Organophosphate Nerve Agent Toxicity in *Hydra attenuata*

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The toxicity for analogues of sarin (GB), soman (GD), and VX was evaluated using *Hydra attenuata* as a model organism. The organophosphate nerve agent analogue simulants used in this investigation included the following: isopropyl *p*-nitrophenyl methylphosphonate (for GB); pinacolyl *p*-nitrophenyl methylphosphonate (for GD); and diisopropyl S-(2-diisopropylaminoethyl)phosphorothioate, diethyl S-(2-diisopropylaminoethyl)phosphorothioate, and diethyl S-(2-trimethylaminoethyl)phosphorothioate (for VX). The toxicity of each organophosphate nerve agent was assessed quantitatively by measuring the minimal effective concentration within 92 h in *H. attenuata*. There is a positive correlation between the molecular hydrophobicity of the compound and its ability to cause toxicity. Results from this study indicate the potential for application of this assay in the field of organophosphate chemical warfare agent detection, as well as for the prediction of toxicity of structurally similar organophosphate compounds. The minimal effective concentration for two of the VX analogues was 2 orders of magnitude more toxic than the analogue for GD and 4 orders of magnitude more toxic than the analogue for GB.

Introduction

Organophosphates have been utilized in various applications since their initial discovery in the early 19th century (*1*). Their most noted employment is usage as agricultural insecticides and chemical warfare agents. Organophosphates were introduced as pesticides after World War II and subsequently incorporated into common agricultural practices ever since (*1*). It is estimated that approximately 200 different organophosphate insecticides are currently used commercially worldwide (*2*). The accumulation of these pollutants has raised environmental concerns due to the inherent toxicity of these compounds (*3*). In general, the mechanism of toxicity involves the inhibition of enzyme activities and the release of neurotransmitters (*2*). Acute toxicity results primarily from the inactivation of the enzyme AChE.[1] However, other direct targets of organophosphate intoxication include the muscarinic and nicotinic acetylcholine receptors. Symptomatic manifestations of organophosphate intoxication leading to death include pinpoint pupils, breathing difficulties, coma, and convulsions. While the mechanism of toxicity is believed to be similar in all species, the chemical dosage directs the intensity of the response (*2, 4*).

From studies on acute toxicity, LD_{50} values of organophosphates for humans have been estimated based on studies using mammals with a variety of chemical warfare agents (*5*). However, most of these studies were conducted between 1940 and 1980, and almost all of these measurements were focused on controlled, acute exposures (*6*). The acquisition of new toxicity data is hampered by multiple regulations and government restrictions. Some of these limitations involve animal sample size and the enormous expense associated with such studies. In this investigation, the toxicities for chiral and achiral analogues of GB, GD, and VX were evaluated using *Hydra attenuata* as the model organism.

H. attenuata is a common freshwater Cnidarian found in slow-moving waters (*7*). Cnidarians are true metazoa with only two body layers, an outer epidermis and the inner gastrodermis. As diploblasts, all of the cells are in close proximity to the aqueous medium and the immediate environment. This particular feature enables *Hydra* to be very sensitive and susceptible to minute amounts of environmental toxicants. In addition to sensitivity, the *Hydra* bioassay is remarkably reproducible because of asexual reproduction, yielding clones of individual organisms. The cost and overall simplicity of this bioassay have contributed to an increase in the number of recent studies using *Hydra* in acute and chronic toxicity tests of water soluble compounds (*8, 9*). Previous studies have exploited the use of the *Hydra* bioassay to assess the toxicity of various compounds including chlorinated phenols (*10*), heavy metals (*7*), and estrogenic compounds (*11*). An

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[1] Abbreviations: AChE, acetylcholine esterase; GB, sarin, isopropyl methylphosphonofluoridate; GD, soman, pinacolyl methylphosphonofluoridate; VX, ethyl S-(2-diisopropylaminoethyl)methylphosphonothioate; tetriso, diisopropyl S-2(diisopropylaminoethyl)phosphorothioate; DEVX, diethyl S-(2-diisopropylaminoethyl)phosphorothioate; MEC, minimum effective concentration; MAP, microsomal activation package; NTE, neurotoxic esterases; OPIDH, organophosphate-induced delayed neuropathy; TES, N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

