of the second world war. By far, the most intensive area of research has centred on finding or choosing an effective oxime to complement the actions of atropine. The ability to compare oxime/atropine, however, depends critically on several variables which are of seldom standardized across laboratories. The critical variables can be divided into four groups: circumstances, for example, the expectations of the medical treatment must be well understood. The question of what percentage of exposed individuals the therapy is expected to save will address components such as upper nerve agent dose limit (e.g. 2 or 4 or 6 LD_{50}s), time and route of exposure, rate of recovery, among others. Similarly, consideration of drug doses and timing of administration along with adjunct drug administration not directly linked to survival from nerve agent poisoning (e. g. diazepam) is important. This presentation explores the therapeutic countermeasures to nerve agent challenges with respect to the categories listed above and presents some ideas on standardizing experimental protocols with the aim of simplifying inter-laboratory comparison of efficacy results.

10. CHANGES IN CHOLINESTERASE ACTIVITY AFTER INTOXICATION WITH CARBAMATES AND THEIR TREATMENT

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In experiment on Wistar albino rats and cats the effect of different pesticides (Sevin, Pirimor) and the drug Physostigmin after single application or after treatment with different pharmacological agents (Atropin, Aprophen, Benactazin, Orocetam) was studied. All these agents were applied in different doses. The activities of brain acetylcholinesterase and serum cholinesterase were followed at different time intervals after treatment. Peculiarities and differences were found in the parameters studied.

11. THE IMPORTANCE OF REACTIVATION RATE OF NERVE AGENT INHIBITED ACETYLCHOLINESTERASE IN DIAPHRAGM AND BRAIN IN VIVO FOR THE EVALUATION OF THE EFFICACY OF OXIMES - A COMPARISON WITH THEIR EFFICACY AGAINST SUPRALETHAL POISONING WITH NERVE AGENTS

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One of the possibilities how to evaluate the therapeutic efficacy of new antidotes against nerve agents is the assessment of reactivation rate of nerve agent inhibited acetylcholinesterase in various tissues. In the present study, the therapeutic efficacy of new oxime BI-6, that has been synthesized in our Department of Toxicology, was compared with other oximes (pralidoxime, obidoxime, methoxime, HI-6, HLö-7) by the method mentioned above. The reactivation rate of nerve agent inhibited acetylcholinesterase in rat diaphragm and brain by oximes in equimolar doses (100 μmol/kg) was compared with their efficacy against supralethal poisoning with soman ($2xLD_{50}$), sarin ($3xLD_{50}$) or GF agent ($3xLD_{50}$). Each oxime was always combined with atropine (21 mg/kg).

HLö-7 appears to be the most efficacious oxime especially against soman. The oxime BI-6 is a little less efficacious than H oximes (HI-6, HLö-7) but significantly more efficacious than conventional oximes (pralidoxime, obidoxime, methoxime). Methoxime seems to be the most efficacious among conventional oximes. On the other hand, pralidoxime is not practically effective against all three nerve agents studied.

Relatively low dose of oxime was sufficient for survival of nerve agent poisoned rats if the reactivation rate of nerve agent inhibited acetylcholinesterase in diaphragm and brain was relatively high. Thus, the reactivation rate of nerve agent inhibited acetylcholinesterase in diaphragm and brain closely corresponds with the therapeutic efficacy of oximes against supralethal poisoning with nerve agent.

12. THE IMPORTANCE OF PANPAL PRETREATMENT FOR SURVIVAL OF RATS POISONED WITH SUPRALETHAL DOSE OF SOMAN

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The effect of pharmacological pretreatment with PANPAL (pyridostigmine in combination with benactyzine and trihexyphenidyle) and antidotal treatment (the oxime HI-6 in combination with benactyzine) on survival of rats poisoned with supralethal dose of soman ($2xLD_{50}$) was evaluated on a rat-model with on-line monitoring of respiratory and circulatory parameters.

