**CHEMISTRY AND PRELIMINARY ENVIRONMENTAL
EFFECTS OF MIXTURES OF TRIISOPROPYL
PHOSPHITE, BIS-(2-ETHYLHEXYL)-PHOSPHONATE,
AND SULFUR**

Final Report

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PREFACE

The work described in this report was authorized under a Related Services contract with the U. S. Department of Energy, Contract No. DE-AC06-76RLO 1830. This work was started in August 1990 and completed in August 1991.

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The authors wish to thank Don C. Klover and Barbara A. O'Brien for technical support during this project.

EXECUTIVE SUMMARY

The present studies were performed to evaluate the reaction chemistry and preliminary biotic impacts of BIS, TIP, and TIPS. Reaction chemistry studies were designed to simulate in-flight mixing characteristics. Aquatic studies were conducted using amendment methods, and potential terrestrial impacts were evaluated using *in vitro* methods for electron transport in isolated chloroplasts.

The final products delivered to the environment by use of this binary system would be TIPS, excess elemental sulfur, and lesser quantities of BIS. The reaction is rapid and nearly complete. No significant quantities of secondary reaction products were observed. In general, it is unlikely that environmental release of these products would have significant or lasting effects based on the preliminary algal tests and electron transport studies.

There is an apparent species sensitivity difference in algae for the simulants BIS, TIP, and TIPS, with *Chlorella* being more sensitive than *Selenastrum* based on cell number studies. However, the extent of adverse effects was not excessive. *Chlorella* exhibited a reduced growth rate in terms of cell numbers (i.e., cell division) in the presence of 20 ppm TIP and TIPS, but not BIS. TIPS produced more extensive metabolic effects, but these effects were not serious. In the *Selenastrum* only a slight lag in growth was evident in the TIPS cultures, and no differences were apparent in the final yield.

There was no apparent effect of TIP or TIPS on the electron transport systems of isolated chloroplasts at the concentration tested (10 ppm).

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1.0 INTRODUCTION

Army Regulation (AR) 200-2 integrates environmental considerations into U. S. Army plans and programs and provides the Army's requirements for documentation of environmental matters. As implemented by the U. S. Army Materiel Command, this regulation requires environmental analyses and documentation for all items developed by subordinate research and development commands.

Chemical simulants are substances whose physical and chemical properties resemble those of other compounds enough that they may be used to mimic those compounds or study-specific attributes of those compounds. Chemical agent simulants are important in U. S. Army chemical defense programs because they can be used in place of more hazardous chemical agents in training personnel and developing chemical defense material.

Environmental impacts from releases of chemical agent simulants depend on many factors (e. g., the routes and rates of release, modes of dissemination and degradation, and toxicities to indigenous organisms). Because these factors vary with the location and nature of the chemical discharge, correlation of specific impacts with release of a specific compound is difficult, if not impossible, to predict from the characteristics of the compound alone. This difficulty is especially pronounced for chemical agent simulants used in different environments (desert, arctic, tropical, aquatic) under a variety of experimental conditions.

The use of chemical agent simulants and decontaminants at both U. S. and foreign training sites has the potential for producing significant environmental impacts. Data are available on the acute toxicity of many of the agent simulants and decontaminants resulting from ingestion, inhalation, or skin contamination in a variety of species, including rats, mice, dogs, rabbits, guinea pigs, and even humans (Captain S. R. Bennett, personal communication). Unfortunately, specific scientific data on the potential terrestrial and aquatic environmental fate and ecological effects of these chemicals, either singly or in combination (e. g., simulant mixed with a decontaminant, simulant binary mixtures), are inadequate to allow the establishment of rational use and cleanup criteria.

This report will address the chemistry of reaction mixtures containing bis-(2-ethylhexyl) phosphonate (BIS), triisopropyl phosphite (TIP), and sulfur (S). The objective of these studies is to determine the chemistry of these mixtures with respect to stable end products that are released to the environment and to assess their preliminary toxicity.

1.1 CHEMICAL PROPERTIES OF MIXTURE COMPONENTS

The mixture components consist of BIS, TIP, and S. The principal reaction product is 0,0,0-triisopropyl-phosphorothiaolate (TIPS). Bis-(2-ethylhexyl) phosphonate is a simulant for the nerve agent VX and is used in simulated decontamination studies. Triisopropyl phosphite is employed as one of the reactants, along with BIS and rhombic S, to simulate binary reactions and dispersion. TIPS represents the major reaction product from BIS/TIP/S.

The chemical simulant BIS, $[\text{CH}_3(\text{CH}_2)_3\text{CH}(\text{C}_2\text{H}_5)\text{CH}_2\text{O}]_2\text{P}(\text{O})\text{H}$, is a neutral dialkyl ester of phosphoric acid. It is liquid at room temperature and is relatively insoluble in water (<1 ppm). Its important physical properties include a boiling point of $>300^\circ\text{C}$ and a vapor pressure of 6×10^{-5} mm Hg at 25°C . The compound is chemically relatively stable and is prone to slow hydrolysis in aqueous environments, yielding phosphoric acid and 2-ethyl-1-hexanol. No chemical data could be found on either TIP or TIPS.

1.2 ENVIRONMENTAL FATE AND EFFECTS

The environmental persistence of BIS, because of its low volatility, is dependent on rates of chemical and biological degradation. No reliable data are available for persistence in either terrestrial or aquatic environments. While a relatively limited data base exists on the fate and effects of BIS, the document of Howard et al. (1985) contains pertinent information concerning its chemical characteristics, stability, and toxicity. No environmental data were found pertinent to either TIP or TIPS.

1.3 OBJECTIVES

The purpose of these studies is to provide information about the environmental behavior and effects of binary simulant reaction products. The scope of these efforts is restricted to establishing the reaction chemistry involved with this simulant mixture and assessing preliminary toxicology of these products based on algal growth and electron transport in isolated chloroplasts of spinach.

2.0 MATERIALS AND METHODS

The following methodology and experimental protocols were employed in evaluating the reaction chemistry and preliminary biotic impacts of BIS, TIP, and TIPS. Reaction chemistry studies were designed to simulate in-flight mixing characteristics. Aquatic studies were conducted using amendment methods, and potential terrestrial impacts were evaluated using *in vitro* methods for electron transport in isolated chloroplasts.

