Catalytic Recruitment in the Inactivation of Acetylcholinesterase by Soman: Temperature Dependence of the Solvent Isotope Effect[1]

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Abstract: 3,3-Dimethyl-2-butyl methylphosphonofluoridate (soman) recruits at least 60–70% of the catalytic power of acetylcholinesterase during phosphonylation of the active-site serine, as compared with the acetylation by acetylcholine. The solvent isotope effect is 1.34 ± 0.11 and is temperature independent within experimental error between 5 and 45 °C. This observation is quite similar to what has been found for the reactions of aryl acetates and anilides. Average activation parameters for the same temperature range are $\Delta H^* = 7.3 \pm 0.6$ kcal/mol and $\Delta S^* = -4 \pm 2$ eu (H_2O) and $\Delta H^* = 7.1 \pm 0.4$ kcal/mol and $\Delta S^* = -6 \pm 1$ eu (D_2O). A rate-limiting process with <50% contribution of general-base-catalyzed heavy-atom reorganization and >50% contribution of an induced-fit conformational change is supported by the data.

Organophosphorus compounds that have a good leaving group inhibit serine hydrolases very effectively and almost irreversibly[2,3] by phosphonylation of the active-site serine. Presumably, through interactions between these compounds and the serine hydrolases, the catalytic potential of the enzyme is recruited[4,5] to effect phosphoryl transfer. This phosphorylation process is similar to acylation in the reactions of natural substrates.[6] We have reported observations on catalytic recruitment in the inactivation of the serine proteases by phosphonate esters.[4] There we found that acid–base catalysis was among the components of catalytic power recruited. Scheme I gives a comparison of elementary steps in the reactions of acetylcholinesterase (AChE) with its natural substrate and with organophosphorus inhibitors.

AChE accelerates[7] the hydrolysis of its natural substrate by a factor of 2×10^{17}, and it is quite effective with phenyl acetate and *o*-nitrophenyl acetate as well.[8] Rosenberry[9] proposed that the rate-determining step in the mechanism of AChE-catalyzed hydrolysis of acetylcholine is the encounter of the enzyme with its natural substrate (acetylcholine shows no solvent isotope effect nor any isotope effects with substrate label and has a rate constant $k_{cat}/K_m \sim 10^8$ M^{-1} s^{-1}). Aryl esters react too slowly with AChE for diffusion to be rate-limiting, but they still show very small solvent isotope effects on k_{cat}/K_m. The rate-determining step is an induced-fit conformational change subsequent to substrate binding and preceding the "chemical steps" in which covalency changes occur. Quinn[8,10] confirmed the correctness of this mechanism recently. He based his conclusions on an extensive study of the catalytic mechanism of AChE with a broad range of substrates. A profound finding of this work is that in its acylation AChE uses only a single protonic center for the removal of the proton on the serine catalytic residue. Both Quinn's group and we[6] found that the deacetylation reaction, which is chiefly rate-limiting at saturation under physiological conditions, also involves general-base catalysis by a single proton.

Here we ask how 3,3-dimethyl-2-butyl methylphosphonofluoridate (soman), one of the most effective of organophosphorus

Scheme I

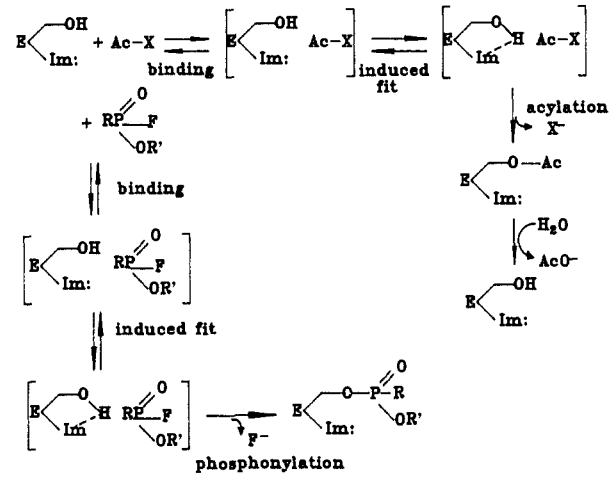

Table I. Temperature Dependence of the Second-Order Rate Constants in Protium and Deuterium Oxides for Inhibition of Acetylcholinesterase from Electric Eel[a] by Soman at pH 7.70 and Equivalent[b]

