CONDUCTING ANALYSIS OF BIOMEDICAL SAMPLES TO ASSESS EXPOSURE TO ORGANOPHOSPHORUS NERVE AGENTS

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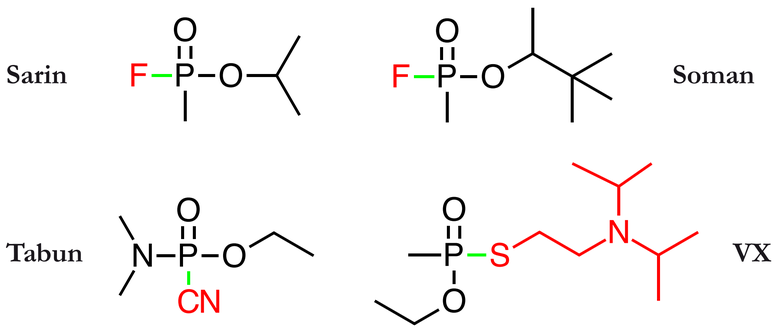
1. INTRODUCTION

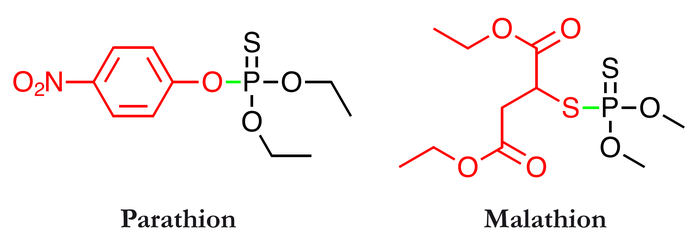
Highly toxic **nerve agents** such as **Tabun**, **Sarin**, **Soman** and **VX** are banned under the Chemical Weapons Convention (CWC) and formed major parts of **large stockpiles** of chemical weapons during the **Cold War**. **Terrorist attacks** carried out by the cult Aum Shinrikyo in **Japan** in 1994/95 employed **Sarin**. The OPCW supported UN mission that investigated the August 2013 chemical attacks in **Ghouta/Syria** determined that the chemical agent used was also **Sarin**. **Sampling and analysis** of **environmental samples** can reveal the presence of absence of these agents (and/or their degradation products) but in order to assess if a **potential victim** was exposed, the analysis of **biomedical samples** is required. **Blood** and **urine** samples are preferred as they are easily collected but the analysis of body **tissues** is also possible. Tissue samples are especially relevant in case of deceased individuals.

2. NERVE AGENTS - CHEMISTRY AND STRUCTURE

Nerve agents are **organophosphorus** compounds and are liquid at room temperature. For understanding their reactions in the human body it is helpful to introduce the concept that the molecules are made up by **two different parts**: A. The **phosphorus containing part** (shown in black) in which a phosphoryl group (P=O) is bonded to an O-alkyl (-O-R) group and a short alkyl group (R) or a small dialkylamino group (-NR_2) in case of Tabun. The other part of the molecule is the so-called **"leaving group"** (shown in red). In case of Sarin and Soman this is a fluorine atom (-F), in case of Tabun a cyano group (-CN) and in case of VX a larger group containing nitrogen and sulphur. Most relevant reactions of the agents involve the chemical bond connecting these two groups (shown in green).

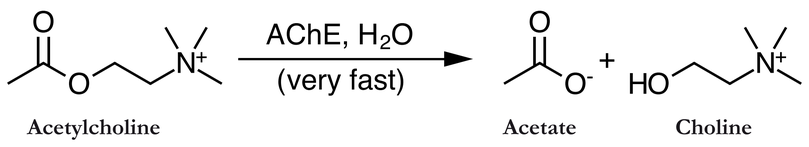


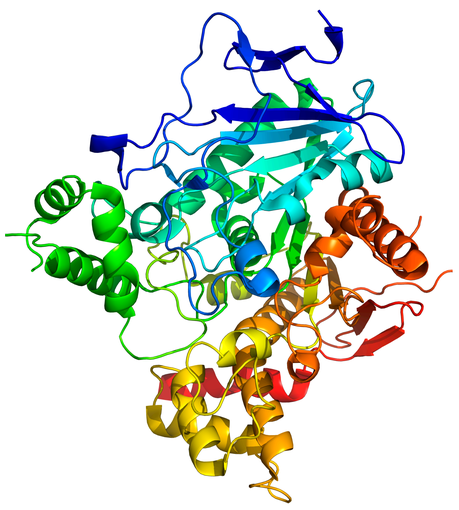
Organophosphorus **pesticides** are **similar** in structure (nerve agents were found while looking for new effective pesticides) and mode of action. Parathion and Malathion are shown as examples below. The substitution of oxygen in the phosphoryl group with sulphur lowers toxicity for humans.



