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Stable adducts of nerve agents sarin, soman and cyclosarin with TRIS, TES and related buffer compounds—Characterization by LC-ESI-MS/MS and NMR and implications for analytical chemistry☆,☆☆

Jürgen Gäb[a,b], Harald John[c], Marco Melzer[a,d], Marc-Michael Blum[a,*]

[a] Blum - Scientific Services, Ledererstrasse 23, 80331 Munich, Germany
[b] Institute of Pharmaceutical Chemistry, Philipps University Marburg, 35032 Marburg, Germany
[c] Bundeswehr Institute of Pharmacology and Toxicology, Neuherbergstrasse 11, 80937 Munich, Germany
[d] Institute of Pathology, Johannes Gutenberg University Mainz, 55101 Mainz, Germany

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ABSTRACT

Buffering compounds like TRIS are frequently used in chemical, biochemical and biomedical applications to control pH in solution. One of the prerequisites of a buffer compound, in addition to sufficient buffering capacity and pH stability over time, is its non-reactivity with other constituents of the solution. This is especially important in the field of analytical chemistry where analytes are to be determined quantitatively. Investigating the enzymatic hydrolysis of G-type nerve agents sarin, soman and cyclosarin in buffered solution we have identified stable buffer adducts of TRIS, TES and other buffer compounds with the nerve agents. We identified the molecular structure of these adducts as phosphonic diesters using 1D ^{1}H-^{31}P HSQC NMR and LC-ESI-MS/MS techniques. Reaction rates with TRIS and TES are fast enough to compete with spontaneous hydrolysis in aqueous solution and to yield substantial amounts (up to 20–40%) of buffer adduct over the course of several hours. A reaction mechanism is proposed in which the amino function of the buffer serves as an intramolecular proton acceptor rendering the buffer hydroxyl groups nucleophilic enough for attack on the phosphorus atom of the agents. Results show that similar buffer adducts are formed with a range of hydroxyl and amino function containing buffers including TES, BES, TRIS, BIS-TRIS, BIS-TRIS propane, Tricine, Bicine, HEPES and triethanol amine. It is recommended to use alternative buffers like MOPS, MES and CHES when working with G-type nerve agents especially at higher concentrations and over prolonged times.

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1. Introduction

Organophosphorus nerve agents like sarin (GB, Fig. 1, **1**), soman (GD, Fig. 1, **2**) or cyclosarin (GF, Fig. 1, **3**) pose a continuing threat to military personnel and civilian populations despite being banned under the Chemical Weapons Convention (CWC) [1]. Some non-signatories to the CWC are still believed to have ongoing chemical weapon programs and the sarin attacks by Aum Shinrikyo in Matsumoto in 1994 and on the Tokyo subway in 1995 demonstrated that non-state actors are capable of producing and using nerve agents [2]. In addition to protection and detection technologies, effective means for the rapid decontamination and detoxification of these agents are required [3]. New decontaminants based on the hydrolytic action of enzymes like diisopropyl fluorophosphatase (DFPase) are current subjects of research and development [3–5].

To monitor the enzymatic hydrolysis of nerve agents an array of techniques including pH-stat titration [6], ion sensitive electrodes [7], substrate mimics with fluorogenic leaving groups [8] and a recently introduced application of *in situ* FTIR spectroscopy [9] are

Abbreviations: ACN, acetonitrile; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Bicine, *N*-bis(2-hydroxyethyl)glycine; BIS-TRIS, bis(2-hydroxyethyl)-imino-tris(hydroxymethyl)methane; BIS-TRIS propane, 1,3-bis(tris(hydroxymethyl)methylaminopropane); CHES, *N*-cyclohexyl-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; FA, formic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GB, sarin; GD, soman; GF, cyclosarin; HSQC, heteronuclear single quantum coherence; IMPA, isopropylmethylphosphonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MRM, multiple reaction monitoring mode; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Tricine, *N*-tri(hydroxymethyl)methylglycine; TRIS, tris(hydroxymethyl)aminomethane; TSP, trimethylsilyl-2,2,3,3-tetradeuteropropionic acid; VX, *O*-ethyl-*S*-2-diisopropylamino-ethylmethylphosphonothiolate.

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* Corresponding author. Tel.: +49 151 50401125.
E-mail address: mmblum@blum-scientific.de (M.-M. Blum).

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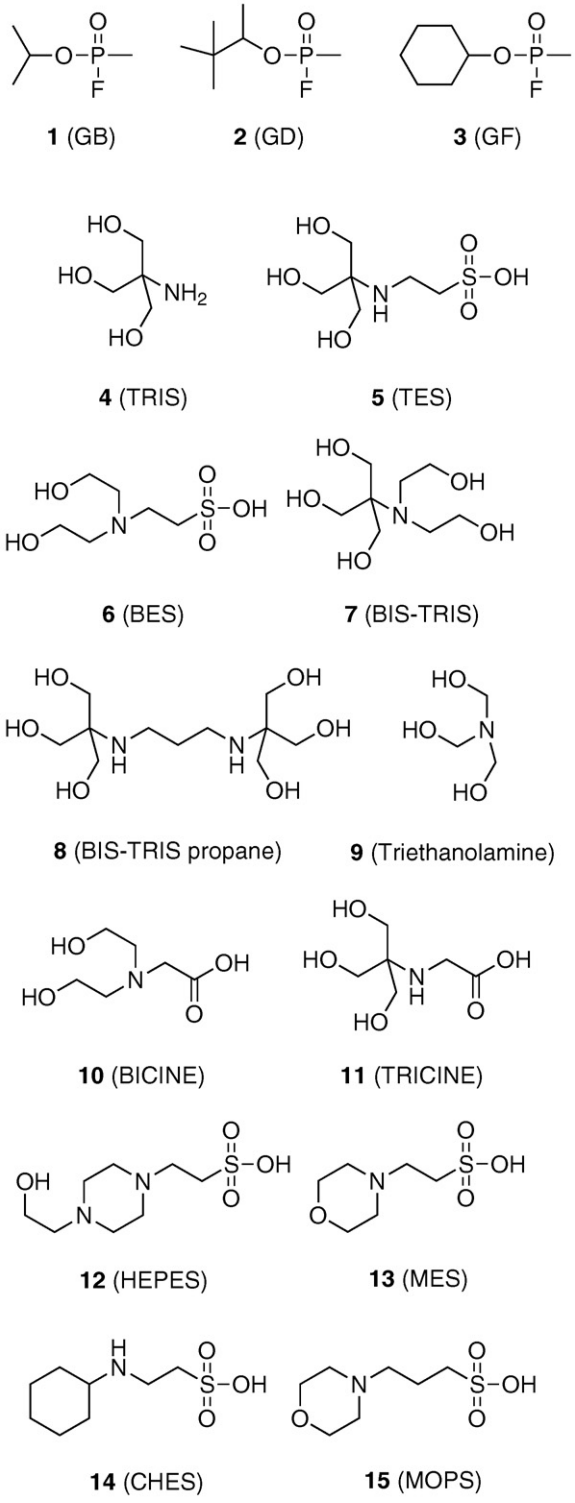

Fig. 1. Chemical structures of the G-type nerve agents sarin (GB), soman (GD) and cyclosarin (GF) and structures of common organic buffer compounds.

employed. The hydrolysis reaction of nerve agents like sarin proceeds via the cleavage of the phosphorus-fluorine bond and leads to the formation of a phosphonic acid, a fluoride anion and two protons. Due to this fact the reaction has to be carried out either by the constant addition of base, like in the pH-stat assay, or in buffered solution. Even though simple inorganic buffers like sodium hydrogen carbonate can be used in technical applications their use in the laboratory is discouraged due to limited pH stability and shelf life.