Non-treated soman poisoning caused a rapid respiratory depression, progressive bradycardia and a short increase in mean blood pressure followed by hypotension. The poisoned rats died within 10 minutes on an average because of respiratory and circulatory insufficiency. PANPAL pretreatment or antidotal treatment alone partially restored respiratory as well as circulatory function disturbed by soman but only for a few minutes. The rats died within 30 minutes on an average because of the failure of respiration and circulation. When rats were pretreated by PANPAL and treated by antidotes, the respiratory and circulatory function were completely restored and the rats survived within 60 minute following soman poisoning.

The results confirm that PANPAL pretreatment seems to be able to enhance the efficacy of antidotal treatment to restore respiratory and circulatory function disturbed by soman and, thus, is very important for survival of rats poisoned with supralethal dose of soman.

13. THE DEVELOPMENT OF IMMUNOASSAYS FOR DETECTION OF CHEMICAL WARFARE AGENTS

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With the advent of enzyme linked immunoabsorbant assays (ELISA) and monoclonal antibodies, there has been considerable effort devoted to the development of antibodies to detect and quantify low molecular weight toxic substances in environmental or biological fluids. Polyclonal antibodies against paraoxon were capable of detecting paraoxon in body fluids at a level of 10^{-9} M (~260 pg/mL) when used in a competitive inhibition enzyme immunoassay (CIELA).

Monoclonal antibodies developed against a structural analogue of the chemical warfare agent soman were capable of detecting soman in buffer solutions at a level of 10^{-6} M (~180 ng/mL). These antibodies were found to be highly specific for soman even in the presence of its major hydrolysis product. Subsequent studies with anti-soman monoclonal antibodies extended the level of sensitivity to ~80 ng/mL. These antibodies did not cross react with other chemical warfare nerve agents such as sarin or tabun. In all cases, the time for a confirmatory test was two hours or less. Additional efforts to develop antibodies against sarin or VX have also been successful. These reagents offer the potential for a sensitive, rapid and low cost approach to the diagnosis or detection of the presence of toxic chemical substances. More recent efforts have focused on developing antibodies specific for the highly reactive vesicating agent sulfur mustard or its adducts to DNA.

14. MICROASSAY-BASED ENZYMATIC DETERMINATION OF SOMAN IN RAT'S BLOOD - A NEW SENSITIVE AND RAPID TECHNIQUE FOR QUANTIFYING RESIDUAL LEVELS FOLLOWING INTOXICATION

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Clinical diagnosis of organophosphate intoxication is traditionally based on the appearance of toxic symptoms and abnormal blood acetylcholinesterase levels. Unfortunately, current diagnostic measures do not permit rapid and definitive confirmation of intoxication. Although sensitive gas chromatography (GC) analytical techniques for determining organophosphate concentration in blood is available, they require laborious sample preparation and long analysis time. To allow for a definitive and quick clinical diagnosis of organophosphate intoxication, a sensitive method for rapid and accurate quantitation of residual soman levels in blood has been developed using a microassay-based enzymatic technique. The new analytical technique is based on the linear correlation between residual eel acetylcholinesterase activities and the inhibitor concentration. Blood samples were quenched with perchloric acid, followed by immediate neutralization after deproteinization. The mixture was centrifuged at 3000 g and the supernatants were assayed directly for its soman content. The accuracy of the assay was checked with four different concentrations of soman and found to be

within ± 10 %. The linear range of the assay, (0.1 - 10) nM, with a typical correlation factor of at least 0.999 (for six standards) has been optimized to span a factor of 100 to facilitate routine analysis. The sensitivity of the technique (18.22 - 1822.1 pg/mL blood) is comparable to that attainable by GC-FID analysis (250 pg/mL of blood). The assay capability in monitoring the pharmacokinetic behaviour of soman was validated for both in-vitro and in-vivo rat models.

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15. THE RELATIONSHIP BETWEEN SERUM CHOLINESTERASE ACTIVITY AND APACHE II SCORE IN ORGANOPHOSPHATE POISONING

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AIM: Serum Cholinesterase Activity is the best criterion for organophosphate poisoning. Namba classification is specific for the same intoxication (1). APACHE II Scoring System is a severity of disease classification system frequently used in intensive care unit (2). In this study, serum cholinesterase activity has been determined in 22 patients with organophosphate insecticide poisoning. The relationship between initial serum cholinesterase activity and APACHE II Scores of the patients were investigated as well as daily activity.