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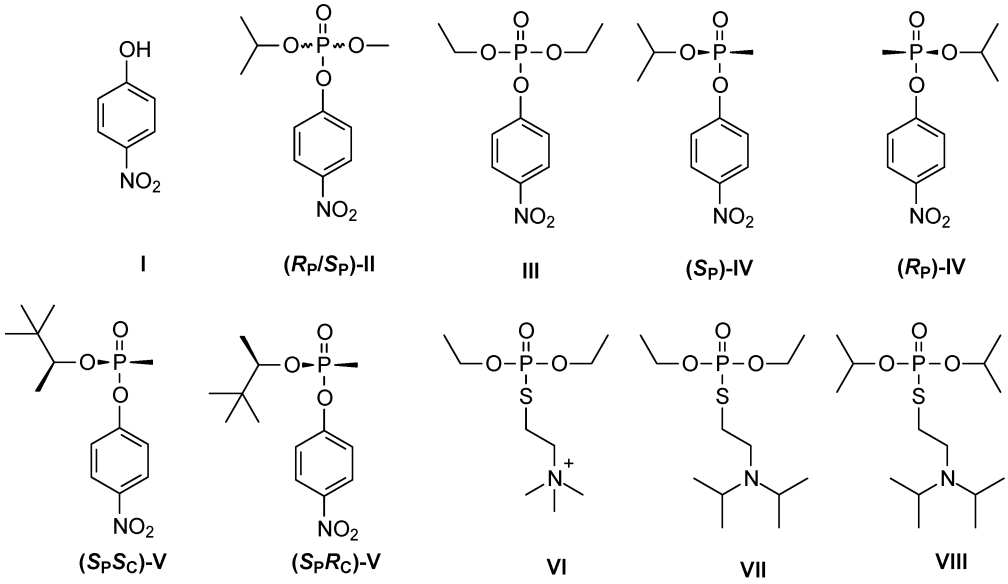
Scheme 1



investigation involving the invertebrate organism, *Daphnia magna*, has also shown a significant correlation with established rat oral LD_{50} values (*12*). Therefore, this system shows great potential as an alternative means to animal testing in prescreening trials for the prediction of toxicity of specific organophosphate compounds.

In this paper, structure−activity relationships were established for toxic properties using a series of chiral and achiral organophosphate analogues of the nerve agents GB, GD, and VX. The toxicity of each organophosphate nerve agent was quantitatively assessed by determining the MEC in *Hydra* after 92 h. The conclusions of this investigation correlate well with organophosphate toxicity studies previously described by Singh (*13*) and Makhaeva et al. (*14*). On the basis of the data from this investigation, there is significant potential for the application of this bioassay in the detection of organophosphate chemical warfare agents, in addition to the prediction of toxicity for structurally similar organophosphate compounds.

Experimental Procedures

H. attenuata. *H. attenuata* were cultured from organisms that were received as a gift from E. Marshall Johnson, Jefferson Medical College (Philadelphia, PA). Cultures of *Hydra* were maintained in shallow bowls of culture solution and stored at 18 °C. The culture solution contained 1.0 mM calcium chloride, 0.45 mM TES buffer, and 0.012 mM EDTA, pH 7.0. Deionized water was used throughout all of the maintenance and assay solutions.

Artemia saliva. Axenic nauplii of brine shrimp (*Artemia saliva)* were prepared on a daily basis, beginning 3 days before each bioassay was conducted. The procedure and conditions for incubation were adapted from Lenhoff (*15*). After the nauplii were hatched in 1% (w/v) NaCl solution, they were treated with iodine (40 ppm) for 15 min and subsequently washed with deionized water. The axenic nauplii were fed to the *Hydra* once a day for a period of 30 min. After feeding, the debris from the feeding was removed by thorough rinsing of the *Hydra* with culture solution. Food was withheld beginning 24 h before initiating the bioassay and throughout the testing period.

Assay Procedures. Procedures for this study were modified from those described by Johnson et al. (*16*). Only *H. attenuata* adults were used in the bioassays, and each test was run in triplicate. For each concentration of the tested compounds, 4.0 mL of culture media was placed in a small Petri dish. The control dish consisted of culture media alone. All of the test dishes were incubated at 18 °C throughout the assay. Adult *Hydra* were observed for symptoms of toxic response at 0, 4, 20, 28, 44, 68, and 92 hours.