temp,[c] °C	$10^{-3}k_i/K_i$ (HOH), M^{-1} s^{-1}	$10^{-3}k_i/K_i$ (DOD), M^{-1} s^{-1}	$[k_i/K_i$ (HOH)]/ $[k_i/K_i$ (DOD)]
5.0	794 ± 66	617 ± 32	1.29 ± 0.12
15.0	1420 ± 41		
20.0	2117 ± 20	1578 ± 79	1.34 ± 0.10
25.0	3020 ± 120	2152 ± 77	1.41 ± 0.07
35.0	4181 ± 300	3159 ± 170	1.33 ± 0.12
45.0	6097 ± 360	4541 ± 313	1.34 ± 0.12
		mean	1.34 ± 0.11
ΔH^*, kcal/mol	7.3 ± 0.6	7.1 ± 0.4	
ΔS^*, eu	−4 ± 2	−6 ± 1	

[a] Sigma V-S, 10 units/L. [b] All solutions contained 0.0434 M K_2HPO_4 and 0.0066 M KH_2PO_4, 1% methanol, and the pH varied ±0.05 unit in the temperature range studied. [c] Precise to ±0.1 °C.

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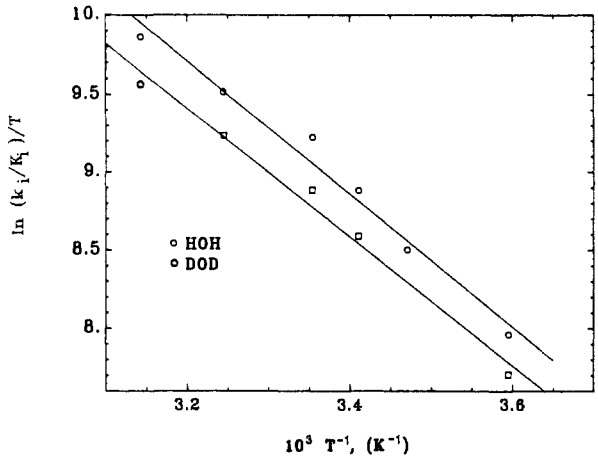
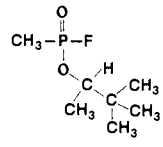


Figure 1. Eyring plot for the inhibition of AChE from the electric eel by soman in H_2O and D_2O buffered at pH 7.65, pD 8.15 with 0.05 M phosphate buffer. The lines were calculated from the data of Table I.

inhibitors ($k_i/K_i \sim 10^6$ M^{-1} s^{-1}), accomplishes its catalytic recruitment and what faculties of the catalytic apparatus are activated during inhibition.



Results

Rapid inhibition of AChE by soman was measured in the presence of the chromogenic substrate phenyl acetate by a technique previously developed and validated.[1,2] The first-order decline of AChE activity was followed by the release of phenol. The inverse of a series of first-order rate constants k_{obsd} at different phenyl acetate concentrations were plotted against the concentration of phenyl acetate [S], while the concentration of soman [I] was held constant at 1×10^{-8} M throughout (eq 1). The

$$1/k_{obsd} = 1/k_i + [(K_i/k_i)/[I]](1 + [S]/K_m) \qquad (1)$$

second-order inhibition constants k_i/K_i were calculated[11,12] from the intercept at [S] = 0 of eq 1 since $1/k_i$ is negligible in our experiments (i.e., k_{obsd} vs [I] is linear at constant [S]).

The bimolecular rate constants for AChE inactivation by soman in protium and deuterium oxides are listed as a function of temperature in Table I. AChE activity at pH 7.6 is constant for at least several hours, even at the temperature extremes of this study. The Eyring plot for the data is shown in Figure 1. The activation parameters obtained by a least-squares fit[13] of the data in Table I to the exponential form of the Eyring equation by a nonlinear least-squares program are also shown in Table I.