METHOD: Patients were grouped as mild, moderate and severely affected according to the Namba Classification. 5 poisoned patients were mild and did not receive antidotes. Intravenous pralidoxime was administered (mean 2 gr.) during continuous atropine infusion (1 gr. in 500 mL. 0.09 % NaCl.). Plasma cholinesterase activity were determined before pralidoxime administration and daily during the treatment.

RESULTS: The relationship between initial serum cholinesterase activity and APACHE II score was not found significant ($p>0.05$). The increase of serum cholinesterase was not significant until the 12th days. The cholinesterase activity reached the significant level on the 12th day ($p<0.05$).

DISCUSSION: APACHE II scores were changed when clinical manifestations became more severe. Previously, we observed serum cholinesterase activity increased three times on the 6th day (3). In another study, we determine that serum cholinesterase activity reached the normal level after the 10th day (4). In this study, we concluded that, the increase of enzyme levels were found significant on the 12th day.

16. A NEW METHOD TO DETECT ORGANOPHOSPHATE EXPOSURE: SERUM ANALYSIS OF VICTIMS OF JAPANESE TERRORISTS

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In principle, organophosphate-inhibited butyryl-cholinesterase in human plasma is the most persistent and abundant source for Biomonitoring of exposure to organophosphate anticholine-sterases. The organophosphate moiety can be remove with a suitable nucleophile from the enzyme. Subsequent quantitation of the latter product with gas chromatography provides a reliable, highly sensitive and retrospective method for detection of exposure to or handling of organophosphates, such as nerve agents and organophosphate pesticides. We applied the new procedure to serum samples from victims of the Tokyo subway attack by the AUM Shinriyko sect and from an earlier attack at Matsumoto. In serum of 10 out of 11 victims from the Tokyo incident and of 2 out the 7 samples from the Matsumoto incident, the procedure yielded sarin equivalents in the range of 0.2-4.1 ng sarin/mL serum. Evidently, these victims had been exposed to an organo-phosphate with the structure i-PrO(Me)P(O)X, presumably with X = F (sarin).

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*Presented by Leo P.A. de Jong

17. STANDARDISATION OF ACETYLCHOLINESTERASE ACTIVITY MEASUREMENTS

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The evaluation of total cholinesterase is still widely used for the determination of an organophosphate (OP) poisoning. Yet from the action of the OP compounds it is clear, that some are stronger inhibitors of the acetylcholinesterase (AChE) and others are inhibiting more the serumcholinesterase (BuChE). Therefore it is of importance, that one measures both activities, especially because the inhibition of the AChE is the dangerous part of such an intoxication. We reported, that the determination of the base line value for AChE of a healthy person is of much importance for deciding if he has suffered an intoxication [A. Wicki, CB MTS I p 1.18-1.24 (1994)]. Since the variation of the 99 % confidence limit is in the order of 31 % for AChE, this could signify, that an individual with a high AChE content could only be taken as suspect, if his AChE value is inhibited by 50 % without having a baseline value. We found that the AChE normalised to the haemoglobin content has a lower variation [A. Wicki et al. CB MTS II p. 282-286 (1996)]. We have worked out a method, which allows to determine the haemoglobin content, AChE- and BuChE-activity in the same cuvette. This has the advantage, that pipetting errors are eliminated to a large extent by this internal standardisation. It enables us to obtain meaningful values indicating an even slightly reduced AChE activity already. This means, that a suspected intoxication can be detected at low expositions.

18. SAMPLE HANDLING AND ANALYTICAL QA AND QC

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As we try to develop a standardized analysis for cholinesterases, there are several data quality issues that should be considered from collection and handling of samples to laboratory quality assurance (QA) and quality control (QC). Many of these concepts have been used and abused in environmental sampling and apportioning responsibility and penalties to companies responsible for pollution. We should expect that the data we present will be contested and we should be prepared to offer documentation that the samples were collected, handled and analyzed in accordance with protocols for forensic evidence.