The MEC at 92 h was determined for each compound tested through a series of assays, consisting of three phases. The first phase involved exposing adult *Hydra* to whole log concentrations of each compound, to determine the range of toxicity apparent within 92 h. The "tulip" stage was defined as the toxic end point of each assay. The lowest concentration of the test compound resulting in the toxic endpoint (e.g., MEC) was carried forward to the second phase of testing. In phase II of testing, the highest and lowest concentrations of the compound were within one log unit of one another. The MEC from the second phase of testing was used as the highest concentration in the phase III testing. The results from the phase III test were used to confirm those of the previous assays.

Chemicals and Reagents. All buffer reagents were purchased from Sigma Chemical Co. Aroclor 1254-induced male rat liver, postmitochondrial supernatant (S-9) was purchased from Molecular Toxicology (Boone, NC) and used as the MAP. Synthesis of the racemic GB analogue (**IV**) and the racemic GD analogue (**V**), along with the chemoenzymatic preparation of the chiral analogues, were prepared as described in Li et al. (*17*). The VX analogues, ecothiophate iodide (**VI**), diethyl VX (**VII**), and tetriso (**VIII**), were synthesized by modifications of procedures described in the literature (*18*, *19*). All of these compounds are toxic and should be used with the appropriate safeguards.

Structure−Activity Analysis. A model for each of the organophosphate compounds was made using ISIS/DRAW. The resulting files were imported into HyperChem Release 7 (Hyper Cube, Inc.), and various calculations were carried out. Molecular properties, such as the octanol−water partition coefficient and polarizability, were obtained for each of the compounds tested. HyperChem calculations were made by a grid method assessment of van der Waals and solvent accessible surfaces (*20*). Determination of the octanol−water partition coefficients was based on implementation of an atom fragment method as described by Ghose et al. (*21*). Polarizability information was deduced using an atom-based method as described by Miller (*22*).

Results

Chemical Compounds. A total of 10 different compounds were synthesized and analyzed for their toxico-

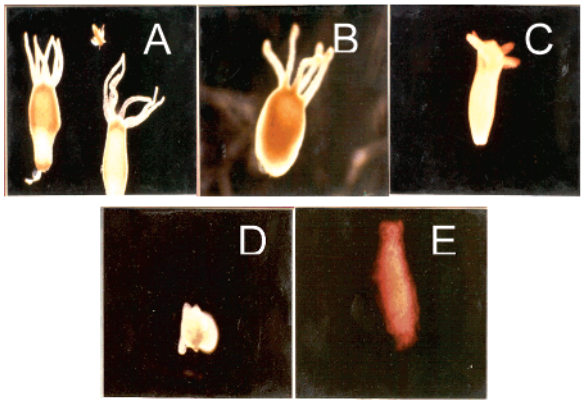


Figure 1. Physical stages of adult *H. attenuata*. (A) Normal state; (B) clubbed tentacles; (C) shortened tentacles; (D) the tulip stage, also defined as the toxic endpoint of the bioassay; and (E) disintegrated.

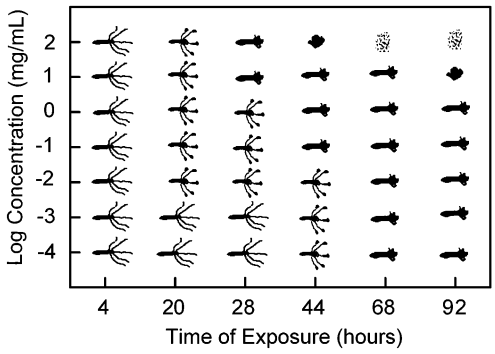


Figure 2. Time course for the toxic effect of various concentrations of paraoxon on *H. attenuata*. The symbols represent the morphologic changes as defined in Figure 1. Additional details are provided in the text.

logical properties using *Hydra* as the test system. Methyl isopropyl *p*-nitrophenyl phosphate (**II**) was utilized as a racemic mixture, while the GB analogue (**IV**) was prepared as individual enantiomers. The GD analogue (**V**) was used as a mixture of four diastereomers, a mixture of the two possible S_P isomers, and as the single S_PS_C enantiomer. The three analogues of VX (**VI**, **VII**, and **VIII**) are achiral. The structures of these compounds are presented in Scheme 1.