2.1 MIXTURE CHEMISTRY

2.1.1 Sources of Chemical Components

Triisopropyl phosphite (95%) and rhombic sulfur (99.999%) were purchased from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin). Bis-(2-ethylhexyl)-phosphonate was obtained from Alpha Products, Thiokol/Ventron Division (Danvers, Massachusetts). High-performance liquid chromatography (HPLC) grade solvents, obtained from J. T. Baker (Phillipsburg, New Jersey), were utilized for HPLC separations and preparation of samples for gas chromatography/mass spectrometry (GC/MS) analysis.

2.1.2 Simulation of In-Flight Mixing

Two reaction approaches were employed to evaluate the kinetics and the final formation of products. The first reaction sequence involved the slow addition of TIP to S followed by the addition of BIS. Sulfur (8.35 g) was placed in a 300-mL round-bottomed flask along with a stir bar. The TIP (36.01 g) was slowly introduced through an addition funnel over a 1-h period. Following the addition of TIP, a 2.0-mL aliquot was removed for analysis by GC/MS. Next, BIS (14.72 g) was slowly added over a 30-min period. Reaction products were assessed by GC/MS.

The second reaction sequence was performed to more closely simulate the conditions of in-flight mixing. The BIS (15.5 g) and S (8.53 g) were slurried in a 300-mL round-bottomed flask. A 2.0-mL aliquot was removed from this mixture for subsequent GC/MS analysis. Next, TIP (31.8 g) was quickly added via an addition funnel. The

entire volume of TIP was added within 35 s. A sample for GC/MS analysis was removed from the reaction pot when the temperature had cooled to 85°C (15 min after the reaction).

2.1.3 Methods for Analysis of Mixture Components and Products

Chemical analyses were performed on both starting materials (TIP) and reaction products (TIPS) using GC/MS methods. Ten microliters of starting material or reaction mixture was diluted to 50.0 mL with hexane. These samples were analyzed by GC (Hewlett Packard Model 5890 gas chromatograph) interfaced to a Mass Selective Detector (Hewlett Packard Model 5970A). Samples were introduced to a J&W DB-Wax column (30 m x 324 µm ID) by 2-µL splitless injections. This polar stationary phase is suitable for the analysis of polar analyses and has the additional advantage of being tolerant toward direct aqueous injections. The components were separated by temperature programming the column from 40 to 250°C at 6°C/min.

The HPLC analysis of BIS employed a Waters model 510 pump and solvent delivery system. Isocratic mobile phase was delivered to either an Ultrasphere-ODS (24 cm x 10 mm ID) or an Ultrasphere-Si (24 cm x 10 mm ID) column, depending on whether the separation mode was reverse or normal phase, respectively. Because the analytes lacked chromophores, it was necessary to use a refractive index detector (Waters model 401 differential refractometer). Analytes were diluted with three parts methanol for reverse phase separations, or with three parts hexane for normal phase separations. A Wisp model 712 automatic injector was used to deliver samples ranging in volume from 10 to 60 µL to the column.

2.2 BIOTIC EFFECTS

2.2.1 Algal Phytotoxicity

The phytotoxic effects of BIS, TIP, and TIPS on algal growth rate and dry matter production were determined for two species of green algae: *Chlorella pyrenoidosa* and *Selenastrum capricornutum*. The algae, obtained as slant cultures from Carolina Biological Supply Company (Burlington, North Carolina) were grown under sterile conditions for two transfers (15 days) in 250-mL Erlenmeyer flasks containing 60 mL of Freshwater Alga-Gro® (Carolina Biological Supply Company, Burlington, North

Carolina) with supplemental sodium bicarbonate (3.0 mg/mL, w/v, filter sterilized) in a PsyCroTherm® Control Environment Incubator Shaker (New Brunswick Scientific Company, New Brunswick, New Jersey) at 20°C with an 18-h photoperiod (300 $\mu\text{E}/\text{min}^2/\text{s}$ Photosynthetically Active Radiation, PAR).

Individual stock solutions at a final concentration of 20 ppm (v/v) of each of the three simulants were prepared composed of Alga-Gro, bicarbonate, and simulant, and filter sterilized. Both algal species were exposed to each of the simulants as follows: 5 mL of algal culture in log growth phase were added to the 60 mL of the simulant solution and the cultures incubated under the conditions given above. At time 0, 3, 5, 9 and 10 days post-inoculation, the cell count per milliliter was determined using a Model ZH Coulter Counter. Upon reaching stationary growth phase, the cultures were filtered through tared 0.5- μ membrane filters (Nucleopore Corporation, Pleasanton, California), the filters were dried at 70°C for 48 h, and a final culture dry weight was taken.

2.2.2 Effects of Electron Transport

Isolated Chloroplast Measurements

Chloroplasts were isolated from commercially obtained spinach (*Spinacea oleracea*) leaves according to the methods of Walker (1980). Approximately 80 g of leaves with the mid-ribs removed were washed with distilled water and chilled before grinding. The leaves were then ground for 10 s with a sorvall tissue homogenizer in 50 mL of grinding medium, consisting of 0.33 M sorbitol; 10 mM $\text{Na}_4\text{P}_2\text{O}_7$; 5 mM MgCl_2 ; and 2 mM sodium ascorbate, pH 6.5, which had been chilled to a slush-like consistency to maintain the grinding temperature around 4°C. The ground material was then filtered through eight layers of cheesecloth and the filtrate immediately centrifuged at 1500 x g for 90 s. The supernate was then decanted and the surface of the pellet washed with 1 mL of resuspension mix, which was then discarded. The pellet was resuspended in a mixture consisting of 0.3 M sorbitol; 2 mM Na_2EDTA ; 1 mM MgCl_2 ; 1 mM MnCl_2 ; 50 mM HEPES; 10 mM NaHCO_3 ; 5 mM PPi ; and 0.5 mM P_i , pH 7.6. Chlorophyll content was determined according to the method of Arnon (1949): 50 μL of the chloroplast suspension was added to 20 mL of 80% (v/v) acetone and filtered (through No.1 Whatman paper), and the absorbance read at 652 nm. Nine divided by the absorbance gives the volume in microliters of the original suspension containing 100 μg of chlorophyll. All procedures were carried out under low light and at 4°C.