All the efforts in analyzing samples are wasted if the samples are not collected, handled, stored and shipped in a way to maintain data integrity. With chemicals as reactive as the chemical warfare nerve agents, the agents will be reacting with the chemical soup that makes up living organisms. It is important to get the samples to the laboratory quickly, but properly preserved. Sample collection and handling issues include documentation of how, when and where the sample was taken and chain of custody (documentation accompanying the samples, signed and dated at every change of hand) documentation. Do the chemicals react with the containers, plastic surfaces or implements?

Analytical issues include documentation of the QA process and specific QC essentials, including certification of analytical reagents and standards and instrument and solution calibrations, verification of detection limits and analytical precision and accuracy (relative percent difference between samples, spikes and spike recoveries).

19. CHOLINESTERASE REACTIVATORS AS PROPHYLACTICS AGAINST OP INTOXICATION

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Several cholinesterase reactivators were tested for their ability to ameliorate the signs of acute OP intoxication. The prophylactic efficacy at different time before OP-challenge and dose regimes of the compounds was assessed following the next parameters: severity score of incapacitation, based on six graduated scale, time to onset of convulsions, survival time. A good protective effect for the most of compounds was found. The possible mechanisms of antidotal action are discussed.

20. ATROPINE AEROSOL SPRAY AAS

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Atropine is the mainstay of therapy for anticholinesterase poisoning. For military and civil personnel who are at the risk for organophosphorus poisoning, an alternate route of administration instead of the commonly executed i.m. injection was claimed for years.

Aerochem/Inter-Cb developed the first nasal spray in 1989, and substantial amounts were used

during the Gulf War. Today the product is using environmentally accepted propellent gas, is adapted in size and newly packed.

The poster gives comparisons of the different administration route, instructions for use, etc. of the improved product.

21. CHANGES OF THE RAT LIVER MICROCIRCULATION FOLLOWING SUBLETHAL INTOXICATION WITH SOMAN

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Vascularization of the rat liver following soman (O-pinacolyl methylphosphono-fluoridate) intoxication was studied using scanning electron microscopy. Corrosion casts were prepared with commercially produced methyl methacrylate monomer which was pre-polymerized and injected into the pre-washed and fixed vascular bed. The injections have been provided through the left cardiac ventricle. Samples of injected organs were treated in 40% KOH. The obtained corrosion casts were sectioned using a stereoscopic light microscope. Normal vascular bed pattern of some organs was studied and compared with vascularization after soman poisoning. Changes in the vascular bed architecture of the liver were described.

22. MONITORING OF THE CHOLINESTERASE STATUS IN ORGANOPHOSPHATE POISONING

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Inhibition of tissue acetylcholinesterase (AChE) is generally thought to be the principal biochemical lesion in organophosphate (OP) poisoning. Depending on the structure of the OPs, various phosphorylated AChEs are formed that differ in the rates for spontaneous and oxime-induced reactivation and for irreversible inhibition by „aging".

The rationale for the use of oximes in the treatment of OP poisoning thus is the potential reactivatability of tissue AChE. Since this enzyme source is not easily accessible erythrocyte AChE (ery AChE) is used as a suitable surrogate marker. In fact, monitoring of ery AChE allows to answer the following questions: (1) Is the patient's enzyme reactivatable at all by the therapeutic oxime *in vitro*? (2) Did the oxime therapy result in reactivation *in vivo*? Oxime therapy can be terminated when anticholinesterases are no longer present in the body. This item can be checked by the following endpoints: (1) The patient's plasma does not inhibit an external enzyme source, e.g. donor eryAChE. (2) The usually depressed plasma cholinesterase (P1ChE) is steadily increasing due to *de novo* synthesis.