***Hydra* Bioassay.** *Hydra* were used to assess the toxicological properties of selected organophosphate nerve agents. In these tests, the *Hydra* were observed to undergo a series of morphological changes as a function of time and these physical changes are illustrated in Figure 1. The tentacles first begin to be "clubbed" at the end (Figure 1B) and then shorten in length (Figure 1C). The *Hydra* continue to shrink to a tulip stage (Figure 1D) and then eventually disintegrate (Figure 1E). The tulip stage is defined as the toxic end point. The MEC was determined for each compound through a series of three tests. In the first test, the compound was assayed in increments of whole log concentrations. During the first stage of testing, the concentration at which the *Hydra* reached the toxic end point within 92 h was used as the upper limit in the second phase of the assay. For example, the toxicity of paraoxon (**III**) on *Hydra* was first examined with a range from 2.5×10^{-4} to 2.5×10^{2} mg/L. From this initial assessment, the toxicity after 92 h occurred from an exposure to paraoxon that was within the range from 2.5 to 25 mg/L. Representative data are presented in Figure 2. The second and third tests (data not shown) confirmed that the MEC_{92h} was 25 mg/L. Throughout each phase of testing, the culture solution was used in the absence of added nerve agent as a negative control to ensure the integrity of the *Hydra* throughout the assay. The MEC_{92h} values for the remaining nine compounds were determined in exactly the same manner, and the final values are summarized in Table 1.

Table 1. Properties of Organophosphate and Organophosphonate Esters

compound	MEC_{92h} (mg/L)	log partition coefficient [octanol/water]
I	9.4×10^{1}	−2.2
(R_P/S_P)-**II**	6.0×10^{1}	−0.92
III	2.5×10^{1}	−0.99
(R_P)-**IV**	1.0×10^{2}	
(R_P/S_P)-**IV**	2.0×10^{-2}	−1.5
(S_P)-**IV**	1.3×10^{-2}	
(R_PR_C/R_PS_C/S_PR_C/S_PS_C)-**V**	2.5×10^{-4}	−0.12
(S_PR_C/S_PS_C)-**V**	2.0×10^{-4}	
(S_PS_C)-**V**	8.0×10^{-5}	
VI	1.0×10^{-4}	2.0
VII	3.0×10^{-6}	3.1
VIII	3.0×10^{-6}	3.9

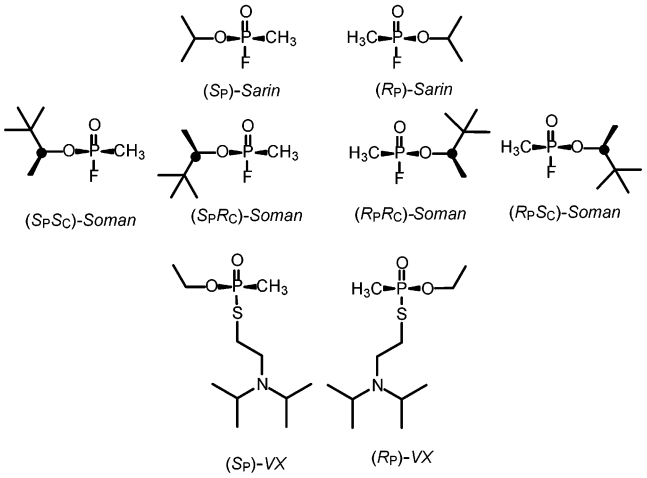
Of the 10 compounds tested for toxicity with *Hydra*, two of the VX analogues (**VII** and **VIII**) were the most toxic. The two S_P isomers of the GD analogue (**V**) were approximately 2 orders of magnitude less toxic than the two most toxic analogues for VX. The most toxic enantiomer of the analogue for GB (S_P-**IV**) was approximately 2 orders of magnitude less toxic than the most toxic analogue for GD (S_PS_C-**V**). In contrast, the R_P enantiomer of **IV** was approximately 4 orders of magnitude less toxic the corresponding S_P enantiomer. Overall, the compounds tested varied in toxicity by approximately 8 orders of magnitude. This order of toxic properties by the various analogues of GB, GD, and VX mirrors the toxicity of the nerve agents GB, GD, and VX (Scheme 2). Shown in Figure 3 is a plot of the MEC_{92h} values measured in this study for the organophosphate nerve agent analogues **IV**, **V**, and **VII** vs the reference dosage (mg/kg/day) for GB (2.0×10^{-5}), GD (4.0×10^{-6}), and VX (6.0×10^{-7}). The reference dosage estimates the daily exposure level that can be tolerated without harmful effects during a lifetime (*23*). The relative toxicity for the analogues is consistent with the relative toxicity displayed by the CW agents themselves.