Photochemical Assays

Assays were conducted on two photosystem reaction centers (PS II and PS I) and whole-chain electron transport, measuring oxygen evolution and uptake with a Clark-type electrode (YSI Instruments, Yellow Springs, Ohio) in a 1.8-mL volume, water-jacketed cuvette (Gilson Medical Electronics, New Hartford, Connecticut) maintained at 18°C. Given the relative insolubility of all three simulants (BIS, TIP, and TIPS) the stock solutions were prepared in absolute EtOH so that addition of 20 µL would equal a final concentration within the cuvette of 1 or 10 ppm. All assays were conducted in paired cuvettes at the same time, with one cuvette serving as a control and the other containing the simulant. A similar volume of absolute EtOH (20 µL) without simulant was added to the control cuvette at the same time. The simulants were added directly to the cuvette before illumination (~5 min). Control chloroplasts were treated in the same manner. Assays were run in triplicate, and all data are expressed in either µMol O₂/h/mg chl or as percent control of the paired assay. The analyses of the three components of the chloroplast electron transport system were performed according to the following methods:

- PS II measurements - Assays were conducted according to the methods of Boyer and Bowen (1970). The assay medium (1.8 mL) consisted of 0.33 M sorbitol; 2 mM Na₂EDTA; 1 mM MgCl₂; 1 mM MnCl₂; and 50 mM HEPES, pH 7.6. Sodium 2,6-dichloroindophenol (DCIP), 0.88 mM, was added just before the addition of chloroplasts (100 µg). The suspension was then illuminated from the side with saturating light (>1200 µEinstein min⁻²/s) at 600 nm, and the rate of oxygen evolution determined from the initial slope of the electrode output as a function of time.
- PS I Measurements - Assays were conducted according to the methods of Keck and Boyer (1974). The assay medium consisted of 1 mM ADP; 1 mM K₂HPO₄; 0.1 M KCl; 5 mM MgCl₂; 0.1 mM DCMU; 80 µM DCIP; 1 mM sodium ascorbate; 0.5 mM methyl viologen (MV), 0.5 mM sodium azide (prepared daily); and 100 µg chlorophyll. Assays were illuminated and measured as above.
- Whole-Chain (Water to MV) Measurements - Two assays were used. In the first, assay conditions were identical to those described for PS I measurements (Keck and Boyer 1974), except that DCMU, DCIP, and sodium ascorbate were omitted from the medium. The second was conducted according to the methods of Allen and Holmes (1986): measured electron transport following illumination through PS I and PS II (following addition of MV) and of PS I alone (following addition of ascorbate and DCIP to DCMU-inhibited thylakoids). The final concentrations in the reaction cuvette were 0.1 mM MV; 10 µM DCMU; 0.1 mM DCIP; 5 mM ascorbate; and 5 mM sodium azide.

3.0 RESULTS AND DISCUSSION

The initial phase of this project evaluated the reaction between S and TIP to form 0,0,0-triisopropyl-phosphorothiolate (TIPS). This reaction sequence (shown in Figure 3.1) is used to simulate the fundamental chemical reaction that occurs in binary nerve-gas weapons. The relative quantities of active components used to produce the reaction simulation are as follows: 23.3% BIS, 54.4% TIP, and 12.8% elemental sulfur. A chemical dye and dispersing agent make up the remaining portions of the mixture. Of these components, only TIP and S react to give the product TIPS. The dispersion pattern of simulant from the burst site is characterized by monitoring the fluorescence of the dye. The dispersing agent promotes the uniform dispersion of S, which is an essential condition for in-flight reaction.

FIGURE 3.1. Reaction Between TIP and Sulfur to Form TIPS

3.1 SYNTHESIS AND CHEMICAL ANALYSIS OF REACTION PRODUCTS

3.1.1 Synthetic Reaction Sequences

To study the TIP/TIPS reaction on the laboratory scale, the quantity of reactants were proportionately reduced. The first reaction sequence (Reaction I) involved the slow addition of TIP to S followed by the addition of BIS. The chemical structure of BIS is shown in Figure 3.2. Sulfur was placed in a 300-mL round-bottomed flask along with a stir bar. The TIP was slowly introduced through an addition funnel over a 1-h period. The temperature of the mixture increased from 25 to 75°C, clearly illustrating the exothermic nature of the reaction. Following the addition of TIP, a 2.0-mL aliquot was

FIGURE 3.2. Chemical Structure of BIS

removed for analysis by GC/MS. Next, BIS was slowly added over a 30-min period. The reaction mixture temperature remained constant during the addition of BIS, and there was no indication of further reaction.

The second reaction sequence (Reaction 2) was performed to more closely simulate the conditions of in-flight mixing. The BIS and S were slurried in a 300-mL round-bottomed flask. A 2.0-mL aliquot was removed from this mixture for subsequent GC/MS analysis. Next, TIP was quickly added via an addition funnel. The entire volume of TIP was added within 35 s. The evidence for reaction was dramatic. The reaction temperature quickly rose to exceed 140°C. Simultaneous with this temperature surge, the reaction mixture assumed a deep-orange color as the temperature exceeded the melting point of S (112°C). The excess S eventually solidified and precipitated out of solution as the reaction mixture slowly cooled. A sample for GC/MS analysis was removed from the reaction pot when the temperature had cooled to 85°C (15 min after the reaction).

3.1.2 GC/MS Analysis of Starting Materials and Reaction Products

GC/MS Analysis of BIS

Initial gas-chromatographic runs examined the purity of the starting materials. The total ion current chromatogram resulting from injection of BIS is shown in the top of

Figure 3.3. The mass spectrum of the single peak is shown below the chromatogram. This spectrum was found to match the reference spectra of 2-ethyl-1-hexanol. The possibility exists that 2-ethyl-1-hexanol was formed as an analytical artifact by the thermal degradation of BIS within the gas-chromatographic injection port. To test this hypothesis, a standard of 2-ethyl-1-hexanol was prepared and run under the same chromatographic conditions as the analysis for BIS. The total ion current chromatogram and mass spectrum resulting from this analysis are presented in the bottom of Figure 3.3. The nearly perfect match in both retention time and mass spectra between injections of BIS and 2-ethyl-1-hexanol confirm that the alcohol is formed by thermal degradation of BIS upon analysis by gas chromatography. To verify the purity of BIS, it became necessary to examine this material by HPLC. The HPLC results are described in a later section of this report.