For use in biochemical and also analytical applications a wide variety of organic buffer compounds is available. The compound tris(hydroxymethyl)aminomethane (TRIS, Fig. 1, **4**, pK_a = 8.1) is among the most used buffers, either alone or in combination with other compounds like EDTA in TAE (TRIS-EDTA) buffer. TRIS is also frequently used during sample isolation and preparation from biological fluids like blood and urine. However, TRIS is not a completely inert buffer substance as it contains a primary amino function that may undergo typical reactions of primary amines. Examples for this are reactions with aldehydes [10] or the recently described reaction with thioester intermediates in intein-mediated protein cleavage [11]. TRIS was also reported to inhibit a number of enzymes [12,13]. A closely related buffer substance used for experiments with the *in situ* FTIR technique is *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES, Fig. 1, **5**, pK_a = 7.5). TES is less problematic than TRIS as it contains a secondary amine, mainly present in its protonated form in the buffering range. It was introduced as one of the "Good"-Buffers by Good et al. [14]. In addition to these two compounds several other organic buffer compounds, which are structurally related to TRIS and TES, are also commonly used. These include BES (**6**), BIS-TRIS (**7**), BIS-TRIS propane (**8**), triethanolamine (**9**), BICINE (**10**), TRICINE (**11**) and HEPES (**12**, see Fig. 1).

We have used some recently described 1D ^{1}H-^{31}P HSQC NMR experiments [15,16] to screen reaction solutions for the organophosphorus compounds present including eventual impurities and side products. The ^{1}H-^{31}P HSQC transfers magnetization from the hydrogen nuclei to the phosphorus, while detection occurs again on the hydrogen. This leads to the disappearance of all proton signals that are more than three bonds away from the phosphorus. Advantages to regular ^{31}P{^{1}H} NMR spectra are the increased sensitivity and information content of the spectra. Unexpectedly, the incubation of GB, GD and GF in TRIS and TES buffered solutions at pH 7.5 led to the formation of unidentified organophosphorus species in large quantities. We report the identification of these compounds as adducts of the nerve agents with the buffer compounds and their characterization by LC-ESI-MS/MS and NMR techniques. In addition to TRIS and TES we were able to show that also structurally related buffers (**6**–**12,** Fig. 1) form adducts if they contain a hydroxyl group in close proximity to an amine function. To the knowledge of the authors this is the first report on adduct formation of commonly used organic buffer compounds with organophosphorus nerve agents. We identified the reaction products as "*O*-adducts" bound to the phosphorus atom via hydroxyl oxygen of the buffer instead of "*N*-adducts" bound via the nitrogen atom of the buffer compound. A reaction mechanism is proposed in which the amine group of the buffer acts as an intramolecular proton acceptor increasing the nucleophilicity of the hydroxyl group. Finally, recommendations for the substitution of TRIS, TES and other hydroxyl and amine group bearing buffers by alternative compounds for work with nerve agents are given.

2. Materials and methods

2.1. Materials

Ethylenediamine (≥99%), 1,1,1-tris(hydroxymethyl) ethane (99%), *N*,*N*-dimethylethanolamine (≥99.5%) and ethanolamine (≥98.5%) and triethanolamine (≥99%) were obtained from Sigma–Aldrich (Taufkirchen, Germany). Anhydrous acetonitrile (ACN, ≥99.5%, stored over molecular sieves) was obtained from Fluka via Sigma–Aldrich. D_2O (99%), tris(hydroxymethyl) aminomethane (TRIS, ≥99.9%), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES, ≥99%), *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES, ≥99%), bis(2-hydroxyethyl)-imino-tris(hydroxymethyl)methane (BIS-TRIS, ≥99%),

1,3-bis(tris(hydroxymethyl)methylaminopropane) (BIS-TRIS propane, ≥98%), *N*-bis(2-hydroxyethyl)glycine (BICINE, ≥98%), *N*-tri(hydroxymethyl)methylglycine (TRICINE, ≥99%), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, ≥99.5%), 2-(*N*-morpholino)ethanesulfonic acid (MES, ≥99%), *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES, ≥99%), 3-(*N*-morpholino) propanesulfonic acid (MOPS, ≥99%), and Schott Economic NMR sample tubes were from Roth (Karlsruhe, Germany).

Acetonitrile (gradient grade) and formic acid (FA, Uvasol) for LC-MS/MS experiments were purchased from Merck (Darmstadt, Germany).

Chemical warfare agents sarin (GB, *O*-isopropyl methylphosphonofluoridate, CAS 107-44-8), soman (GD, *O*-pinacolyl methylphosphonofluoridate, CAS 96-64-0), cyclohexylsarin (GF, *O*-cyclohexyl methylphosphonofluoridate, CAS 329-99-7) and VX (*O*-ethyl-*S*-2-diisopropylamino ethylmethylphosphonothiolate, CAS 50782-69-9) were supplied by the German Ministry of Defense. Purity of the agents was >95% as determined by NMR.

2.2. NMR spectroscopy

All NMR measurements were conducted on a Bruker Avance III Ultrashield 400 MHz NMR-spectrometer equipped with a 5 mm inverse Z-gradient broad-band probe head at 298 K. Topspin (Bruker) was used for data processing and analysis. Unless stated otherwise all spectra were collected without X nucleus decoupling to easily assign the protons coupling to phosphorus and to prevent sample heating during the measurement. The proton pulse length was calibrated by the 360° pulse method whereas the phosphorus pulse was calibrated through optimization in the 1D ^{1}H-^{31}P HSQC experiment. For all experiments the 90° pulse length for the ^{1}H pulse was set to 9.4 μs and to 8.5 μs for the phosphorus. The frequency offset for the proton pulse was set to 4 ppm and 32 ppm for the phosphorus pulse and a spectral width of 12 ppm was applied. The used pulse sequence for the 1D ^{1}H-^{31}P HSQC experiment was the one published by Koskela et al. [15].