The test compounds were modeled in ISIS/DRAW, and the resulting files were analyzed using HyperChem Release 7 (Hyper Cube, Inc.). Molecular dynamics calculations including geometry optimization energy, volume, and surface area were obtained for each compound, in addition to the polarizability and the partition coefficient for a mixture of 1-octanol and water. Of these molecular properties, the partition coefficient in octanol−water gave the best correlation relative to the MEC_{92h} values determined here, as illustrated in Figure 4.

Discussion

Bioassays. Exposure to each organophosphate compound tested led to toxicity in *Hydra*. In terms of the MEC within 92 h of exposure, the leaving group product, *para*-nitrophenol (**I**), was among the least toxic compounds whereas two of the VX analogues, diethyl VX (**VII**) and tetriso (**VIII**), were the most toxic (Table 1). The toxicity elicited from *p*-nitrophenol was significantly

Scheme 2



lower than that of the various GB and GD analogues tested; thus, it can be concluded that the lethality from exposure to these organophosphates was not due to the presence or accumulation of the leaving group product. However, the GB analogue (**II**) and paraoxon (**III**) induced lethality only at relatively high concentrations. From this observation, it can be concluded that the organophosphate triesters have less of an inhibitory effect than the organophosphonate diesters on AChE in vivo and are thus tolerated at higher concentrations. In contrast, organophosphonate diesters, containing a P−CH_3 substituent, cause significantly greater toxicity, which is supported by the data from exposure to the GB (**IV**) and GD (**V**) analogues. In comparing the two GB analogues (**II** and **IV**), the racemic phosphonate GB analogue (**IV**) was significantly more toxic than that of the corresponding phosphate analogue of GB (**II**), yielding a 3000-fold difference in MEC_{92h}.

A notable effect on toxicity was observed on the stereochemistry at the phosphorus center for the two isomers of the GB analogue (**IV**) and the stereoisomers of the GD analogue (**V**). Exposure to the S_P isomer of **IV** led to a 7600-fold greater toxicity than that observed for the R_P isomer. The differential toxicity has also been noted in studies with rodents exposed to individual nerve agent isomers of GB (*24*). The rate constant for the inactivation of AChE for the S_P isomer of GB was shown to be 5000-fold greater than that of the R_P isomer (*25*). The difference in the rate of inactivation of AChE is even greater between the most toxic S_PS_C isomer of GD and the relatively nontoxic R_PR_C isomer of GD (*25*).

The MEC for the GB analogue (**IV**) is 100-fold greater than for the GD analogue (**V**). From the literature, it has been noted that GD is more toxic than GB and that a differential toxicity occurs among the isomers of both nerve agents (*25*). The MEC in *Hydra* revealed that the most toxic isomer within the racemic GD analogue (**V**) is of the S_P configuration. The MEC of the isolated S_PS_C isomer is ∼3 times lower than that of the racemic mixture. The results from this study are similar to those of Benschop et al., which indicate that the most toxic isomer in mouse studies is the S_PS_C isomer of GD (*25*). Experimental evidence also indicates that the S_PS_C isomer of GD is the most reactive with human AChE and that the chirality at the phosphorus center is the most important element in determining the stereoselective toxicity (*26*).