GC/MS Analysis of TIP

The GC/MS analysis of TIP revealed the presence of three peaks. Results of this analysis are shown in Figure 3.4. The mass spectra of the three compounds are shown below the total ion current chromatogram in order of increasing retention time. The first eluting and most abundant peak is due to TIP, as evidenced by the molecular ion at m/e of 208. The identity of the later eluting peaks remains elusive. It is not clear if these compounds represent impurities in TIP or whether these compounds arise from the thermal degradation of TIP in the gas-chromatographic injection port.

Reaction Products

The GC/MS studies of the reaction mixtures served to confirm the original temperature observations recorded during the reactions. Analysis of TIP mixed with S (Reaction 1), a reaction which proved to be exothermic, showed no detectable TIP; however, a single product of TIPS was observed. The chromatogram and corresponding mass spectrum of this product are shown in Figure 3.5. The mass spectrum clearly shows the molecular ion of TIPS at m/e of 240. Analysis of the reaction mixture after the addition of BIS showed TIPS and a small peak caused by the presence of BIS. Analysis of the BIS and S mixture (Reaction 2) showed only the peak expected for BIS, indicating the lack of any reaction.

FIGURE 3.3. Total Ion Current Chromatograms and Spectra of BIS (top) and 2-Ethyl-1-Hexanol (bottom)

FIGURE 3.4. Total Ion Current Chromatogram of TIP. Spectra from top to bottom are in order of increasing retention time.

FIGURE 3.5. Total Ion Current Chromatogram and Mass Spectrum of the Reaction Product of TIP Mixed with Sulfur

Analysis of Reaction 2 after addition of TIP, a reaction which was decidedly exothermic, is presented in Figure 3.6. This analysis shows a small amount of BIS present in the final reaction mixture amidst a prominent quantity of TIPS. Because this reaction mixture reached high temperatures, it is not clear whether the mixture contains BIS or the thermal degradation product, 2-ethyl-1-hexanol. Studies to clarify this question involved analysis of Reaction 2 by HPLC and are described in a later section of this report.

Interestingly, of the three peaks which were observed during the analysis of TIP, only the compound with the longest retention time showed persistent presence after reaction with S. This persistence suggests that the second eluting peak (retention time of 14.45 min, Figure 3.4) was due to the thermal degradation of TIP in the injection port, while the least abundant component (retention time of 16.96 min, Figure 3.4) was due to an impurity present in the TIP stock solution. Several of the major fragments found in the spectrum of the suspected impurity (m/e of 99, 125, 141, and 167) were found offset at 16 higher mass units in the spectrum of TIPS (m/e of 115, 141, 157, and 183). These

spectral characteristics suggest that the impurity may be the oxygenated analog of TIPS shown in Figure 3.7.

3.1.3 HPLC Analysis of Reaction Products

As a result of the shortcomings of gas-phase analytical techniques for analyzing non-volatile and thermally labile compounds, the starting materials and Reaction 2 were examined by HPLC. If high-molecular-weight compounds form under the reaction conditions, these polymeric products should be detected by HPLC.

A chromatogram of Reaction 2 separated on a silica column is shown in the top of Figure 3.8. The mobile phase used for this separation was 20% ethyl acetate and 80% hexane.

FIGURE 3.6. Total Ion Current Chromatogram of Reaction 2. The mass spectrum of BIS is shown below the chromatogram. The major component is due to TIPS.

FIGURE 3.7. Tentative Structure of the Impurity Contained in TIP

FIGURE 3.8. Normal Phase Chromatograms of Reaction 2 (top), BIS (center), and 2-Ethyl-1-Hexanol (bottom)

The chromatogram shows a major component eluting shortly after the column dead volume; this component is due to TIPS as well as a component with a retention time of 10.63 min. The chromatogram in the center of Figure 3.8 resulted from an injection of BIS. It was shown by co-injection experiments that BIS and the component with a retention time of 10.63 min co-elute. Therefore, the results obtained during this experiment complement the results of GC/MS analysis. Both studies show Reaction 2 to consist of TIPS and BIS. Furthermore, HPLC analysis failed to show the presence of any high-molecular-weight material that could have formed through polymerization reactions at the high temperature achieved during reaction.

The analysis of BIS (Figure 3.8 center) showed only one peak, which is testimony to the purity of this starting material. An analysis of 2-ethyl-1-hexanol under identical conditions is shown in the bottom of Figure 3.8. It is immediately obvious that the BIS stock solution does not contain any 2-ethyl-1-hexanol. Because 2-ethyl-1-hexanol is not an impurity in BIS, this experiment provides decisive evidence for the thermal conversion of BIS to 2-ethyl-1-hexanol during gas-chromatographic analysis. Additionally, 2-ethyl-1-hexanol was not present in Reaction 2 (Figure 3.8 top), indicating that the temperature achieved during the reaction was not sufficient to promote thermal degradation of BIS to 2-ethyl-1-hexanol.

The compositions of Reaction 2 and BIS were also examined under reverse phase conditions. A chromatogram of Reaction 2 obtained with a mobile phase of 100% methanol is shown in the top of Figure 3.9. For comparison, an injection of BIS is shown in the bottom of Figure 3.9. This analysis further demonstrates that Reaction 2 contains predominantly TIPS (retention time of 4.84 min) and BIS (retention time of 6.33 min). The lack of nonvolatile components, which was observed under normal phase conditions, is further verified under reverse phase conditions. Because TIPS elutes shortly after the column dead volume under both reverse and normal phase chromatography, and because many components may co-elute at this retention time, it became desirable to search for chromatographic conditions that would retain TIPS. A chromatogram obtained on the octadecyl silica column of Reaction 2 with a mobile phase of 80% methanol and 20% water is shown in Figure 3.10. The mobile phase strength was not strong enough to elute BIS; however, the high purity of the product is apparent from the single peak caused by TIPS, which elutes with a retention time of 13.32 min.

FIGURE 3.9. Reverse Phase Chromatograms of Reaction 2 (top) and BIS (bottom)

FIGURE 3.10. Reverse Phase Chromatogram of Reaction 2 Illustrating the Purity of TIPS

3.1.4 Persistence of Reaction Products

Further GC/MS studies examined the stability of TIPS in the Reaction 2 matrix at 1, 20, 44, and 140 h after the reaction. There was no indication of any alteration or loss of TIPS from the reaction mixture as based on examination of the integrated peak areas.