We used this test battery to monitor the course of organophosphate poisoning and the influence of obidoxime therapy. Before transfer to the intensive care unit (ICU), all patients (6 cases) received primary care by an emergency physician. In the ICU, atropine sulphate was continuously administered i.v. upon demand according to the endpoints: no bronchorrhoea, dry mucous membranes, no auxiliary sweating. Obidoxime (Toxogonin®) was given as an i.v. bolus (250 mg) followed by continuous infusion at 750 mg/24 h. Obidoxime was effective in life-threatening parathion poisoning (n = 4), particularly impressive when the dose absorbed was comparably low. In mega-dose poisoning net reactivation was not achieved until several days after ingestion when the concentration of active poison in plasma had declined. The reactivatability *in vivo* was longer lasting than expected from *in vitro* experiments. Therapy with obidoxime allowed a marked reduction of the atropine demand (usually 0.5 to 1 mg/h).

Obidoxime was quite ineffective in oxydemeton-methyl poisoning when the time elapsing between ingestion and oxime therapy was longer than one day. When obidoxime was administered shortly after ingestion (1 h), the reactivation was nearly complete.

23. DEVELOPMENT OF A STANDARD OPERATION PROCEDURE FOR DETERMINATION OF ACETYLCHOLINESTERASE (ACHE) ACTIVITY IN THE BLOOD OF ORGANOPHOSPHATE (OP) POISONED PATIENTS

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Erythrocyte AChE determination is preferable to that of plasma cholinesterase because of its lower biological variability and closer correlation with the functionally important tissue AChE. Nonetheless, routine measurements of AChE activity in clinical toxicology is quite unusual. Hence, we have tried to provide simple, rapid and reliable protocols for sample preparation and AChE determination.

Sample handling is critical since, prior to determination, spontaneous and oxime-induced reactivation may occur along with further (re-)inhibition in the presence of OP. These effects can be almost completely prevented by immediate (bedside) dilution (1:100) of the blood sample in ice-cold phosphate buffer (0.1 M, pH 7.4) followed by rapid freezing. With this procedure, bimolecular reactions are slowed down (0.01%) and erythrocytes are hemolyzed, thereby facilitating precise measurements of enzyme activity in simple photometers. Storage of the diluted blood samples over 2 weeks did not significantly alter enzyme activity in the presence of paraoxon (10 nM) and obidoxime (100 nM).

AChE activity is determined by a modified Ellman procedure in 0.1 M sodium phosphate, pH 7.4, with 0.45 mM acetylthiocholine (5 x K_m) at 37 °C. Lowering pH and substrate concentration decreases significantly the blank reaction and thus increases the sensitivity (lower level: 2% of normal activity). To exclude interference with plasma cholinesterase, ethopropazine (10 μM) is present in the assay. Measurement of the developing color at its absorbance maximum (412 nm) is not feasible because of the interfering oxyhemoglobin. Since simple filter photometers with mercury lamps are usually available even in low-tech laboratories it appears reasonable to exploit the strong emission wavelength at 436 nm. Here, oxyhemoglobin absorption is roughly one fifth compared to 412 nm while the indicator absorption is reduced by 20% only. Moreover, thermochromism of the colored indicator is weak at this wavelength. AChE activity is normalized to the hemoglobin content of the sample to compensate for variations in sample dilution and red blood cell count. Total hemoglobin in completely (!) hemolyzed samples can be determined either as carbon monoxide hemoglobin after reduction with dithionite or as cyanomethemoglobin after oxidation with ferricyanide. Both hemoglobin derivatives can be monitored at 546 nm (filter photometer) or at 419 nm (HbCO) and 540 nm (HbCN). AChE activity in blood is most accurately expressed as U/mmol Hb (Fe). This standardized procedure has been applied to monitor the AChE status in several organophosphate poisoned patients.