All samples were shimmed by automated gradient shimming and automatically tuned. Spectra were weighted with an exponential function and a line broadening of 0.1 Hz was applied.

2.3. LC-ESI-MS/MS

The binary LC system (two pumps, autosampler, column oven and controller, all PE 200 series, PerkinElmer, Rodgau-Jügesheim, Germany) was coupled to an electrospray ionization triple quadrupole mass spectrometer (API 4000 QTrap, Applied Biosystems, Darmstadt, Germany) controlled by the accompanying software Analyst 1.4.2. Chromatographic separations were performed on Atlantis T3, 5 μm, 150 mm × 2.1 mm I.D. (Waters, Eschborn, Germany) using 0.1% (v/v) formic acid as eluent A and acetonitrile–water (80:20, v/v) with 0.1% (v/v) formic acid as eluent B with a flow rate of 200 μl/min at 30 °C. Preparations containing the TRIS-adducts were analyzed in gradient mode injecting a 100 μl sample volume. The following gradient was used for GB-TRIS, time [min]/B[%]: 0/8; 7/50; 8/80; 11/80; 12/8; 13/8, whereas a slightly different gradient was applied to the less polar GF-TRIS, time [min]/B[%]: 0/8; 7/60; 8/90; 14/90; 15/8; 17/8.

Mass spectrometric detection following positive electrospray ionization was carried out in the product ion scan mode as well as in the multiple reaction monitoring (MRM) mode using nitrogen as collision and spray gas. The following parameter settings were applied for both methods: curtain gas 15 psi, ionization spray voltage 3000 V, entrance potential 10 V, temperature 300 °C, heater gas (GS1) 30 psi, and turbo ion spray gas (GS2) 30 psi.

In product ion scan mode only TRIS-adducts were detected using the precursor ions at *m*/*z* 242.3 of GB-TRIS and *m*/*z* 282.4 of GF-TRIS applying 30 V collision energy (CE), 66 V declustering potential (DP) and 10 V cell exit potential (CXP). Fragment spectra were extracted from chromatographic peaks.

For simultaneous detection of adducts, corresponding nerve agents and TRIS analysis was performed in MRM mode using transitions to the most abundant product ions: GB-TRIS (242.3 → 104.3); GF-TRIS (282.4 → 104.3); GB (141.0 → 99.1; DP 26 V, CE 13 V, CXP 4 V), GF (180.9 → 99.1; DP 31 V, CE 13 V, CXP 4 V), and TRIS (122 → 56.2, CE 24 V). Dwell time was set to 50 ms for each transition and additional parameters were used as described above.

2.4. Sample preparation

Samples obtained from studies on the fate of GB and GF stored in TES and TRIS buffer at room temperature were prepared to be analyzed by NMR as well as LC-MS/MS techniques.

2.4.1. NMR sample preparation

All NMR samples contained 140 μl of D_2O in a total volume of 700 μl to obtain a lock signal in the experiments. The amount of TRIS or TES was varied in the range from 50 mM to 300 mM by adding 35 μL to 210 μL of a 1 M buffer stock solution (pH 7.5) to the samples. For a final concentration of 0.1 vol% nerve agent samples were spiked with 70 μl of the stock solution (1 vol% in ACN) respectively and the samples were added up to 700 μl with H_2O. To elucidate the impact of buffer concentration and the pH dependency, the reactions were followed at 298 K by recording 6 1D ^{1}H-^{31}P HSQC spectra during the first 30 min. Peak area of nerve agents, phosphonate hydrolysis products and buffer adducts was plotted versus reaction time to obtain a concentration–time profile.

2.4.2. LC-ESI-MS/MS sample preparation

For the preparation of buffer adducts in large amounts for LC-ESI-MS/MS studies samples contained 200 mM TRIS adjusted to pH 7.5 and 0.1% (v/v) of nerve agent (GB 7.1 mM, GF 5.5 mM). The samples were allowed to react at room temperature for 48 h and were subsequently checked for complete nerve agent degradation by NMR spectroscopy. Subsequently, samples were diluted in two steps: First the sample was diluted with HPLC eluent A to obtain a concentration of approximately 2.5 μM of the adduct (total dilution 1:1000). Second, 10 μl of this solution and 10 μl of a nerve agent solution in eluent A (GB 71 nM, GF 55 nM) were mixed and diluted subsequently with 0, 50 or 100 μl of HPLC eluent B and added up to 1000 μl with eluent A to obtain final solutions containing 0 vol%, 5 vol% or 10 vol% of eluent B (0 vol%, 4 vol% and 8 vol% ACN). The concentration of TRIS, nerve agents and the adducts was 2 μM, 5–7 nM and 25 nM, respectively.

For the preparation of the GF-TRIS adduct at low agent concentrations the GF stock solution (1 vol%, 54 mM) was diluted twice 1:50 with ACN by mixing 20 μl of the respective solution with 980 μl ACN. The resulting solution had a GF concentration of 21.6 μM. 20 μl of this solution were mixed with 50 μl of a 1 M TRIS solution (pH 7.5) and 930 μl H_2O (final GF concentration 432 nM). The reaction mixture was incubated for 6 h at RT. Afterwards 100 μl of the mixture were diluted 1:10 by the addition of 900 μl HPLC eluent A. To adjust the pH of the solution to pH 3–4, 3 μl of formic acid were added.

3. Results and discussion

3.1. Identification of sarin and cyclosarin adducts with TRIS and TES buffer by 1D ^{1}H-^{31}P HSQC NMR

After a few minutes of incubation of a sample of 0.1 vol% (7.1 mM) GB in 100 mM TES (pH 7.5) a 1D ^{1}H-^{31}P HSQC NMR

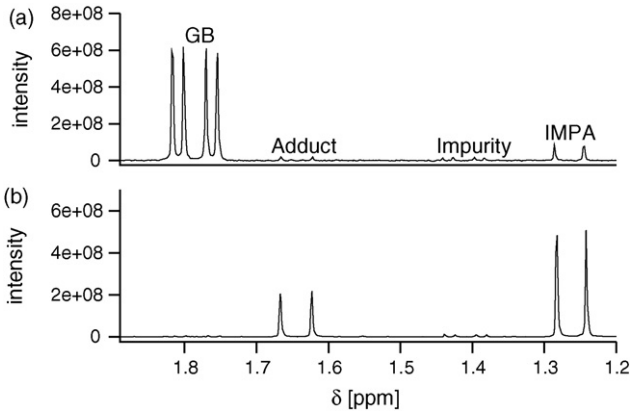

Fig. 2. 1D ^{1}H-^{31}P HSQC NMR signals of protons in the phosphorus bound methyl group of GB, the hydrolysis product IMPA, the GB-TES buffer adduct and an unidentified impurity after (a) 10 min of incubation and (b) 48 h of incubation.

