Several parameters of the Novichok nerve agents A230, A232 and A234 were determined. Hydrolysis rates were approximately one to three orders of magnitude slower than G-type nerve agents and approximately zero to two orders of magnitude slower than V-type nerve agents. A230 was the most labile Novichok compound followed by A232 then A234. Activation energies ($E_a$) and frequency factors ($A$) were determined for all three compounds. The organophosphorus acid anhydrolase (OPAA) enzyme had catalytic efficiencies on the Novichok compounds ranging between $10^4$ and $10^5$ M$^{-1}$ min$^{-1}$ with the highest $k_{cat}/K_m$ value for A230, then A232 and lastly, A234.

1. Introduction

In 2018 a report in Chemical and Engineering News presented a family of three Novichok nerve agents (Figure 1) including a compound designated A234, alleged by the British government to have been used to poison Sergei and Yulia Skripal in Salisbury, England on March 4, 2018 (Peplow, 2018). The other structurally-related analogs in this family are designated A230 and A232 (Nepovimova and Kuca, 2018) and (Kloske and Witkiewicz, 2019) published different structures for A234 but the identification of the compound used against the Skripals was confirmed by the Organization for the Prohibition of Chemical Weapons (OPCW) on April 12, 2018 and the compound identified by OPCW is herein referred to as A234 (Poplow, 2018). The compounds referred to herein as A230 and A232 are respectively the methyl and methoxy versions of the ethoxy compound A234. Even given the heretofore lack of availability of these compounds for study, there has been speculation as to the nature and effects of the Novichok compounds, including a theoretical evaluation of their potential for enzyme-catalyzed hydrolysis (Lyagin and Efremenko, 2019), a theoretical study of A234 chemistry (Bhakhoa et al., 2019), a theoretical detection scheme based on vibrational spectroscopy (Tan et al., 2019), theoretical medical diagnostics (Pohanka, 2019), (Chai et al., 2018) and theoretical toxicological studies (Carlsen, 2018). Of course it is very difficult to study, even theoretically, compounds for which the structure is not certain and in fact, several of these studies are based on compounds other than that identified by the OPCW as having been used in Salisbury. Here we sought to determine the hydrolysis rates of the three racemic compounds published in Chemical and Engineering news, their energies of activation, and their susceptibility to catalysis by the organophosphorus acid anhydrolase (OPAA) enzyme. The OPAA enzyme has previously been shown to catalyze the hydrolysis of G-agents and, to a lesser extent, V-agents (Daczkowski et al., 2015; Bae et al., 2018).

2. Materials and methods

2.1. Novichok compounds

A230, A232 and A234 were synthesized by in-house methods and characterized by multinuclear nuclear magnetic resonance (NMR) spectroscopy and gas chromatography mass spectrometry (GC-MS). All were greater than 95% pure. A232 has a CAS number of 2308498-31-7. A230 and A234 have no known CAS numbers.

2.2. Fluoride assays

Fluoride measurements for Arrhenius plots and pH comparisons were made in a temperature-controlled vessel, calibrated with three known standards (10, 100 and 1000 μM F$^-$) independently at each temperature and pH value. Details were as described (Bae et al., 2018) pH was controlled with 50 mM bis-tris-propane buffer. For reactions where the initial readings were below 10 μM F$^-$, small amounts of NaF were added to raise the starting concentration into the lower portion of the calibrated range then normalized to a starting value of 10 μM F$^-$ for clarity of
presentation. Working stock dilutions were 300 mM in anhydrous isopropanol for all compounds.

2.3. Enzyme purification

The OPAA enzyme was prepared as described previously (Bae et al., 2018). Briefly, the Escherichia coli host cell containing the cloned OPAA gene was grown to late log phase in 1 L of LB broth in a bioreactor. Cells were harvested and the enzyme was purified by ammonium sulfate fractionation and the 45–65% pellet obtained was redissolved, desalted and purified to apparent homogeneity on a 10 ml Q Sepharose column by elution with a 0–0.6 M NaCl gradient. Protein purity was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with OPAA migrating with near homogeneity.