24. AN IMPROVED MICROASSAY PROTOCOL FOR SPECTROPHOTOMETRIC MEASUREMENT OF ERYTHROCYTE AND PLASMA CHOLINESTERASES

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Microassay-based spectrophotometric technique for measurement of cholinesterase activities is ideal for carrying out rapid, economical large scale studies to determine population baseline cholinesterase level. However, the precision and accuracy of microassay spectrophotometric techniques are limited by inherent problems of pathlength variability. To overcome this problem and exploit its potential for carrying out large scale cholinesterase measurements, we have developed an improved microassay protocol. The new protocol corrects for pathlength variance by normalizing the determined cholinesterase activities with relevant blood parameters. In the case of acetylcholinesterase, total haemoglobin content is used as the internal standard while plasma cholinesterase activities is normalized by plasma protein content. In a minor but important departure from current assay protocols, both total haemoglobin and plasma protein contents were determined in the same sample wells only after kinetic determinations of cholinesterase activities have been completed. By taking the quotients of cholinesterase activities over the normalizing factors, we would nullify errors arising from pathlength variances. The resultant quotient will also serve as a common term for comparison of results obtained from different method of cholinesterase measurements. To permit total haemoglobin determination after cholinesterase activities have been measured, 4,4'-dithiodipyridine was used as the chromophore in our protocol instead of DTNB. The choice of chromophore permitted the use of a lower dilution factor leading to faster analysis time. The choice of substrates for acetylcholinesterase and plasma

cholinesterase determinations were also investigated in this study and found to have a significant influence on the % CV of the final results. Two different spectrophotometric means of determining total haemoglobin content were also investigated in this study and their respective accuracies and precisions are reported with reference to values obtained from a clinical co-oximeter (OSM3) designed for haemoglobin measurements.

25. THE PUSH-PULL MECHANISM OF DEALKYLATION IN SOMAN-INHIBITED CHOLINESTERASES IS UNIQUE[1]

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The pH-dependence and solvent isotope effects of dealkylation in diastereomeric adducts of *Electric eel (Ee)* and fetal bovine serum (FBS) acetylcholinesterase (AChE) inactivated with P(-)C(+) and P(-)C(-) 2-(3,3-dimethylbutyl) methylphosphono-fluoridate (soman) were studied at 4.0 ± 0.2 °C. The log rate constant versus pH profiles are best described by a sigmoid and a hyperbolic component for all adducts with pKs 4.0 for *Ee* AChE and 4.3-4.8 for FBS AChE. Maximal rate constants (k_{max}) are 13-16 x 10^{-3} s^{-1} for *Ee* AChE and 8 x 10^{-3} s^{-1} for FBS AChE. Catalytic participation of the Glu199 anion is consistent with the pH profile below pH 6. The solvent isotope effects at the pH maxima are 1.1-1.3 indicating negligible transfer of protons at the enzymic transition states for the dealkylation reaction. Slopes of log rate constant versus pH plots are near one at 25.0 ± 0.2 °C between pH 7.0 and 10.0. This implies that if $HisH^+440$ is the general acid catalyst, its pK at the transition state for dealkylation is greater than ~ 7, but probably closer to the pK in a phosphonate diester than in a phosphonate monoester anion enzyme adduct. In stark contrast, the corresponding adducts of trypsin are very stable even at 37.0 ± 0.2 °C: The diastereomers of soman-inhibited trypsin were studied at 37.0 ± 0.2 °C by reactivating trypsin from the adducts with 0.1 M 2-PAM. The rate constants are pH independent and ~ 10^4 times smaller than k_{max} for analogous adducts with AChE. S_N1 dealkylation seems negligible or nonexistent in soman-inhibited enzymes outside the cholinesterase family. The catalysis of dealkylation in soman-inhibited AChEs is estimated to occur at > 10^{10} times faster than a plausible nonezymic reaction: nearly half of it can be attributed to an electrostatic push and an electrostatic pull provides some of the balance of transition state stabilization. The results of this work together with result of a product analysis by Michel et al. (1969 can be explained by an initial and rate-determining methyl migration from Cβ to Cα. This is driven by the high electron density of residues (Glu199, Trp84) at a crowded active site and may be concerted with C-O bond breaking. The positive charge at the rate determining transition state is distributed between Cβ and His 440. A tertiary carbo-cation may have a fleeting existence before it is trapped by water of neighboring electrons which is likely to be promoted by Glu199 as the proton acceptor.