3. ACETYLCHOLINESTERASE - THE TARGET

The primary **toxicity** of nerve agents is due to their ability to **inhibit** the action of an **enzyme** (protein with catalytic activity) crucial in the process of **conducting nerve signals**. **Acetylcholinesterase** (**AChE**) is responsible to break down the **neurotransmitter acetylcholine** at neuronal junctions by hydrolysis (reaction with water, see figure below). In a simplified view this switches a nerve signal from on to off. If the enzyme is **blocked**, acetylcholine will **accumulate** and signal transmission cannot be terminated. This leads to **cholinergic crisis** and typical symptoms including **sweating, salivation, miosis (pinpoint pupils), paralysis, respiratory failure** and eventually **death**. Because **AChE** is a very **fast and efficient enzyme** (one enzyme molecule can break down 25000 molecules of acetylcholine per second) and is not present in very large amounts, blocking of the enzyme quickly leads to fatal consequences.

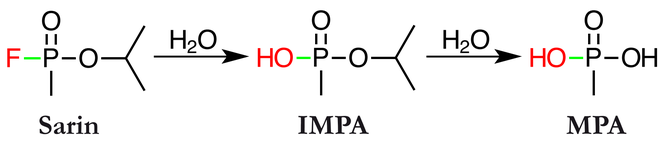




Human AChE consists of **640 amino acids**. In the human body most of the AChE is found as units of two (**dimer**) or four (**tetramer**) AChE molecules that are **anchored** to a **membrane**. The figure to the left shows the complicated folding of the protein leading to its three dimensional **structure**. Helical substructures and so called beta-sheets (thick arrows) can be identified. The catalytic active site is buried deep inside the enzyme. It contains three amino acids crucial for catalytic activity: Serine 200, Histidine 440 and Glutamate 327. The nerve agents attach to Serine 200 to block the enzyme.

4. ANALYSIS OF METABOLITES

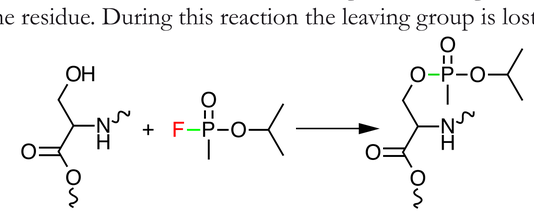
Nerve agents that are not interacting with AChE or other proteins in the human body (see below) normally **hydrolyze** quite rapidly. This is especially the case of **hydrophilic** agents such as Sarin while **lipophilic** agents such as VX can form **depots** of intact agent **in fatty tissues**. In case of Sarin the **primary hydrolysis product** (which is unable to block AChE) is **isopropyl methylphosphonic acid (IMPA)** that can further degrade to **methylphosphonic acid (MPA)**. Other indicators for the presence of the agent are t**ypical sideproducts** formed during Sarin synthesis such as **diisopropyl methylphosphonate (DIMP)**.



These compounds can be detected in **urine** and **blood** samples using **liquid** or **gas chromatography**. Due to the **low concentrations** in body fluids (in the **parts per billion** range) **GC-MS/MS** or **LC-MS/MS** methods employing **single ion monitoring (SIM)** or **multiple reaction monitoring (MRM)** modes are commonly used. This requires **targeted analysis**, meaning that one has to specifically analyize for a specific compound such as IMPA.

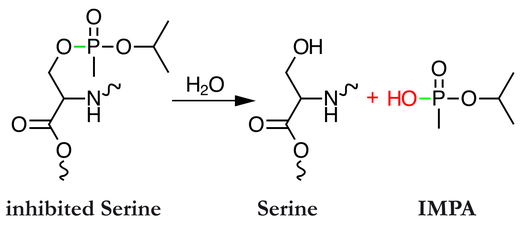
5. PROTEIN ADDUCTS AND THEIR FATE

Nerve agents do not only react with AChE but also with other proteins. One highly similar to AChE is **Butyrylcholinesterase (BChE)**. In contrast to the membrane anchored AChE, BChE is found in **blood serum** and can be used for analysis more easily. The active site of BChE also contains a **catalytic triad** of serine, histidine and glutamate and the **molecular mechanism of inhibition** is **identical** with AChE with the agent attaching itself to the serine residue. During this reaction the leaving group is lost.