Discussion

Catalytic Recruitment. The second-order rate constant for inactivation of AChE from the electric eel by soman is 3×10^6 M^{-1} s^{-1} at 25 °C, while the rate constant for the neutral hydrolysis of soman is estimated from ref 5 to be 3×10^{-6} M^{-1} s^{-1}. The rate constant for phosphonylation of the active-site serine of AChE by soman is thus 10^{12}-fold the rate constant for its neutral hydrolysis. The rate constant for acetylation of the active-site serine of AChE by acetylcholine is 10^{17}-fold the rate constant for its hydrolysis.[7] Thus, AChE accelerates its own phosphonylation by soman with 12/17 = 60–70% of the catalytic power AChE employs in accelerating its own acetylation by acetylcholine.

Table II. Activation Parameters[a] for Acetylcholinesterase Reactions: k_{cat}/K_m for Substrates and k_i/K_i for Inhibitors

compd, rate-limiting process	ΔH^*, kcal/mol	ΔS^*, eu	k(HOH)/k(DOD)	ref
acetylcholine, diffusion	2.5 ± 0.6	−14 ± 0.6	1.1 ± 0.1	9
4-methoxyphenyl formate, induced fit	3.8 ± 0.2	−18.1 ± 0.3	(1.0)[c]	8
4-methoxyphenyl formate, displacement at carbonyl	24.1 ± 0.3	51.8 ± 0.7	(1.9)[c]	8
aryl acetates and anilides, induced fit	0–2	−40 to −30	(1.0)[c]	8
aryl acetates and anilides, displacement at carbonyl	10–15	−12 to +10	(2.3)[c]	8
paraoxon,[b] overall	10.6 ± 0.2	−3 ± 2		16
soman, overall	7.3 ± 0.6	−4 ± 2	1.34 ± 0.11	this work

[a] At the molar standard state. [b] Maximal estimated errors. [c] Calculated values taken from ref 8.

Solvent Isotope Effects. The solvent isotope effect for inactivation of AChE by soman is 1.3–1.4 between 5 and 45 °C, very similar to what has been observed with acylation by anilides and some aryl acetates.[7,9] It is consistent with the proposition in Scheme I, that at least two components contribute to the rate-limiting process. These two components are envisoned as a "physical step", such as the induced-fit conformational change (isotope effect of unity), and a "chemical step", the general-base-catalyzed phosphonylation (isotope effect greater than two).

In contrast, inactivation of serine proteases such as chymotrypsin, trypsin, elastase, and subtilisin by phosphonate esters involves solvent isotope effects[4] of 1.7–2.1. This shows that enzymic general-base catalysis of the phosphonylation step (Scheme I) is rate-limiting to a greater degree in serine proteases than it is with AChE.

Temperature Dependence. Figure 1, the Eyring plot for the inhibition reaction, appears somewhat curved within the 5–45 °C range. However, the activation parameters calculated from data points at the two extremes[14] do not differ enough to allow for a meaningful resolution of the curve into two intersecting lines. Therefore, no attempt was made to evaluate the two enthalpy and two entropy values for each isotopic solvent. Instead, the data were fit to a *single* Eyring equation for each isotopic solvent by nonlinear regression (Table I). Since the solvent isotope effect is small, the activation parameters in H_2O and D_2O overlap within experimental error. The solvent isotope effect itself does not vary over this temperature range by more than the experimental error of a little less than 10%. Any systematic variation of the solvent isotope effect that may be present is therefore buried in the experimental noise.

One notable feature of the activation parameters themselves is that they are rather similar (Table II) to the values we calculated for the activation parameters for the inactivation of AChE by diethyl 4-nitrophenylphosphate (paraoxon) from earlier data of Aldridge.[15] For comparison, we have also calculated from data published by Wilson[16] for the acetylation of AChE by acetylcholine: $\Delta H^* = 2.5$ kcal/mol and $\Delta S^* = -12.6$ eu, characteristic for a nearly diffusion-controlled reaction. As explained above, the small or absent isotope effects and large rate suggest that diffusion is rate-limiting for this reaction.[9]