Enzyme activity and hydrolysis rates of G-series and Novichok agents were determined as described (Bae et al., 2018). V-series hydrolysis was measured at 412 nm in a Jasco V-730 spectrophotometer using quartz cuvettes and the Ellman’s Reagent assay (Ellman, 1959). Kinetic parameters were calculated using Biosoft EnzFitter® software (Biosoft.com). Data were generally collected at substrate concentrations 1/3 to three times the \( K_m \) (the substrate concentration required for the reaction to occur at 1/2 \( V_{\text{max}} \)) under conditions that consumed less than 10% of the substrate.

3. Results

3.1. Ultraviolet-visible spectra

Ultraviolet-visible spectra of the neat compounds (Figure 2) showed no obvious wavelength at which to follow the hydrolysis of any of the compounds by spectrophotometry, so a fluoride electrode was used to measure hydrolysis via defluorination.

3.2. Hydrolysis rates

Hydrolysis rates of G-series and Novichok nerve agents including A230, A232 and A234, all containing a single fluoride leaving group, were measured in 50 mM bis-tris-propane buffer pH 7.2 at 25 °C with a fluoride electrode. Hydrolysis of the V-series compounds was determined with the Ellman’s reagent assay to measure free thiol from the cleavage of the P-S bond. Compounds were used at a final concentration of 1 mM, diluted into the buffer from 300 mM stocks in anhydrous isopropanol. The G-series and V-series agents (Figure 3) were not the specific focus of this study but were included for reference, since they have been studied more extensively than the Novichok compounds. The hydrolysis reaction profiles are shown in Figure 4 and the corresponding hydrolysis rates are shown in Table 1.

3.3. Arrhenius plots: determination of \( E_a \) and \( A \) values

The Arrhenius equation (Eq. 1) gives the dependence of the rate constant of a reaction on the temperature, a pre-exponential factor and other constants of the reaction.

\[
K = Ae^{-\frac{E_a}{RT}}
\]

In the equation, \( k \) is the rate constant, \( T \) is the temperature in Kelvin, \( A \) is pre-exponential factor which defines the rate of reaction due to frequency of collisions in the correct orientation, \( E_a \) is the activation energy in Joules and \( R \) is the universal gas constant.

By measuring hydrolysis rates at four different temperatures and plotting the respective rates as \( \ln K \) vs \( 1/T \) (K) it was possible to
determine the $E_a$ (energy of activation) and $A$ (frequency factor) for each compound. $E_a$ is derived from the slope of the plot and the natural log of the frequency factor is equal to the Y-intercept of the $\ln K$ vs $1/T$ ($K$) plot. These values are summarized in Table 2 below. The actual measurements and calculations shown in Supporting Information.

3.4. Enzymatic catalysis

As mentioned above, theoretical studies have been conducted to evaluate the potential for enzymes to catalyze the hydrolysis of Novichok compounds. Hydrolytic enzymes offer the potential of rate accelerations of several orders of magnitude with a relatively very small amount of protein as the catalyst. The activity of these various enzymes on G and V-type agents has been reported in the literature and was recently reviewed (Bigley and Rauschel, 2019). However, to our knowledge there have been no previous reports of enzymatic activity on A230, A232 or A234. Table 3 shows the kinetic constants of the OPAA enzyme with the Novichok compounds as substrates.

4. Conclusion

There has been speculation in the literature about the stability or decontamination of these compounds but to our knowledge no actual data have been published. Nepovimova and Kuca (2018) reference “low stability in the environment” and Franca et al. (2019) speculate about possible methods of decontamination. The known facts of the poisoning incident in Salisbury, U.K. suggest that A234 might be relatively stable in the environment since it was evidently used to intentionally contaminate a doorknob some time prior to the actual poisoning (Peplow, 2018). Here we have shed some light on these issues through a determination of the hydrolysis rates of these agents and a comparison of those rates to other known chemical nerve agents.