The stability of both TIPS and TIP in aqueous solutions was examined in a preliminary manner under light and dark conditions. Solutions were made by diluting 10 μL of TIP or Reaction 2 solution to 50 mL of water. These solutions were sonicated to facilitate dissolution; however, it was apparent that all of the the TIPS did not dissolve in the water. The solutions were either exposed to 400 $\mu\text{Einstein}$ s of PAR (400 to 700 nm) or shielded from light by wrapping the volumetric flasks in aluminum foil. It was found by subsequent GC/MS analysis that there was no detectable TIP in either the light or dark experimental samples analyzed after 24 h. Evidently, TIP was rapidly destroyed in aqueous solutions. Interestingly, the impurity tentatively identified in the TIP stock solution was observed to persist in both the light and dark aqueous solutions. This blatant difference in chemical reactivity reinforces the conclusion that this component is

an impurity in the TIP stock solution. The results from the TIPS experiment indicate that TIPS is present in approximately one-tenth the concentration of a corresponding hexane solution of Reaction 2 after 24 h. These results are inconclusive because solubility problems were encountered when attempting to dissolve Reaction 2 in water. There was only a slight decrease in the peak area of TIPS in the light-exposed Reaction 2 solution (area of 52,660) when compared to the solution maintained in the dark (area of 62,398) after 24 h. Again, these results are difficult to interpret because the limited solubility of TIPS in water.

In summary, the TiP/TIPS reaction is extremely fast and goes to completion without the formation of detectable by-products. As based on this laboratory study, the components delivered to the environment by use of this binary system are the product TIPS, excess elemental sulfur, and BIS. Actual tests would be expected to disperse dye as well as the above-mentioned compounds.

3.2 POTENTIAL ENVIRONMENTAL EFFECTS

3.2.1 Effects of Binary Components on Algal Growth

The concentrations of TIPS, BIS, and TIP employed in the algal effects experiment were set at 20 ppm. This concentration was chosen to reflect both the concentrations reported at approximately mid-plume in field tests^(a), as well as their low solubility in water. Under these conditions, differences between the growth rate (number of cells per milliliter) of two algal species was determined.

Inoculum from log growth phase cultures were added to growth media containing the individual simulants. The data provided in Figure 3.11 are averages plus or minus standard deviation of three replicate cultures for each of the three simulants and controls. Microscopic examination and Coulter Counter oscilloscope patterns of the interim and 10-day-old cultures showed *Chlorella* to have significantly larger cell size and lower cell number per milliliter than *Selenastrum*. Precise quantitative sizing measurements were not possible, as the instrument was not configured for this type of data output; therefore, data regarding individual cell size will not be included.

(a) J. Allen, Program Manager, Dugway Proving Ground; personal communication with R. J. Fellows, Research Scientist, Pacific Northwest Laboratory.

The growth rate of control cultures of *Selenastrum* was substantially greater than that of *Chlorella*. When the inoculated cultures were exposed to simulants, there appeared to be a greater effect on *Chlorella* growth rate (Figure 3.11A) than on that of *Selenastrum* (Figure 3.11B). In *Chlorella*, exposure to TIP and TIPS resulted in a 50%

FIGURE 3.11. Growth of (A) *Chlorella* and (B) *Selenastrum* in the Presence of BIS, TIP, and TIPS

mean reduction in cell number after 10 days incubation compared to controls ($P \leq 0.1$). Although cultures were less affected by BIS, a 25% reduction in cell number was observed. The growth rate of BIS-treated cultures was not significantly different from that of the controls ($P > 0.1$). It should be noted that cultures had reached stationary phase by Day 9.

The *Selenastrum* cultures grew at a much greater rate than those of the *Chlorella* (Figure 3.11B versus 3.11A). There was a slight but significant ($P \leq 0.05$) lag in the initial growth rate of the TIPS cultures when compared to the others over the first 5 days of growth, but this lag was lost by the time the cells achieved stationary phase at 10 days post-inoculation (Figure 3.11B). The average cell number of cultures treated with simulant was actually slightly higher than that of the control at the end of the experiment, but this difference is not significant ($P > 0.1$). As noted above, there appears to be a rapid decomposition of TIP in an aqueous environment, yielding phosphoric acid. There are also reports (Captain S. R. Bennett, personal communication) that BIS is capable of producing a similar decomposition product in water. The availability of an additional phosphorus source in the media of these cultures from the phosphoric acid may have been partially responsible for the apparent slight stimulation in cell number.

Although growth rate (cell division), expressed in number of cells per milliliter, is one method of determining the effects of the simulants, overall dry-matter accumulation in the algal cultures may be affected, and thus provide indications of any adverse metabolic effects of these simulants. Dry-matter production was assessed in the 10-day cultures following filtration and drying. The dry-weight results are provided in Table 3.1. Dry-matter production results for *Chlorella* indicate no significant difference ($P \geq 0.2$) for BIS and TIP, compared to the controls. Only TIPS was significantly different from the control ($P \leq 0.1$) as determined by a two-tailed t-test. The dry-weight data, in conjunction with the cell number data, indicate that, although there were fewer cells in the TIPS cultures, the cell size was apparently larger (Coulter Counter oscilloscope patterns did indicate this but not significantly) and contained a higher specific weight. There were no significant effects on dry-matter production ($P > 0.1$) in the simulant-treated *Selenastrum*.

TABLE 3.1. Final Dry Weight of *Chlorella pyrenoidosa* and *Selenastrum capricornutum* Cultures Grown in Freshwater Media^(a) Containing 20 ppm of Either BIS, TIP, or TIPS. Data are averages.

Species	Simulant			
	BIS	TIP	TIPS	Control ^(b)
Avg. Dry Wt. (g) $\pm$ SD (n=3)				
<i>Chlorella</i>	0.032 $\pm$ 0.012 ^(c)	0.039 $\pm$ 0.004 ^(c)	0.034 $\pm$ 0.00 ^(d)	0.042 $\pm$ 0.005
<i>Selenastrum</i>	0.041 $\pm$ 0.003 ^(c)	0.040 $\pm$ 0.007 ^(c)	0.036 $\pm$ 0.004 ^(c)	0.042 $\pm$ 0.001

(a) Sixty milliliters of Alga-gro®-Freshwater, Carolina Biological Supply Co., Burlington, North Carolina, containing 3.0 mg/mL (w/v) Na-bicarbonate.