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(14) $\Delta H^* = 6.8$ kcal/mol and $\Delta S^* = -7$ eu/mol between 278 and 288 K and $\Delta H^* = 9.7$ kcal/mol and $\Delta S^* = 3$ eu/mol between 313 and 318 K.
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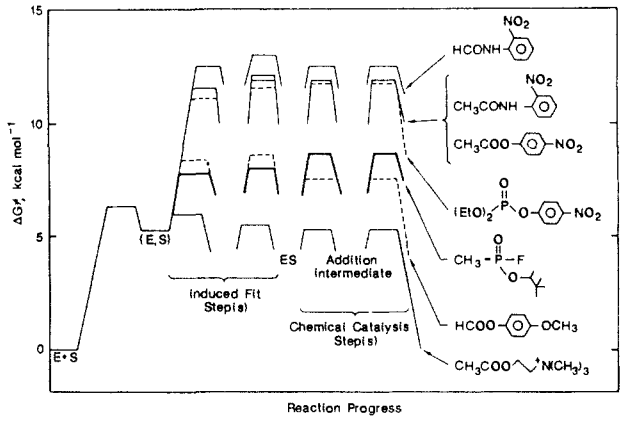

Figure 2. Free energy diagram for a comparison of recruitment of the catalytic power of AChE from the electric eel by the following species: acetylcholine,[9] 4-methoxyphenyl formate,[10] soman (heavy line), 2-nitrophenyl acetanilide,[10] 4-nitrophenyl acetate,[18] paraoxon,[16] and 2-nitrophenyl formanilide.[10] The highest barrier is calculated from the corresponding bimolecular rate constant at 25 °C for each reaction. Relative heights of the barriers for the induced-fit and chemical steps for the anilides and for the aryl acetates are adopted from ref 10 and applied to the others. The two steps indicated for induced fit with barriers of ~1 kcal/mol are arbitrary. They are based on $k \sim 10^{12}$ s^{-1} for the decomposition of conformational isomers of the enzyme. A stepwise "chemical catalysis" is also assumed with equal chance for a rate-limiting barrier for the transition state for bond breaking and making (see text).

Quinn's thorough investigation[10] of the mechanism of acylation of AChE with the use of *o*-nitroanilides and aryl acetates as substrates provides ample support, by several probes, for the view that the overall acetylation reaction comprises multiple steps in series. One (at least) of the contributing rate-limiting steps does not involve proton transfer but, perhaps, as induced-fit conformational change. The multistep character of the acylation reaction is manifested in strongly curved Eyring plots for the anilides. The precision in the kinetic measurement of these substrate reactions is inherently greater than that in the competitive inhibition reactions we have studied. Correspondingly, the authors were able to resolve these curves into two contributions, and we have included results of their calculations in Table II. The data are not very sensitive to the assumption that the solvent isotope effect for the induced-fit step is 1.0. Clearly, however, it must be less normal than the observed effects, although a reasonably small nonunit effect (either normal or inverse) cannot be excluded. The overall solvent isotope effect that is in the range 1.3–1.5 for the anilides can then be accounted for by a 55–70% contribution of the induced-fit step with no solvent isotope effect (1.0) and a 30–45% contribution of the step involving proton transfer with an intrinsic solvent isotope effect of 2.3.

The enthalpy of acylation by unnatural substrates is similar to that of phosphonylation, whereas the entropy for rapid acylation and phosphorylation is less negative than the entropy for acylation by poor substrates.

Dynamics of Acylation and Phosphonylation. Free energy diagrams are compared in Figure 2 for reactions of AChE with different carboxyl and phosphoryl compounds. The natural substrate portrayed on the lowest line of diagram reacts with near diffusion-controlled rate, exceptional catalytic rate enhancement; and chemical steps do not contribute to its rate-controlling process. Some aryl acetates react at rates only 10- to 50-fold below the diffusional rate. The rate-determining step for these reactions is presumably an induced-fit conformational change ($k_H/k_D < 1.2$). Phosphonylation of AChE by soman and acetylation by the anilides and aryl acetates seem to succeed in inducing an at least adequate fit for catalysis but fall short of (100–10000 times smaller second-order rate constants) what acetylcholine with the evolutionarily expected structure can accomplish. Likewise, chemistry does not take place with the kind of efficiency experienced by the natural substrate during phosphonylation or acetylation by unnatural substrates, which is reflected in a larger contribution of the covalent rearrangement to the rate-limiting process. This contention is supported by the slightly larger solvent isotope effects and similar activation thermodynamics for these compounds. It appears also that the balance among the rate-limiting steps shifts slightly between the two groups of processes toward a greater contribution from the chemical steps involving proton transfer in the case of inhibition by soman. This is shown by the heavy line on Figure 2.