spectrum revealed the unexpected appearance of an unidentified doublet (d) at 1.65 ppm, J_{HP} = 17.6 Hz (Fig. 2a) in addition to the expected doublet of isopropyl methylphosphonic acid (IMPA) (δ = 1.27 ppm, J_{HP} = 16 Hz), the hydrolysis product of GB, and the doublet of doublets (dd) of GB (δ = 1.79 ppm, J_{HP} = 19.0 Hz, J_{HF} = 6.4 Hz). Both IMPA and GB were identified via reference spectra using trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) as the internal reference standard. After 48 h the signal for GB had completely vanished while the signal of the unidentified compound had grown significantly and remained stable with a signal peak area ratio of nearly 1:2 compared to the phosphonate IMPA signal indicating a 33% yield under these conditions (Fig. 2b). An aqueous sample of GB without buffer compounds displayed only the appearance of IMPA but not of the unidentified signal at 1.65 ppm upon incubation. The fact that the aqueous sample only contained TES, GB, and ACN and the appearance of a signal in the 1D ^{1}H-^{31}P HSQC spectra with a coupling constant of 17.6 Hz at 1.65 ppm (typical for protons with $^{2}J_{HP}$ coupling to phosphorus) are indicative of the formation of a covalent adduct between the nerve agent and the buffer.

Inspection of the structural features of the buffer substance revealed three possible types of nucleophilic groups: a sulfonate, a secondary amine and three primary alcohols (Fig. 3, **5**). Performing an analogous reaction with TRIS as the buffering substance also led to the formation of a potential adduct that gave rise to the same NMR signal with almost the same chemical shift. Thus, the sulfonate was excluded as the possible nucleophile since the structure of TRIS contains no sulfonate group (Fig. 3, **4**) and sulfonates are also known to be very poor nucleophiles. Only the hydroxyl group and the amine remained as possible nucleophiles for the attack on the nerve agents. Greenhalgh and Weinberger showed in the 1960s that GB reacts with ethanolamine to yield exclusively the *O*-phosphonylation product, while the phosporochloridate analogue chlorosarin yields mainly the *N*-phosphonylation product [17]. However, the relevance of these findings for the present study is limited because these reactions had been carried out in a water-free medium (i.e., chloroform in the presence of an excess of triethylamine).

If the buffer would act as an oxygen nucleophile the formed adduct would be a phosphonic acid diester (Fig. 3, **17a**). The fact that no doublet for the methylene group adjacent to the phosphorus bound oxygen and the quaternary carbon of the buffer moiety was observed and the observation that the reaction with TES was much faster than with TRIS could be seen as arguments in favour of the buffer acting as a nitrogen nucleophile leading to the formation of a phosphonoamidate (Fig. 3, **17b**). The difference in reaction rates could be explained by the different pK_a values for TRIS and TES and the resulting different concentrations of free base at pH 7.5. TRIS exhibits a pK_a of 8.1 and TES a pK_a of 7.4. Therefore, the amount of free base is higher for TES than for TRIS, which would result in higher reaction rates.

Additional nerve agents of the G-type (GD and GF) were tested and they also led to the formation of NMR signals indicative of adduct formation. Even with VX, a V-type nerve agent with a thiol leaving group, which is known to hydrolyze only slowly in aqueous solution, an adduct was formed although the appearance of the NMR signal was significantly slowed down because of the lower

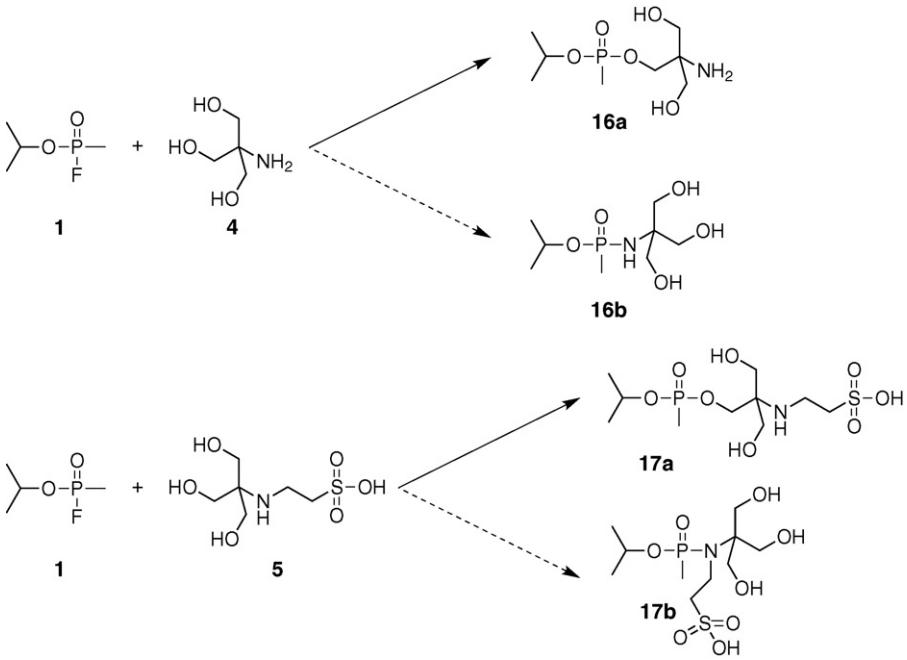

Fig. 3. Scheme of the reaction of GB (**1**) with TRIS (**4**) and TES (**5**) yielding either the *O*-bound ester adducts (**16a** and **17a**) or the *N*-bound phosphonoamidates (**16b** and **17b**). As shown by NMR and LC-MS only the *O*-bound ester adduct is found experimentally.

Table 1
Formation of buffer-adducts as determined by NMR between nerve agents GB, GD, GF, VX and organic buffer compounds.+, adduct formation; (+), slow adduct formation; 0, no adduct formation; n.d., not determined.

	GB	GD	GF	VX
TRIS (**4**)	+	+	+	(+)
TES (**5**)	+	+	+	(+)
BES (**6**)	+	+	+	n.d.
BIS-TRIS (**7**)	+	+	+	n.d.
BIS-TRIS propane (**8**)	+	+	+	n.d.
Triethanolamine (**9**)	+	+	+	n.d.
BICINE (**10**)	+	+	+	n.d.
TRICINE (**11**)	+	+	+	n.d.
HEPES (**12**)	+	+	+	n.d.
MES (**13**)	0	0	0	n.d.
CHES (**14**)	0	0	0	n.d.
MOPS (**15**)	0	0	0	0

reactivity of this class of nerve agents against nucleophiles. Finally a group of buffers structurally related to TRIS and TES (**6**–**12,** see Fig. 1) were tested for adduct formation with GB, GD and GF and all of them tested positive (Table 1). The stability of the formed adducts was checked by regular NMR measurements of the reaction solutions up to two weeks. No adduct degradation was observed. Also three buffers without free hydroxyl groups (**13**–**15**, see Fig. 1) were tested and no adduct formation could be detected over a time of up to 8 h.