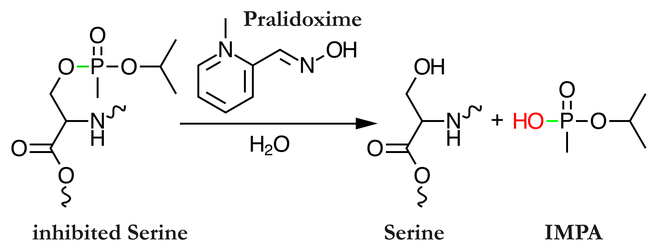


After the attachment of the agent to the serine residue, the enzyme is **blocked** and cannot perform its normal activity. This primary protein adduct can react further in a number of ways:

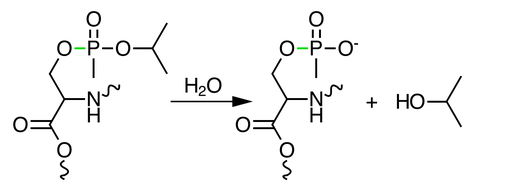
Spontaneous reactivation:
The inhibited Serine might react with water to **produce the original and functional serine residue** plus the hydrolysis product of the agent (IMPA in case of Sarin). While this process plays a role for certain pesticides, it is **too slow** to be of relevance in case of nerve agent poisoning.



Reactivation with a nucleophile
Nucleophilic compounds such as **oximes** can be used for induced **reactivation**. Such oximes are commonly used as **therapeutics** in case of nerve agent poisoning. They include compounds such as 2-PAM (Pralidoxime), Obidoxime, HI-6, MMB-4 and TMB-4.

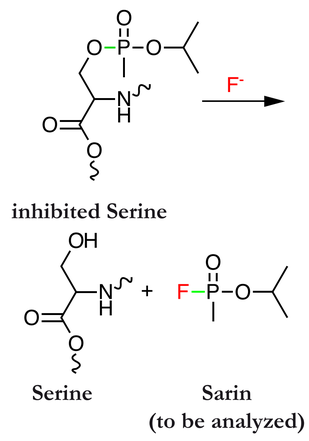


Ageing
The inhibited serine can **loose an additional group** from the phosphorus atom leading to a structure with a negative charge at an oxygen connected to the phosphorus (a process called ageing). This structure **cannot be reactivated** using oximes. While some agents age relatively slow ly (over hours and days) others are much faster. **Soman ages within minutes**, making medical therapy even more difficult.



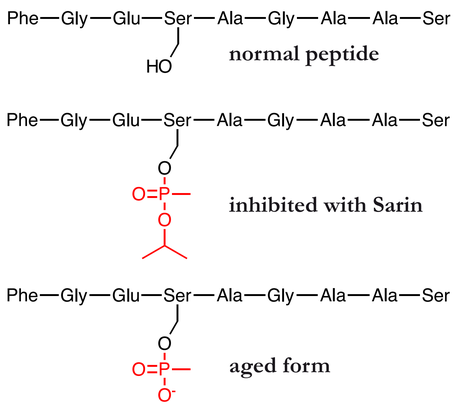
6. FLUORIDE REACTIVATION

One advantage of analysing protein adducts over free metabolites in blood is that they persist for much longer times. While **free metabolites** are cleared from blood in a **couple of days**, **protein adducts** may persist for **several weeks**. One approach for analysis that does not require a look at large protein molecules or fragments is **fluoride regeneration**. Sodium fluoride solution is added to the blood or plasma sample and the **fluoride ions** react with the protein adducts to **release the agent** again. In case of Sarin, Soman and Cyclosarin the original agent is regenerated. In case of Tabun, Fluorotabun is produced and in case of VX the product of fluoride regeneration is Ethylsarin. The one problem that exists with this procedure is that **aged protein does not react with fluoride** and **these molecules escape detection**.



7. DIRECT ANALYSIS OF ADDUCTS

When a nerve agent binds to AChE or BChE there is a **characteristic mass change in the protein** that can be used to identify the agent. The established procedure is relying on BChE in human blood plasma. Instead of using the intact protein (consisting of 574 amino acids) the **protein is cut into smaller pieces** (so called peptides) by using the **digestive enzyme Pepsin**. The fragment of interest is a **peptide of nine amino acids** that contains the serine residue inhibited by nerve agents:



The different peptides generated by the Pepsin digest are **separated** using **liquid chromatography (LC)** and analysed using **tandem mass spectrometry (MS/MS)**. As the **leaving group** of the agent is **lost** when binding to AChE or BChE, this analysis **can not reveal the absolute identity** of the used agent (the same is true for fluoride regeneration and any other analysis that does not identify the intact agent). For example, an adduct that is identical to the one produced upon exposure to Sarin might actually come from an agent that featured a leaving group similar to that of VX. Aged adducts caontain less information, but these peptides contain **more information** than just finding free MPA, as MPA is also a degradation product of some legitimate chemicals such as the flame retardant dimethyl methylphosphonate (DMMP). The **aged adduct is clear proof** that the body was **exposed** to a **toxic methyl-phosphonic chemical** that is **able to bind to and block AChE and BChE**. DMMP, for example, is unable to do this.
An alternative source for protein adducts is **serum albumin**. After digestion with **Pronase** adducts with the amino acid **Tyrosine** can be detected.