In its full complexity, the balance governing a multitude of steps is conveniently thought of as two manifolds of processes, one physical and one chemical. The conformational induction of AChE seems to be substrate driven.[8,10] In the chemical manifold, a stepwise or a concerted mechanism may prevail, and for the sake of generality, the former is indicated on the diagram. In this case then, the balance between formation and breakdown of an intermediate is expected to be different in acyl transfer from phosphoryl transfer. In the reaction of anilides, for instance, enzymic general-acid–base catalysis of both serine attack and removal of the aniline leaving group may occur. Organophosphorus fluoridates, on the other hand, may utilize enzymic general-base catalysis of the attack by serine,[4] but the departure of fluoride may only be accompanied by extensive solvent reorganization, i.e., a solvent isotope effect of 1–1.5 instead of 2–3. The overall solvent isotope effect for the chemical steps alone could originate from these two components. One may then need to consider a balance between a conformational step(s) and the contribution of both bond breaking and bond making with the associated general-acid–base catalysis or solvent reorganization steps characteristic for each type.

In conclusion, the inhibition of AChE by soman recruits the enzyme catalytic power very effectively in both inducing the correct fit for catalysis and in using the chemical apparatus at the active site of serine for phosphonylation. The energy requirement is very likely to be in the same order of magnitude for the two, physical and chemical, manifolds. Both formation of the serine O–P bond and rupture of the soman F–P bond may contribute within the chemical phase.

Experimental Section

Materials. Inorganic salts and buffer components were reagent-grade chemicals, which were used as purchased. Protium oxide was distilled, from a copper-bottom still, passed through a Barnstead mixed-bed ion-exchange column, boiled for 20 min, and cooled suddenly. Deuterium oxide (Norell Inc. 99.9% deuterium) was distilled (100–102 °C fraction) and stored under nitrogen. Phenyl acetate (Eastman) was purified by column chromatography and vacuum distillation.

AChE from *Electrophorous electricus* was obtained with an activity of 1000 μmol units/mg (Sigma type V-S) lyophilized powder. Solutions of AChE were prepared in 0.1 M phosphate buffer at pH 6.9, and 46 ng/mL by dilution of the Sigma preparation and were frozen until use. Reactions were conducted at enzyme concentrations of 0.005–0.01 unit/mL of AChE based on the Ellman assay.[17] The enzyme was introduced into the reaction mixture in 50 μL of a stock solution. pH 7.60 was maintained in protium oxide (0.0066 M KH_2PO_4, 0.0434 M K_2HPO_4) and the corresponding pD in deuterium oxide. Aliquots of phenyl acetate stock solutions were hydrolyzed in NaOH, and the liberated phenoxide ion was measured at 286.9 nm (ϵ 2544 M^{-1} cm^{-1}) to verify actual substrate concentrations. Nonenzymic hydrolysis of phenyl acetate was completely negligible under our conditions.

Kinetics. Rate measurements involved the automated acquisition of 500–1000 data points with a Perkin-Elmer Lambda-7 spectrophotometer interfaced to a Zenith Z-100 microcomputer. The temperature was controlled with a thermoelectronic (Peltier) device or a Lauda K4/DR circulating water bath, both furnished with a thermistor probe attached to a digital readout.

In a typical kinetic experiment the appropriate volume of buffer was equilibrated within 0.05 °C of the working temperature (as monitored with a thermistor probe) in a quartz cuvette in the cell compartment of the instrument. Soman was injected in a 50-μL volume from a (2–5) × 10^{-7} M cold H_2O or D_2O (pH 4, HCl) solution, and phenyl acetate was delivered in a 10-μL volume from 0.05–0.20 M methanolic stock solu-

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tions. The absorbance was monitored after each injection to assure that phenyl acetate was intact before the enzymic reaction began. The reaction was initiated with the injection of the enzyme in a 50-μL volume to bring the total to 1 mL.

The irreversible inhibition of AChE in the presence of the substrate was studied under pseudo-first-order conditions with >1000-fold excess of inhibitor over enzyme concentration to obtain a signal equal to <10% decomposition of the substrate. First-order rate constants were calculated by a least-squares fit of absorbance time coordinates. Substrate-independent, second-order inhibition constants were calculated by a linear least-squares fit of the inverse observed rate constants to substrate concentration.