### Table 1. Measured rates of hydrolysis at 25 °C in 50 mM bis-tris-propane, pH 7.2.

| Compound | Rate (μM/min) |
|----------|---------------|
| GB       | 6.68          |
| GD       | 4.31          |
| GF       | 6.54          |
| GP       | 5.39          |
| VR       | 0.237         |
| VX       | 0.246         |
| A230     | 0.17          |
| A232     | 0.061         |
| A234     | 0.0032        |

### Table 2. $E_a$ and $A$ values for respective compounds.

| Compound | $E_a$ (kJ/mol) | $A$ (per min) |
|----------|----------------|---------------|
| A230     | 39.28          | $8.22 \times 10^9$ |
| A232     | 47.39          | $8.10 \times 10^7$ |
| A234     | 37.98          | $1.91 \times 10^6$ |

Figure 3. GB (propan-2-yl methylphosphonofluoridate), GD (3-methylbutan-2-yl methylphosphonofluoridate), GF (cyclohexyl methylphosphonofluoridate), GP (2,2-dimethylcyclopentyl methylphosphonofluoridate), VX (N,N-diisopropyl-2-(methyl-(2-ethoxy)phosphoryl)sulfanylenethanamine), VR (N,N-diethyl-2-(methyl-(2-methylpropoxy)phosphoryl)sulfanylenethanamine).

Figure 4. Log scale comparison of nerve agent hydrolysis rates at 1 mM and 25 °C. Novichok and G-series measurements are fluoride electrode data; V-series measurements are absorbance data from Ellman’s reagent assays at 412 nm, converted to molarity with Beer’s Law using the extinction coefficient of 14,150 M$^{-1}$ cm$^{-1}$. All values were normalized to a starting point of 10 μM product for purposes of comparison.
Hydrolysis of the Novichok agents at 25 °C was two to three orders of magnitude slower than the G-series agents and zero to two orders of magnitude slower than the V-series agents. The hydrolysis data should be relevant to the effort to determine the persistence of Novichok agents in the environment or in the body although other factors such as binding to inorganic materials or proteins may play a role.

The catalytic activity of OPAA on the Novichok compounds was approximately two to three orders of magnitude less than its activity on G-type agents and two orders of magnitude greater than its activity on V-type agents (Bae et al., 2018) (Li et al., 2016) (Daczkowski et al., 2015). The stereochemistry of these catalytic reactions still needs to be explored because other nerve agents have been shown to exhibit stereospecific toxicity and the OPAA enzyme has been shown to exhibit stereospecificity in its interaction with other nerve agents. It will also presumably be interesting to investigate the potential these enzymes might have for protein engineering with respect to the activity or stereochemistry of their interactions with Novichok compounds.

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Declarations

Author contribution statement

Steven P Harvey: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Leslie R McMahon: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data.

Frederic J Berg: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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Table 3. Kinetic values of wild-type OPAA enzyme on A230, A232 and A234 at 25 °C in 50 mM bis-tris-propane buffer at pH 7.2. mM = millimolar, $k_{cat} = \text{number of substrate molecules each enzyme site converts to product per unit time}$; min = minute; M = molar.

| Substrate | $K_m$ (mM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (M$^{-1}$ min$^{-1}$) |
|-----------|-------------|------------------------|----------------------------------|
| A230      | 9.12 ± 0.90 | 870 ± 52               | $9.54 \times 10^4$ ± $1.52 \times 10^4$ |
| A232      | 13.1 ± 3.28 | 900 ± 151              | $6.67 \times 10^5$ ± $2.87 \times 10^5$ |
| A234      | 1.57 ± 3.00 | 547 ± 74               | $3.49 \times 10^6$ ± $1.14 \times 10^6$ |