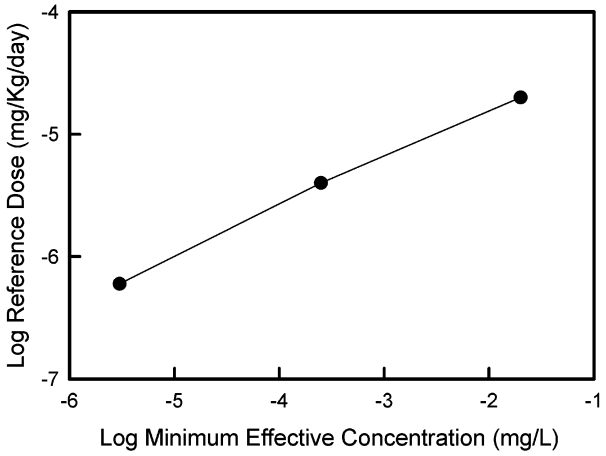


Figure 3. Comparison of the MEC_{92h} (mg/L) for the organophosphate nerve agent analogues **IV**, **V**, and **VII** determined in this investigation vs the reference dose (mg/kg/day) for the chemical warfare agents GB, GD, and VX.

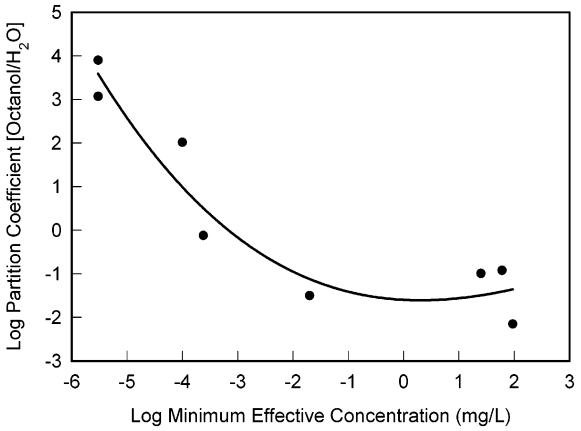


Figure 4. Correlation between the log of the partition coefficient for a mixture of octanol/water and the MEC_{92h} for the compounds assayed in this investigation. The solid line through the data is for comparison purposes only.

The compounds with the lowest values for MEC were the VX analogues, **VI**, **VII**, and **VIII** (Table 1). While the MEC for **VI** was only about half of that for the GD analogue (**V**), the toxicity elicited from **VII** and **VIII** was ∼100-fold greater than that for **V**. Both of these VX analogues caused lethality in *Hydra* at a concentration that was ∼7 orders of magnitude less than that of paraoxon. However, the toxicity of diethyl VX (**VII**) was reduced by 10 000-fold (data not shown) in the presence of MAP, consisting of enzymes present within the S9 fraction from Aroclor 1254-induced male rats. From these data, it appears that the various enzymes present in MAP aid in the detoxification of diethyl VX and perhaps structurally similar compounds as well. Overall, the VX analogues were more toxic than the GD analogues, which in turn were more toxic than the GB analogues, as expected from the known order of toxicity (*27*).

Molecular Modeling. All of the compounds used in the bioassays were modeled with HyperChem. In many studies, it has been reported that hydrophobicity is a significant factor in determining toxicity and drug reactivity (*14*). Molecular hydrophobicity was determined for each compound in terms of the partition coefficient for an octanol−water mixture. The leaving group for four of the compounds tested here, *p*-nitrophenol, was the most water soluble compound while tetriso was the most

soluble in octanol. The most hydrophobic compounds exhibited greater toxicity as illustrated in Figure 4. These findings are in agreement with the hypothesis that the hydrophobic organophosphorus compounds quickly enter the nervous system and become readily available to attack multiple targets involved in organophosphate-induced toxicity (*2*). It has been previously established that there is a good correlation between the partition coefficient for organophosphate compounds and the NTE inhibition (*13*) and that an increase in OPIDN is observed as the hydrophobicity of a compound is increased (*14*).

Potential Applications. The *Hydra* bioassay may be incorporated into an array of other applications. The ability of this organism to detect ppm to ppt levels of organophosphate nerve agents enhances the degree of sensitivity demanded by biosensors. In addition, the cost, efficiency, and ease of this assay permit it to be run multiple times in a variety of formats. Because *H. attenuata* are sensitive to the organophosphates and organophosphonates tested here, it is assumed that this organism will be sensitive to related compounds. The bioassay used here could be applied as a prescreening tool in determining the relative toxicity of related organophosphorus nerve agents, as well as individual stereoisomers that have yet to be screened for toxicity.

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