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26. STRUCTURAL REQUIREMENTS FOR ADJUVANT, NON-SPECIFIC INTERACTIONS OF CHOLINESTERASE REACTIVATORS

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The preventive and curative properties of cholinesterase usual reactivates (PAM, Obidoxime, Toxogonin), oximes from the Ilse Hagedorn series and benzoyl derivatives of pyridinium aldoxime, were tested in soman, sarin and V_x poisoning.

Although the topology of the receptor and consequently, that of the reactivator active centers to be clear, the efficiency hierarchy *in vivo* and even *ex vivo,* on the blood samples, seems to be very different from the expected one from the theory.

To escape from this rub, a more general pharmacokinetic-pharmacodynamic approach was tried. The cholinesterase reactivators dynamic interaction with the postsynaptic membrane was analyzed in the frame of the author's theory on the drugs molecules - membrane interfaces finite interactions[1]. The theory considers not only drug-receptor interactions but also reversible interactions between drug molecule on the one part and spatial distribution of molecules and field of forces at the level of membranal interfaces. Such a way, the action mechanism of cholinesterase reactivators was decomposed in some basic components; pharmacokinetic interactions, hydrophobic interaction with the adjacent areas, interaction with the electric field in the double layer of the membranal interfaces and finally, the ionic interaction with the receptor charges and dipoles.

As an in vitro model of the interaction reactivator molecule - membrane as a whole, it was studied the effect of PAM-Cl, Toxogonin and HI-6 added in the aqueous subphase on the monolayers of cholesterol. The superficial pressure isotherms indicated the presence of weak interactions with

effects on the cohesion of films.

As pattern of the interaction with the electric field in the double layer of the membranal interfaces, it was studied the Acetylcholine effects on the interfacial tension at the mercury/electrolytic solution interface at different external fields.

Finally, although the problems were not solved, the nightmare was somewhat removed, since it was understood that more specific interaction with the receptor implies less favorable chemical stability and pharmacokinetic properties, less adjuvant non-specific but necessary interactions.

[1]V. Voicu, C. Mirciolu: Mocanisme farmacologico in interface membrane, Ed. Academioi. Bucuresti 1994.

27. PRETREATMENT AGAINST NERVE AGENT POISONING: THE VALUE OF PYRIDOSTIGMINE

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Despite great research efforts in the past decades the antidotal therapy against nerve agent poisoning and especially soman poisoning is unsatisfactory. Due to this fact the concept of pre-exposure partial inhibition of acetylcholinesterase (AChE) by a reversible AChE inhibitor (carbamate) was developed. In the meantime several NATO and other countries stockpiled pyridostigmine bromide tablets to provide a pretreatment compound for their soldiers. During the Gulf War II many thousand allied servicemen were ordered to take pyridostigmine tablets to be prepared against an Iraqi chemical attack, which fortunately did not occur.

The theoretical concept of partial protection of AChE by pyridostigmine was examine by in vitro experiments with isolated AChE and by a number of animal investigations using different species and protocols. These data indicate that there are marked differences in the efficacy of pyridostigmine pretreatment (and atropine plus oxime treatment) depending on the species used and on the dose of the various compounds. In soman or tabun poisoned rodents pyridostigmine increased the protective ratio of antidotal treatment while in sarin or VX poisoned rodents a missing or negative effect of pyridostigmine pretreatment was observed. In soman poisoned primates some protective effect of pyridostigmine was recorded, however, the database is insufficient for a final assessment. After reviewing the open literature pyridostigmine pretreatment can be recommended in face of an impending soman exposure but it cannot be recommended for sarin, cyclosarin, tabun or VX.