Least-squares fit of the second-order rate constants to the Eyring equation was computed with the use of a BMDP statistical program.[13]

Oxidation and Reduction of Hemoproteins by Trioxodinitrate(II). The Role of Nitrosyl Hydride and Nitrite

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Abstract: The reactions of hemoglobin (Hb), oxyhemoglobin (HbO_2) and oxymyoglobin (MbO_2), methemoglobin (Hb^+), metmyoglobin (Mb^+), and ferricytochrome *c* (Cyt c^+) with the monoanion of trioxodinitrate(II) were investigated in phosphate-buffered solutions at pH 7.0 and 25 °C. Oxymyoglobin is oxidized to Mb^+ by the decomposition products of $HN_2O_3^-$ in a stoichiometric transformation that requires a 2:1 $[MbO_2]$:$[HN_2O_3^-]$ reactant ratio and produces nitrite, nitrate, and peroxide. Methemoglobin undergoes reductive nitrosylation with nitroxyl formed by $HN_2O_3^-$ decomposition, and its kinetic complexity, identified as due to a competing reaction of HNO with β-93 cysteine sulfhydryl groups of Hb^+, has been unraveled. Nitroxyl causes sulfhydryl oxidation to disulfide and, in an example of the entrapment of nitroxyl by reduction, is converted to hydroxylamine. Ferricytochrome *c* is reduced to ferrocytochrome *c* by apparent outer-sphere electron transfer from HNO. Hemoglobin is converted to Hb^+ and HbNO by $HN_2O_3^-$, and evidence is presented that HNO, through coordination with Hb, is a catalyst for nitrite oxidation of hemoglobin. The composite data demonstrate that dissociation of nitrosyl hydride from nitrite occurs in $HN_2O_3^-$ decomposition, and they suggest a greater diversity of transformations for nitroxyl than has been previously recognized.

We recently reported that decomposition of trioxodinitrate ($HN_2O_3^-$) in the presence of deoxyhemoglobin resulted in the production of methemoglobin and nitrosylhemoglobin.[1] This unprecedented transformation led us to suggest that decomposition of trioxodinitrate occurred by dissociation of nitric oxide from the nitrous acid radical anion (eq 1), and results obtained from

$$HN_2O_3^- \rightarrow NO + (HONO)^{\bullet -} \quad (1)$$

reactions with oxymyoglobin and oxyhemoglobin, in which the ferrihemoprotein was formed together with nitrite and nitrate, were interpreted similarly. Extensive studies by Bonner,[2-6] Hughes,[7,8] and others[9-11] had previously defined trioxodinitrate decomposition by a pathway involving the production of nitrite and nitrosyl hydride (eq 2) that adequately explained available data but appeared to be unsuitable to account for the transformations observed with hemoproteins.

$$HN_2O_3^- \rightarrow HNO + NO_2^- \quad (2)$$

As a result of this report, with its conflicting interpretation of the mechanistic pathway for trioxodinitrate decomposition, Hollocher and co-workers reinvestigated the reaction of the monoanion of sodium trioxodinitrate with deoxyhemoglobin and deoxymyoglobin, confirming the rate law and hemoprotein product ratios but differing from us in the stoichiometry for production of nitrite and nitrous oxide.[12] Their results, and particularly the reaction stoichiometry, were consistent with the direct intervention of an activated form of $HN_2O_3^-$ or its primary breakdown products. Complimentary studies of trioxodinitrate decomposition in the presence of nitric oxide were also performed to evaluate eq 2 as the only viable dissociative pathway, and together with Gratzel's prior report of nitroxyl association with nitric oxide,[13] Bazylinski and Hollocher concluded that eq 2, and not eq 1, was the primary pathway for trioxodinitrate decomposition.[14] More recently, the photochemical decomposition of trioxodinitrate was shown to produce triplet nitroxyl and nitrate in apparent agreement with eq 2.[15]

We have investigated trioxodinitrate decomposition in the presence of oxyferrohemoproteins and ferrihemoproteins with the expectation that results obtained from this study would further clarify their redox mechanisms. Our results with methemoglobin correspond to those reported by Bazylinski and Hollocher[16] with some relevant exceptions that are described in this publication. That methemoglobin is reduced during $HN_2O_3^-$ decomposition while oxyhemoglobin and deoxyhemoglobin are oxidized by the same reagent is one reason for our interest in the mechanism of

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