3.2. Dependency of the formation of adducts on buffer concentration, pH and functional groups

To gain a deeper understanding of the molecular structure of the buffer adducts and the potential reaction mechanism leading to their formation GB was incubated with different concentrations of TES ranging from 50 mM to 300 mM. The reaction was followed by recording 6 1D ^{1}H-^{31}P HSQC spectra within a reaction time of 30 min. The results (Fig. 4a) indicate a significant dependency of the adduct formation on the buffer concentration with higher reaction rates at higher buffer concentrations. In a similar experiment the incubation of GB with TES was repeated at different pH. The pH of a 100 mM TES solution was adjusted near the pK_a of TES to values of 7.0, 7.5 and 8.0, so that a sufficient buffer capacity was maintained (Fig. 4b). The changes in pH led to a change in the ratio of non-protonated versus protonated amino group in TES from approximately 1:3 at pH 7.0 to approximately 3:1 at pH 8.0. While the reaction was comparatively slow at pH 7.0 (low amount of free amine) the reaction was significantly accelerated at pH 8.0 (high amount of free amine).

To test for the requirement of functional groups in the buffer compound the incubation of GB was repeated with 1,1,1-tris(hydroxymethyl)ethane (Fig. 5, **18**) and ethylene diamine (Fig. 5, **19**) bearing only one of the two potential nucleophilic groups: hydroxyl or amine. Compound **18** was buffered with 100 mM MOPS (a buffer lacking hydroxyl groups) as protons would be released during the course of the reaction and **18** lacks a protonable functional group. Both compounds were incubated with GB at pH 7.5 and at pH 8.5 but the recorded 1D ^{1}H-^{31}P HSQC NMR spectra did not reveal the formation of any kind of adduct (Fig. 5). Based on these results it seems to be a requirement for adduct formation that a hydroxyl group is present in close proximity to a basic amine function in the buffer molecule.

3.3. Characterization of the GB-TRIS and GF-TRIS adducts by LC-ESI-MS/MS

To separate the buffer adducts of GB and GF from other components in the reaction mixture and to obtain fragmentation mass

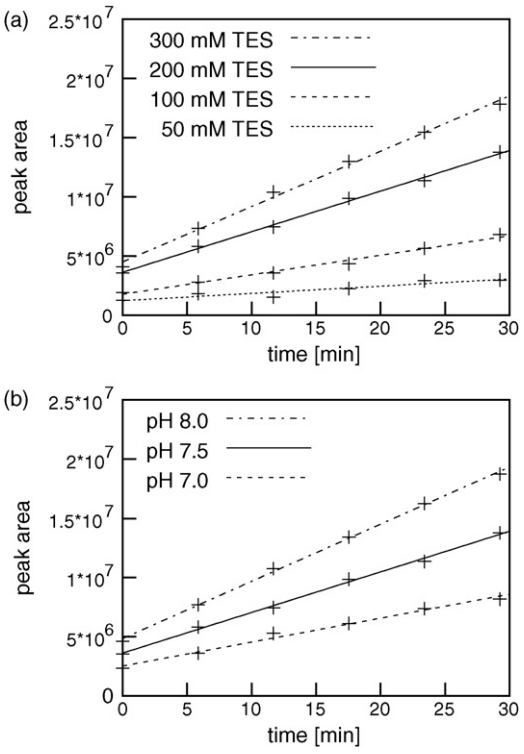

Fig. 4. Production of GB-TES adducts at different buffer concentrations and pH (a) Plot of the peak area of the phosphorus bound methyl group protons of the adduct versus reaction time at different concentrations of TES buffer showing a clear dependency of the rate of adduct formation on the buffer concentration. (b) Same plot as in (a) but at constant buffer concentration and different values of pH indicating faster reaction rates at higher pH.

spectra, liquid chromatography (LC) was used in combination with ESI-MS/MS. Due to the simpler structure of TRIS and easier generation of positively charged ions caused by the lack of a sulfonate group, the experiments were conducted with this buffer compound. A sample of GB incubated with 200 mM TRIS (pH 7.5) was checked by NMR for complete reaction after 48 h. After this time the peak area ratio for the methyl group protons bound to the phosphorus atom of the hydrolytic degradation product and the adduct was determined in a regular ^{1}H NMR experiment showing a 2:3 ratio, indicating a synthetic yield of 40% of adduct (approx. 2.5 mM). The reaction mixture was diluted without further purification with eluent A – eluent B (90:10, v/v) to a final adduct concentration of approximately 25 nM and an ACN concentration of 8 vol%. The diluted sample was spiked with 7 nM of nerve agent. This mixture containing agent, buffer and buffer adduct was separated by LC and the detection of analytes was achieved by ESI-MS/MS. The overlaid chromatograms of the multiple reaction monitoring (Fig. 6a) of

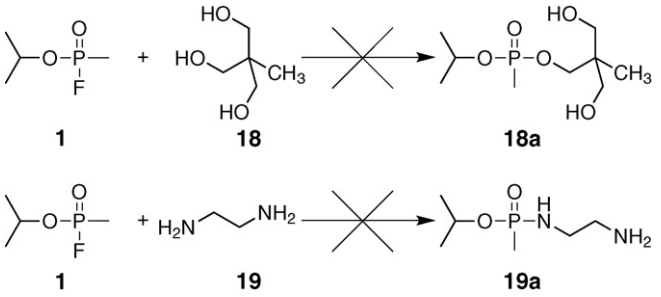

Fig. 5. Scheme of potential reactions of GB (**1**) with 1,1,1-tris(hydroxymethyl) ethane (**18**) and ethylenediamine (**19**). None of the products (**18a** and **19a**) was experimentally observed.