(b) Inoculated with sterile distilled water.

(c) Not significant in two-tailed t-test, $P > 0.2$.

(d) Significant in two-tailed t-test, $P \leq 0.1$.

3.2.2 Effects on Plant Electron Transport

Isolated Chloroplasts

Preliminary studies to assess the potential adverse effects of these simulants on higher plant metabolism were performed using isolated chloroplasts. Chloroplasts from spinach (*Spinacea oleraceae*) were employed as a surrogate system because procedures exist for the routine isolation of high-quality chloroplasts. This *in vitro* system, which measures the effects of chemicals on photosynthetic electron transport processes, can provide useful information related to potential metabolic toxicity and has been used previously for simulant evaluation (Cataldo et al. 1988; Cataldo et al. 1989^(a); Van Voris et al. 1987), as a representative plant species.

The electron transport chain of the light-reaction photosystems consists of two separate photoacts, or photosystem reaction centers (PS I and PS II), with accompanying light-harvesting pigment/protein complexes, both apparently located on the interior of the thylakoid membrane, although PS I may be closer to the outer, or

(a)Cataldo, D. A., M. W. Ligothke, B. D. McVeety, R. J. Fellows, S. W. Li, and P. Van Voris. 1989. Draft Final Report, Acute Environmental Toxicity and Persistence of BIS, a Chemical Agent Simulant: BIS, (2-Ethylhexyl) Phosphonate.

stromal, side. The water-splitting site is located on the inner thylakoid membrane, while the other end of the redox potential gradient, the site of NADH₂ production, is located on the outer stromal surface. A number of intermediate electron carriers span the membrane and aid in the transfer of protons to the interior of the thylakoid. In previous studies with BIS(a), no significant effects were observed on any of the electron transport systems of the isolated chloroplasts, so this compound was omitted from the experiments for these exposures. To determine possible site(s) of action by the TIP and TIPS, measurements were taken from both photosystems as well as the intact chain. The results, expressed as percent of measurements obtained from controls in paired experiments, are given in Table 3.2.

TABLE 3.2. Interaction of 10-ppm TIP and TIPS with Electron Transport Systems of Isolated Spinach Chloroplasts(a)

Simulant	Electron Transport System		
	Whole Chain	Photosystem I	Photosystem II
	% Control $\pm$ SD (n=3)		
TIP	99.41 $\pm$ 13.1(b)	99.43 $\pm$ 10.2(b)	93.96 $\pm$ 10.5(b)
TIPS	100.3 $\pm$ 8.9(b)	100.5 $\pm$ 25.0(b)	91.1 $\pm$ 11.7(b)

(a) In vitro amendment of simulant, exposure duration approximately 5 min. Data are avg $\pm$ SD, n=3.

(b) Not significant based on two-tailed t-test, P>0.1.

At TIP and TIPS concentrations of 10 ppm, no significant effects (P>0.1) were noted on either the two photosystems separately or the coupled, whole chain. There was a slight indication of a potential uncoupling of transport to PS I with the addition of ascorbate and DCIP to TIPS-treated chloroplasts, but this was not significant (P>0.2) at these exposure levels. A higher exposure level for the TIP and TIPS was not attempted because of their low water solubility.

(a) Cataldo, D. A., M. W. Ligothke, B. D. McVeety, R. J. Fellows, S. W. Li, and P. Van Voris. 1989. Draft Final Report, Acute Environmental Toxicity and Persistence of BIS, a Chemical Agent Simulant: BIS, (2-Ethylhexyl) Phosphonate.

4.0 CONCLUSIONS

The final products delivered to the environment by use of this binary system would be TIPS, excess elemental sulfur, and lesser quantities of BIS. The reaction is rapid and nearly complete. No significant quantities of secondary reaction products were observed.

There is an apparent species sensitivity difference in algae for the simulants BIS, TIP, and TIPS, with *Chlorella* being more sensitive than *Selenastrum* based on cell number studies. However, the extent of adverse effects was not excessive. *Chlorella* exhibited a reduced growth rate in terms of cell numbers (i.e., cell division) in the presence of 20-ppm TIP and TIPS, but not BIS. This may indicate an effect on the cell division process in this species caused by the TIP portion of both simulants. Only the TIPS produced a more extensive metabolic effect, however (reduced yield, dry weight), and even this was not dramatic. In the *Selenastrum* only a slight lag in growth was evident in the TIPS cultures, and no differences were apparent in the final yield.

There was no apparent effect of TIP or TIPS on the electron transport systems of isolated chloroplasts at the concentration tested (10 ppm). While higher concentrations may promote an uncoupling of the system, these may not be observed within the cell because of the low water solubility of both simulants and the apparent short half-life of the TIP in an aqueous environment.

The binary mixture undergoes rapid and nearly complete reaction. The final products released to the environment are TIPS and excess elemental sulfur. In general, it is unlikely that environmental release of these products would have significant or lasting effects, based on the preliminary algal tests and electron transport studies.

5.0 REFERENCES

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APPENDIX A

QUALITY ASSURANCE AND QUALITY CONTROL

APPENDIX A

QUALITY ASSURANCE AND QUALITY CONTROL

At the Pacific Northwest Laboratory (PNL), quality control (QC) is the system of activities that ensures a quality product. Quality assurance (QA) is the system of activities that ensures that the quality control system is being performed adequately. The prime responsibility for QA/QC is placed on the Program Manager, Principal Investigator, and Task Leaders; however, the QA audit function is maintained outside of the project and is not directly supported by the project. This audit function was performed by Rob Cuello, the Earth and Environmental Sciences Center's senior Quality Assurance Officer, who was outside the control of the Program Manager or the Principal Investigator.

QUALITY ASSURANCE

The objective of the QA program for this study was to ensure the production of precise and accurate (within the stated acceptance criteria), representative, comparable, traceable, and defensible data. To ensure that these conditions were met, PNL's QA program consisted of the following elements:

- review and approval of standard operating procedures
- personnel training
- pretest preparation
- QC of sampling function and analysis function
- QA audits
- feedback and corrective action (if necessary).