28. A CATALYTIC ANTIBODY CAPABLE OF HYDROLYZING THE NERVE AGENT SOMAN

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Pinacolyl alpha-hydroxy-p-aminobenzylphosphonate was proved to be a competitive inhibitor of the somanase isolated from the serum of Bufo bufo gargarizans. This compound was coupled with proteins to form artificial antigens. Antisera and monoclonal antibodies were prepared using these antigens. Soman-binding activity was found in both the antisera and monoclonal antibodies. One of the monoclonal antibody was found to be capable of catalyzing the hydrolysis of soman with a rate enhancement of 920-fold.

The conclusion that the catalytic activity belongs to the antibody but not to contaminating enzymes is supported by the fact that some single chain Fv regions, which were generated using the same immune procedure and phage-displayed techniques by a colleague of our institute, catalyzed also the hydrolysis of soman.

29. EFFECT ON AGING OF SOMAN INHIBITED ACETYLCHOLINESTERASE

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According to Shafferman et al. (Biochem. J., (1996) 318, 833-840) aging of phsphylated acetylcholinesterase (AChE) is mediated by aromatic and polar residues of the active center. If this is indeed the case it should be possible to affect the rate of aging by suitable ligands. Consequently we tested the influence of oximes on the rate of aging. First we adopted the previously described ezyme antigen immunoassay for measuring (AChE) to determine the aging rates. In our experimental setup they were as follows: GD 14.6 h^{-1}, GB 0.052 h^{-1}, GA 0.025 h^{-1} and DFP 0.07 h^{-1}. Then we tested the influence of oximes on the rate of aging and found that HLö-7 was able to reduce the rate of aging in a concentration dependent manner. HI-6 was also effective but to lesser extent than HLö-7 while the other oximes tested (HS-6, HS-3, Toxogonin, TNMB-4, PAM and P2S) had no influence on the aging rate. Tacrine, another bis-quaternary ligand of AchE had no effect of the

aging rate. Our results thus corroborate the findings of Shafferman et al. and show than aging can indeed be influenced by suitable ligands.

30. NEUROPSYCHIATRIC SYNDROMES AND OCCUPATIONAL EXPOSURE TO ZINC PHOSPHIDE IN EGYPT

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Eighty six workers exposed to zinc phosphide pesticide were studied, for evidence of neuro-psychiatric manifestations. They have been evaluated clinically as well as, by electro-encephalography (EEG) and by electro-myography (EMG) in some cases. All were males of mean age 35.8 years and mean duration of exposure to zinc phosphide 11.3 years.

Most of them presented with one or more of neuropsychiatric symptoms including fear of poisoning, anxiety, impotence and easy fatigue.

About half of them showed evidence of neuropsychiatric signs, including hyper-reflexia, polyneuropathy, lumber radiculopathy, cervical myelopathy and anxious mood, impaired attention and psychomotor stimulation.

EEG recording of the exposed group, showed abnormal records in (17.4%) who had a mean age of 39.1 years and duration of exposure to zinc phosphide 15.1 years.

EMG studies showed evidence of partial denervation of anterior tibial group of muscles and flexor digiti minimi in two out of the 30 workers, subjected to EMG examination (6,7%).

Serum levels of Zn and Ca were significantly higher in exposed workers than in the controls ($p<0.005$). Serum Cu, Fe, P, Mg were significantly lower than of the controls.

Electrophoretic pattern of globulin showed that gammaglobulin fraction was significantly increased ($p<0.005$), a_2 and b-globulin decreased ($p<0.005$) in exposed workers.

Lipoprotein pattern showed that the total lipids, B-lipoprotein as well as B/a ratio were significantly encreased ($p<0.005$), while the a_1 lipoprotein was decreased in exposed workers. Triglycerides and cholesterol were significantly increased ($p<0.001$) and phospholipids and phospholipid/cholesterol ration were significantly decreased ($p<0.005$) in those exposed in comparison to the controls.

It was found that exposure to Zn_3P_2 not only causes mild acute and subacute liver cell damage but also affects renal function and may be B-cells of pancreas. 68.6% of the exposed worker had chest symptoms and only 24.4% presented with chest or cardiac signs

Ventilatory functions were abnormal in 70% and abnormal E.C.G. findings were present in 12.8% among the exposed group.