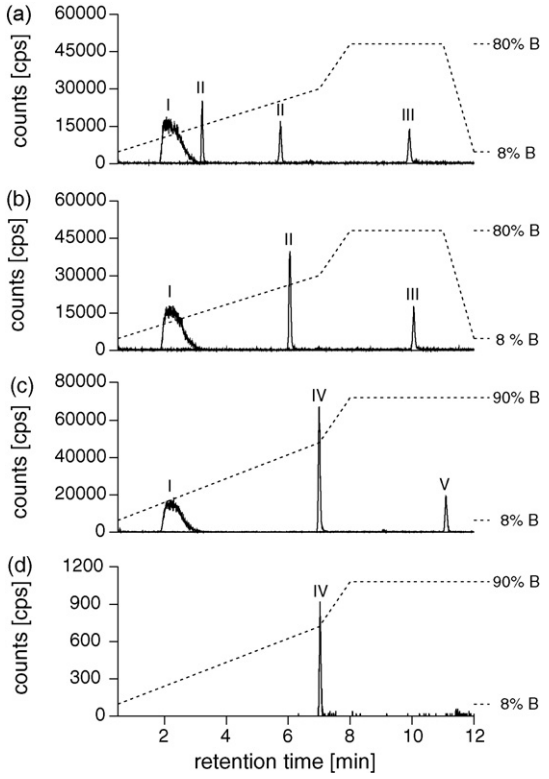

Fig. 6. LC-ESI-MS/MS analysis of nerve agent adducts with TRIS. (a) Overlay of chromatograms from MRM of the separation of GB-TRIS from a sample with surplus TRIS that was spiked with GB. ACN concentration in the sample was 8%. (b) Same chromatograms as in (a) but sample matrix only contained 4% of ACN. (c) Overlay of chromatograms from MRM of the separation of GF-TRIS from a sample with surplus TRIS that was spiked with GF. (d) Single MRM trace for the GF-TRIS adduct after incubation of very low concentrations of GF (trace for TRIS omitted due to much higher intensity). Roman numbering indicates the following compounds: **I** TRIS, **II** GB-TRIS adduct, **III** GB, **IV** GF-TRIS adduct, **V** GF. The dashed line indicates the solvent gradient used in % of eluent B. Compounds were detected in the multiple reaction monitoring mode using the following transitions: TRIS (122 → 56.2); GB-TRIS (242.3 → 104.3); GF-TRIS (282.4 → 104.3); GB (141.0 → 99.1), GF (180.9 → 99.1). Separation was performed on Atlantis T3, 5 μm, 150 mm × 2.1 mm I.D. at 200 μl/min and 30 °C.

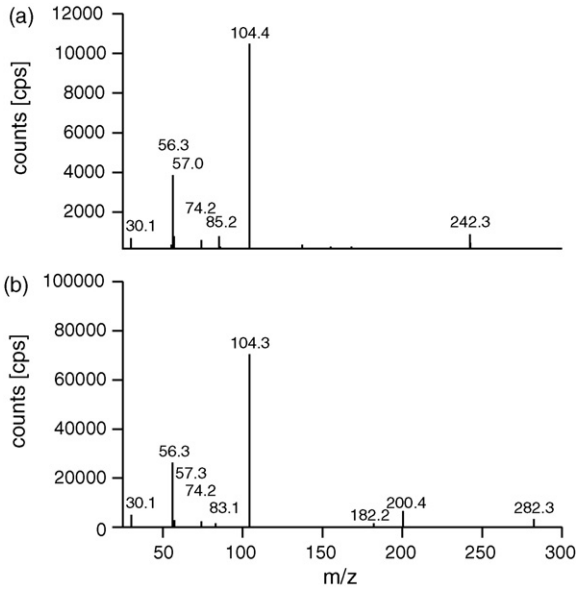

Fig. 7. MS/MS spectrum of (a) GB-TRIS and (b) GF-TRIS. Structures of molecular fragments assigned to the peaks in the spectrum can be found in Fig. 8. Spectra were extracted from LC-ESI(+)-MS/MS runs in the product ion scan mode, monitoring fragments of *m*/*z* 242.3 for GB-TRIS and 282.3 for GF-TRIS.

this separation showed an unexpected phenomenon. After about 2 min a typical broad TRIS peak appeared, which is only weakly retained by the column due to the polar groups of TRIS and the positive charge on the nitrogen under the experimental conditions. At the retention time (t_R) 9.9 min a sharp peak identified as GB was detected. Two additional peaks corresponding to the GB-TRIS adduct were detected at t_R 3.2 min and t_R 5.7 min exhibiting identical fragmentation patterns in product ion scan mode. These results were initially explained by the existence of both proposed GB-TRIS isomers (*N*- and *O*-bonded adducts of TRIS). The first peak was assigned to the more polar ester (Fig. 3, **16a**) bearing a positive charge due to the protonation of the free amine. The second peak was assigned to the phosphonoamidate (Fig. 3, **16b**) bearing no charge and therefore being less polar. The chromatography was reproducible but this observation was in marked contrast to the NMR results where only one adduct was observed. In contrast to these chromatographic results the adduct of TRIS with GF only revealed one peak for the GF-TRIS adduct (t_R 7.0 min) (Fig. 6c). This was in agreement with the NMR results showing only one possible adduct. Therefore, we assumed that the first peak of the GB-TRIS adduct, which was close to the solvent front, was due to a possible matrix effect and samples were diluted into solvents of lower ACN content (0% and 4%, v/v). Corresponding chromatograms revealed only a single peak at t_R 6.1 min (Fig. 6b) exhibiting a peak area identical to the sum of the former two peaks (Fig. 6a). These results verified the proposed phenomenon of a matrix effect and confirmed the existence of only a single buffer adduct (Fig. 6b). However, occurrence of this matrix effect was quite unexpected as sample matrix and eluent composition were of very similar ACN content (8% and 6.4%).

The assignment of the different peaks in the MS/MS spectra of GB-TRIS (Fig. 7a) and GF-TRIS (Fig. 7b) is shown in Fig. 8. It should be noted however that the *m*/*z* values of the molecular ions of GB-TRIS (*m*/*z* 242) and GF-TRIS (*m*/*z* 282) would be identical for both the *O*-bound and the *N*-bound buffer type. The two spectra are very similar in the lower mass range (≤*m*/*z* 104) with identical mass fragment at *m*/*z* 104, 74, 57, 56 and 30. These peaks are assigned to fragments from the TRIS moiety as they were also obtained from MS fragmentation of protonated TRIS molecules. In the higher mass range the most apparent difference between GB-TRIS and GF-TRIS apart from the different molecular ions are the appearance of two additional fragments at *m*/*z* 200 and 182. These fragments serve as a further indication for the existence of the *O*-bound ester adduct of TRIS. The fragment at *m*/*z* 182 was assigned to the adduct after the loss of the cyclohexyl ring (leading to the fragment at *m*/*z* 200) and of H_2O. The loss of H_2O should occur in the TRIS moiety. This fragmentation pathway must proceed via cyclization between the amine and the methylene group of one hydroxymethyl side chain resulting in an aziridine structure. Such aziridine structures were also reported in ESI-MS/MS spectra of phosphoethanolamine species [18]. Aziridine formation can take place only if a free nucleophilic amine is available. This is the case for the oxygen bound adduct but not for the phosphonoamidate. However, the corresponding signal is not observed for GB-TRIS adduct. Therefore additional evidence for the structure of the buffer adducts was required.