Quality Assurance Training

The activities of the QA training program included 1) introducing personnel to the QA program, 2) identifying training elements, 3) preparing and training supervisory and test personnel, 4) implementing QA audits, and 5) conducting refresher QA training or on-the-job training as needed.

The project team reviewed the procedures that governed the collection and interpretation of field and/or laboratory data and familiarized themselves with what audit procedures were to be used. Supervisory personnel required some training to learn the QA procedures that were employed.

Standard Operating Procedure Review and Approval

Standard operating procedures (SOPs) for this program were developed by the Task Leaders, approved by the Principal Investigator, and reviewed to determine what quality control steps would be incorporated. Each team member used the procedures outlined in the SOPs to

- prevent contamination,
- provide the proper sample size,
- ensure proper taxonomic identification,
- provide the proper kind and number of blanks,
- maintain standardization of measuring equipment, and
- guarantee the keeping of usable records.

Quality assurance also ensured that current SOPs were maintained for the program. Whenever it became evident that the data being collected were not sufficiently accurate or appropriate for the intent of the program, the SOP was modified and approved by the Program Manager.

Laboratory Record Books

Work performed on the project was documented in laboratory record books by project personnel. A signature sheet that identified the personnel contributing data to the record book and an assignment form (located inside the front cover of the record book) that indicated the project number and the individual to whom the book was assigned was completed. At the end of the project, the record books and forms were returned to Document Control to be archived.

Sample Identification and Traceability

A system of accountability was used to control the number and variety of samples and the quality of data generated on the research project. The Principal

Investigator helped to design the initial system and, because he worked most closely with the experiments, helped monitor the system's effectiveness. Quality assurance, at this stage of the project, is used to ensure that the samples are completed and are appropriate. Therefore, the QA Officer had the following responsibilities:

- participated in the development of a system for keeping records of samples and for introducing proper QA steps to ensure dependability of the system
- reviewed sample log system and implementation forms that provide information on samples collected and indicate the work to be done on the samples
- monitored the sample identification system to ensure proper labeling of samples, proper dispersements of samples for analysis, and proper quality control of spikes, blanks, and duplicates.

The Principal Investigator and the Task Leaders were responsible for seeing that the documentation was completed either by themselves or by the team members. A team member was usually assigned the responsibility of handling the samples and storing, retrieving, dispersing, and maintaining records of those samples.

QUALITY CONTROL

The key QC operations emphasized in the program were procurement QC, standardization/calibration, sampling, and analysis.

Procurement Quality Control

Task Leaders were responsible for ensuring that all procured materials (e.g., samples, collection containers) conformed to appropriate specifications. They were also responsible for ensuring that reagents and chemicals with limited shelf-life were identified and used before the specified expiration date.

Sampling Quality Control

A sampling-information document was developed by the Task Leader and used to detail the kind of samples that were taken, the locations where the samples were taken, the time and duration of sampling, the size of samples to be taken, and other pertinent information on the conditions that are useful to the sampling team. From this information, the sampling team selected the labor hours and apparatus necessary to carry out the sampling task, and followed the appropriate SOP.

The sampling team then developed a QA plan that included 1) sampling information forms; 2) lists of apparatus, reagents, and supplies; 3) presampling calibrations; 4) onsite checks of apparatus; and 5) post-sampling calibrations.

Analytical Quality Control

Analytical quality was monitored through function checks and control checks. Function checks are performed by the analyst who verified the stability and validity of the sample and the performance of the analytical equipment. Sample validity was assessed in terms of spoilage, container integrity, amount of specimen, sample identification, sample blanks, and general appearance issues, such as condition of filters or uniformity of sample collection across a filter.

The analytical equipment was checked in terms of calibration and performance of calibrating standards; the latter is part of the permanent record of the analysis. It was recommended that calibration standards that span the working range in factors of two should be run through the entire analysis system at least four times. This develops information on precision and detection limits where appropriate.

Control checks were made by analyzing samples provided by the Task Leader. These samples included blanks, duplicates, spikes, and, if available, standard reference materials in quantities that depended on the total number of samples assigned and on the level of accuracy needed in the analysis. These control samples were introduced into the system in such a manner that the analyst did not give them particular attention.

As a rule, when processing large batches of samples (25 samples or more), a control (spike, blank, or replicate) sample should be run after every five samples. A

small batch of samples (up to five samples) may have more control samples than test samples.

For large and continuous amounts of samples, control charts of performance of duplicates and spikes must be maintained. This allows the analyst and Task Leader to know when the system is out of control, which part of the system is the probable cause, and when and what corrective action is to be taken.

APPENDIX B

EXPERIMENTAL PROTOCOL

STUDY COMPONENT: CHEMICAL ANALYSIS OF BIS, TIP, AND TIPS

APPENDIX B

EXPERIMENTAL PROTOCOL

STUDY COMPONENT: CHEMICAL ANALYSIS OF BIS, TIP, AND TIPS

PURPOSE

Analytical procedures were needed to evaluate the reaction between TIP and sulfur to produce TIPS. Requirements of an appropriate methodology were documentation of the purity of starting materials as well as the ability to characterize the composition of the final reaction mixture. Initial analyses were performed by gas chromatography combined with mass spectrometry (GC/MS). Because of the thermal lability of several analytes and the need to analyze for nonvolatile high-molecular-weight analytes, HPLC procedures were implemented in conjunction with the GC/MS methods.

ANALYTICAL METHOD

The GC/MS method employed a Hewlett Packard 5880 capillary gas chromatograph coupled with a Hewlett Packard 5970A mass selective detector. Samples were introduced to a J&W DB-Wax column (30 m x 324 μ m ID) by 2- μ L splitless injections. The injection splitter was opened 0.4 min after injection and remained open for the remainder of the chromatographic run. The fused silica column contained a 0.25- μ m film of polyethylene glycol stationary phase. The stationary phase was immobilized by cross linking and further stabilized by covalent bonding with the silica surface. This polar stationary phase is suitable for the analysis of polar analytes and has the additional advantage of being tolerant of direct aqueous injections. The components were separated by temperature programming the column from 40 to 250°C at 6°C/min. The injection port and transfer line to the mass spectrometer were maintained at 150 and 250°C, respectively. Nominal resolution mass spectra were generated by scanning the quadupole mass spectrometer from 400 to 600 amu at a rate of 200 amu/s. Before each sample set, the mass spectrometer was tuned with perfluorotributylamine (PFTBA).

The HPLC analysis employed a Waters model 510 pump and solvent delivery system. Isocratic mobile phase was delivered at a flow rate of 3.0 mL/min to either an

Ultrasphere-ODS (24 cm x 10 mm ID) or an Ultrasphere-Si (24 cm x 10 mm ID) column, depending on whether the separation mode was reverse or normal phase, respectively. Because the analytes lacked chromophores, it was necessary to use a refractive index detector (Waters model 401 differential refractometer). A Wisp 712 automatic injector was used to deliver samples ranging from 10 to 60 μ L to the column.

METHOD CALIBRATION

Absolute quantitation was not an objective of the present study; however, semi-quantitative comparisons of the peak areas generated during GC/MS runs were used to assess the stability of reaction materials and products in hexane and water solutions. To ensure the validity of these studies, the comparisons were of solutions prepared in an identical manner (10.0 μ L of solution diluted to a final volume of 50.0 mL with water or hexane). These samples were analyzed under identical GC/MS conditions including reference to the same PFTBA tune.

SAMPLE PREPARATION

Aliquots of starting materials or reaction products (10.0 μ L) were diluted to a total volume of 50.0 mL in either hexane or water for GC/MS analysis. The HPLC samples were diluted with three parts methanol for reverse phase separations, or with three parts hexane for normal phase separations. All samples were transferred to amber vials fitted with Teflon-lined caps and analyzed immediately.

APPENDIX C

EXPERIMENTAL PROTOCOL

STUDY COMPONENT: QUANTIFICATION OF PLANT METABOLIC EFFECTS OF CHEMICAL SIMULANTS

APPENDIX C

EXPERIMENTAL PROTOCOL

STUDY COMPONENT: QUANTIFICATION OF PLANT METABOLIC EFFECTS OF CHEMICAL SIMULANTS

PURPOSE

The phytotoxicity of TIP and TIPS on whole plants was investigated using *in vitro* systems. These included 1) the effects of the simulant on photosynthesis (oxygen evolution) and dark respiration (oxygen uptake) in intact leaf segments, and 2) the effects of the simulant on specific photochemical reactions and electron transport chains in isolated chloroplasts.

ANALYTICAL METHODS

Isolated Chloroplast Measurements

Chloroplasts were isolated from commercially obtained spinach (*Spinacea oleracea*) leaves according to the methods of Walker (1980). Approximately 80 g of leaves with the mid-ribs removed were washed with distilled water and chilled before grinding. The leaves were then ground for 10 s with a sorvall tissue homogenizer in 50 mL of grinding medium, consisting of 0.33 M sorbitol; 10 mM $\text{Na}_4\text{P}_2\text{O}_7$; 5 mM MgCl_2 ; and 2 mM sodium ascorbate, pH 6.5, which had been chilled to a slush-like consistency to maintain the grinding temperature around 4°C. The ground material was then filtered through eight layers of cheesecloth and the filtrate immediately centrifuged at 1500 x g for 90 s. The supernate was then decanted and the surface of the pellet washed with 1 mL of resuspension mix, which was then discarded. The pellet was resuspended in a mixture consisting of 0.3 M sorbitol; 2 mM Na_2EDTA ; 1 mM MgCl_2 ; 1 mM MnCl_2 ; 50 mM HEPES; 10 mM NaHCO_3 ; 5 mM PPi ; and 0.5 mM P_i , pH 7.6. Chlorophyll content was determined according to the method of Arnon (1949): 50 μL of the chloroplast suspension was added to 20 mL of 80% (v/v) acetone and filtered (through No.1 Whatman paper), and the absorbance read at 652 nm. Nine divided by the absorbance gives the volume in microliters of the original suspension containing 100 μg of chlorophyll. All procedures were carried out under low light and at 4°C.

Photochemical Assays

Assays were conducted on PS II, PS I, and whole-chain electron transport, measuring oxygen evolution and uptake with a Clark-type electrode (YSI Instruments, Yellow Springs, Ohio) in a 1.8-mL volume, water-jacketed cuvette (Gilson Medical Electronics, New Hartford, Connecticut) maintained at 20°C. Stock solution of the BIS was prepared so that addition of 100 µl would equal a final concentration within the cuvette of 1 or 10 ppm. All assays were conducted in paired cuvettes at the same time, with one cuvette serving as a control and the other containing the simulant. The TIP or TIPS was either added directly to the cuvette before illumination (~1 min) or to a chloroplast suspension in a test tube for 1 h before transfer to the cuvette for assay. Control chloroplasts were treated in the same manner. Assays were run in triplicate, and all data are expressed in either µMol O₂/h/mg chl or as percent control of the paired assay. The analyses of the three components of the chloroplast electron transport system were performed according to the following methods:

- PS II Measurements. Assays were conducted according to the methods of Boyer and Bowen (1970). The assay medium (1.8 mL) consisted of 0.33 M sorbitol; 2 mM Na₂EDTA; 1 mM MgCl₂; 1 mM MnCl₂; and 50 mM HEPES, pH 7.6. Sodium 2,6-dichloroindophenol (DCIP), 0.88 mM, was added just before the addition of chloroplasts (100 µg). The suspension was then illuminated from the side with saturating light (>1200 µEinstein s⁻¹) at 600 nm, and the rate of oxygen evolution determined from the initial slope of the electrode output as a function of time.
- PS I Measurements. Assays were conducted according to the methods of Keck and Boyer (1974). The assay medium consisted of 1 mM ADP, 1 mM K₂HPO₄, 0.1 M KCl, 5 mM MgCl₂, 0.1 mM DCMU, 80 µM DCIP, 1 mM sodium ascorbate, 0.5 mM methylviologen (MV), 0.5 mM sodium azide (prepared daily), and 100 µg chlorophyll. Assays were illuminated and measured as above.
- Whole-Chain (Water to MV) Measurements. Assay conditions were identical to those described for PS I measurements (Keck and Boyer 1974), except that DCMU, DCIP, and sodium ascorbate were omitted from the medium.

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