3.4. Final identification of the adduct structure by NMR

Results from the LC-ESI-MS/MS study were indicative of the oxygen bound type of adduct but a final proof was still missing. If the proposed structure of the adduct is correct, NMR signals for the two protons of the methylene group adjacent to the phosphorus bound oxygen and the quaternary carbon of the buffer moiety should be

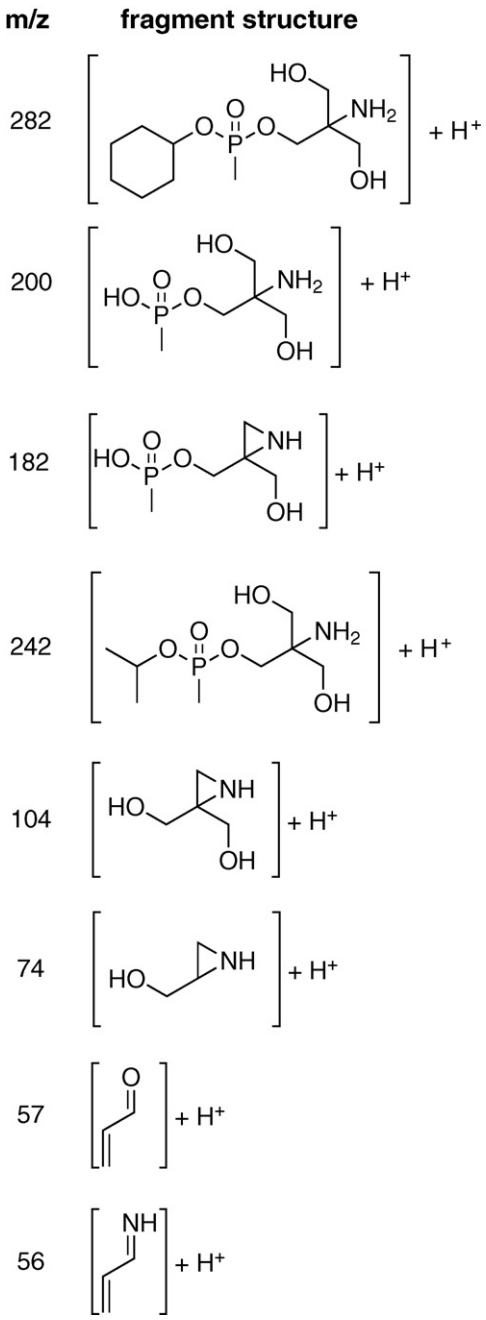

Fig. 8. Assignments of molecular structures of fragments identified in the MS/MS spectra of GB-TRIS (Fig. 7a) and GF-TRIS (Fig. 7b).

visible in NMR experiments. These NMR signals should show a $^3J_{HP}$ coupling to the phosphorus and should therefore appear in a HSQC spectrum while signals were obscured in a classical ^{1}H NMR experiment due to overlapping background signals and could not be assigned. The J_{HP} constant in the 1D ^{1}H-^{31}P HSQC NMR experiment was tuned and varied between 2 Hz and 18 Hz. At J_{HP} = 6.25 Hz three unidentified multiplet (m) signals in the range from 3.8 ppm to 4.8 ppm were visible (Fig. 9a). To assign these signals reference samples containing only IMPA or GB were prepared and reference spectra were recorded. The three spectra were aligned and are depicted in Fig. 9. No GB signals are visible in the NMR spectrum of the sample containing the adduct showing a complete reaction. The IMPA signal at 4.43 ppm was assigned to the C**H**$(CH_3)_2$ proton of the isopropyl group and the multiplet signal at 4.72 ppm was assigned to the C**H**$(CH_3)_2$ proton of the adduct as it has the same shape as the C**H**$(CH_3)_2$ signal in IMPA and is only slightly shifted.

The unidentified signal at 4.06 ppm appeared in the apparent shape of a doublet of quartets. The signal was also identified in a conventional ^{1}H experiment and integrated for 2 protons. This is the expected value for the methylene group. Still the question remains why the signal shows a complex multiplet instead of a simple doublet. The explanation is that the two methylene protons are anisochronous leading to a coupling with each other. Consequently the signal does not appear as a doublet of quartets as it seemed on a first visual inspection but a set of two doublets of doublets with one coupling between the geminal protons ($^2J_{HH}$ = 10.4 Hz) and one to the phosphorus atom ($^3J_{HP}$ = 4.76/5.04 Hz) as known from ABX systems. This kind of coupling has been described in the literature for diethyl phosphonates and has been reviewed [19,20]. To test this assumption a phosphorus decoupled NMR experiment was carried out, which resulted in two doublets as expected. The residual coupling constant for the two unequal protons is $^2J_{HH}$ = 10.4 Hz, which is in the typical range for geminal coupling constants of hydrogen atoms on saturated carbon. This is the required evidence to identify the buffer adduct as the oxygen bound species (**16a** and **17a**) as this kind of signal would not be visible in the nitrogen bound phosphonoamidate.

With the structure of the buffer adduct known it has to be explained why there is no reaction between 1,1,1-tris(hydroxymethyl) ethane and the nerve agents even at the same pH as in the experiments with the buffers and how a plausible mechanism for the reaction would look like. Weinberger et al. [21] demonstrated that the reaction of GB with methanol in the presence of amines (yielding methyl isopropyl methylphosphonate) in an inert organic solvent proceeds via nucleophilic attack of the hydroxyl group on the phosphorus with the amines acting as a general base, facilitating the removal of the hydroxyl proton in the transition state. In the present case, we propose the amine to function as an intramolecular proton acceptor receiving the proton from a hydroxyl group via the formation of an intramolecular five membered ring structure as shown in Fig. 10. This kind of arrangement would significantly increase the nucleophilicity of the oxygen atom allowing a nucleophilic attack on the phosphorus atom of the nerve agent with subsequent release of the leaving group and formation of the buffer adduct. This would explain the strong pH dependency of the reaction in the region of the pK_a of the buffer substance, as a protonated buffer species would no longer be able to efficiently act as a nucleophile. To verify this conclusion we tested the reaction of GB with *N*,*N*-dimethylethanolamine, ethanolamine and triethanolamine and all of these compounds resulted in adduct formation supporting our proposed mechanism.

3.5. Detection of adduct formation at low agent concentration by LC-ESI-MS/MS

Even though the agent concentrations employed in the experiments that lead to the identification of the buffer adducts are realistic for decontamination trials with enzymes, they are unrealistically high for what could be expected in biomedical samples (with the exception of skin samples that were directly exposed to the agent). Therefore, we conducted qualitative experiments to check for adduct formation at significantly lower agent concentration. The agent concentration employed was 432 nM. As the LOD of the NMR method [15,16] and also of the FTIR method [9] is in the mid to low micromolar range, LC-ESI-MS/MS is the method of choice to detect buffer adducts at this concentrations. As an exemplary example the agent GF and the TRIS buffer were chosen. The incubation of 432 nM GF in 50 mM TRIS (pH 7.5) at room temperature for 6 h and subsequent LC-ESI-MS/MS analysis of the reaction mixture did unequivocally reveal the formation of the adduct even

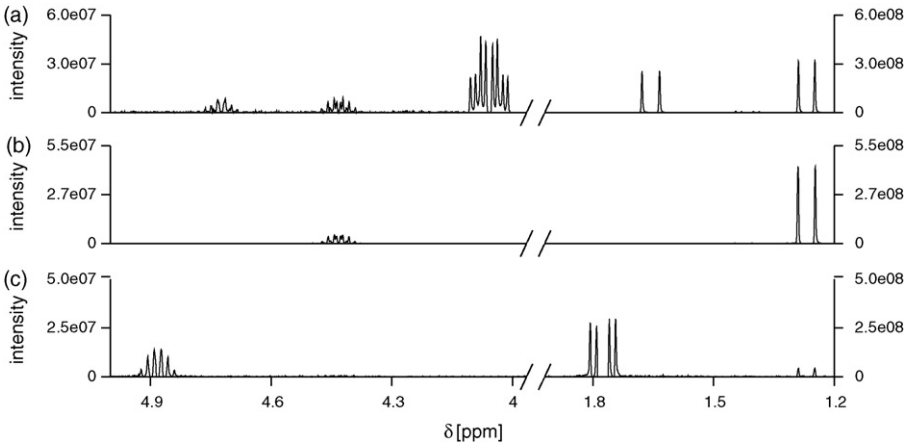

Fig. 9. ^{1}H-^{31}P HSQC spectra (signals >4 ppm are zoomed by a factor of 10 for better visibility) of (a) a reaction mixture of GB and TRIS after complete degradation of GB containing the GB-TRIS adduct and isopropyl methylphosphonic acid (IMPA) (b) a reference spectrum of IMPA (c) a reference spectrum of GB. The complex signal at 4.06 ppm in (a) is only found in the spectrum containing the buffer adduct but not in the reference spectra of IMPA and GB. This signal is assigned to the two methylene protons in the *O*-bound ester type GB-TRIS adduct adjacent to the phosphorus bound oxygen and the quaternary carbon of the buffer moiety. The complexity of the signal arises due to the fact that the two protons are anisochronous and thus give rise to geminal coupling.

at this low agent concentrations as can be seen in the overlaid chromatograms of the MRM (Fig. 6d). We conclude that the formation of buffer adducts is a relevant phenomenon even at lower agent concentrations.

3.6. Implications of buffer adduct formation for analytical chemistry

Previous studies on the stability of G-type nerve agents in aqueous solution revealed that hydrolysis is slowest in the pH range from 4–6 [22]. Also, lowering the temperature of the solution below room temperature slows down the rate of hydrolysis [23]. On the other hand, the presence of nucleophiles can lead to significantly increased degradation rates. This is used for detoxification using the hypochlorite ion at high pH [24], but the presence of nucleophilic compounds can also lead to faster agent degradation in the lower pH range. This was demonstrated with the acetate anion in acetate buffer at pH 4.5 [25]. As outlined above, the formation of buffer adducts is competing with agent hydrolysis in solution yielding significant amounts of adduct, but hydrolysis remains the major pathway for agent degradation. We would argue that problems related with the use of reactive buffers would arise less with solutions where residual agent concentrations should be determined as one would try to reduce hydrolysis by adjusting pH to an appropriate range, minimizing not only hydrolysis but also buffer adduct formation. We see problems rather in analytical approaches where the total initial agent concentration should be determined by assuring complete hydrolysis of remaining agent and subsequent determination of the total concentration of the primary hydrolysis products. With the reaction half-time of the rather stable nerve agent GD at physiological pH of 7.4 being 6.6 h at 27 °C and 4.8 h at 37 °C [23] one would have to use rather long incubations times. If this incubation would happen in reactive buffers a significant amount of the residual agent would react to form the buffer-adducts and escape detection as the phosphonate anion.

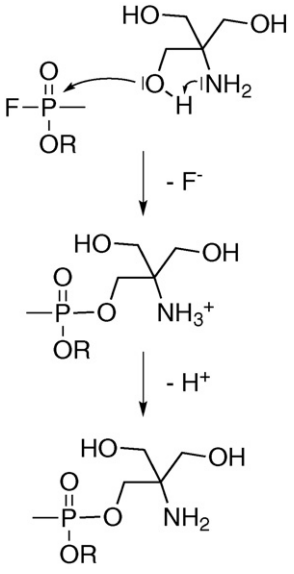

Fig. 10. Scheme of the proposed nucleophilic attack of a TRIS hydroxyl oxygen atom on the phosphorus atom of a nerve agent molecule. The TRIS amino group functions as an intramolecular base, accepting a proton from the hydroxyl group and rendering the oxygen atom more nucleophilic. A fluoride ion is released and the proton transferred from a hydroxyl group to the amine group can be abstracted by a solvent or buffer molecule depending on pH.

4. Conclusion

Nerve agents sarin and cyclosarin react with buffer compounds TRIS and TES to form stable buffer adducts. The same kind of adduct was also detected with soman and we observed buffer adducts with a variety of frequently used buffer compounds. It seems justified to generalize the result, so that agents with a fluorine leaving group undergo quite fast reactions with buffer compounds that contain hydroxyl groups with a neighboring nitrogen moiety functioning as a proton acceptor group. Agents like VX also yield buffer adducts but at much lower reaction rates. An example for a suitable alternative buffer with a pK_a close to physiological pH that does not react in this way is MOPS (pK_a, 7.2). A suitable buffer at slightly acidic pH is MES (pK_a, 6.15) and for mildly basic pH CHES (pK_a, 9.3) (Table 1).

It was clearly shown that TRIS, TES and a number of structurally related buffer compounds are “non-innocent” (as TRIS was recently termed in another investigation where it acted as a reactive component [11]) but reactive buffers when working with nerve agents in aqueous solution. It is advised to select these buffers carefully with respect to reaction conditions (time, temperature, pH) or preferably choose a non-reactive alternative. In case of our recently described work on *in situ* FTIR spectroscopy [9] the enzymatic

activities determined are still valid as the formation of the buffer adducts is subtracted from the enzymatic hydrolysis and is subsumed under spontaneous hydrolysis in aqueous solution. A more severe scenario would be the quantification of agents in samples where TRIS, TES or similar compounds would be added during sample preparation. Here significant deviations from the real values are possible not only when measuring residual agent concentration but also when quantitative conversion to the primary hydrolysis products is expected. This is especially true when incubation times in the buffer are long and buffer concentrations are high.

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