Isolation, Characterization, and Toxicity of (*O,O'*-Dimethyl Phosphorodithioato)copper Complexes

The products of reaction between *O,O'*-dimethyl phosphorodithioate (DMPDT), a degradation product of malathion and dimethoate, and copper(II) have been identified. (*O,O'*-Dimethyl phosphorodithioato)copper(I), Cu_2DMPDT_2, is the major product of reaction, and it has been isolated and characterized, and its toxicological properties have been evaluated. Cu_2DMPDT_2 exhibits unusual behavior when dissolved in chloroform; it immediately oxidizes and forms bis[(*O,O'*-dimethyl phosphorodithioato)]copper(II), $CuDMPDT_2$. This has been verified using electron spin resonance and visible spectroscopy. Since mollusks are very sensitive to even small amounts of copper(II), the toxicity of Cu_2DMPDT_2 has been evaluated. Cu_2DMPDT_2 exhibits no toxicological effects toward the snail (*Australorbis glabratus*) and, therefore, would be ineffective in the control of bilharzia (schistosomiasis). $CuDMPDT_2$, another product of reaction, cannot be isolated from a mixture of phosphorodithioates. ^{31}P NMR unequivocally demonstrates a mixture of four phosphorodithioate and two phosphonate reaction products in acetone.

Malathion [*O,O'*-dimethyl *S*-(1,2-dicarboethoxyethyl) phosphorodithioate] and dimethoate [*O,O'*-dimethyl *S*-(*N*-methylcarbamylmethyl) phosphorodithioate] are two organophosphorus pesticides which are widely used and exhibit low mammalian toxicity. Malathion is registered in Puerto Rico for use on sugar cane and pepper. In addition, it is used in San Juan for the control of mosquito larvae. Dimethoate is registered for peppers and tomatoes.

The degradation products of malathion have received considerable recent attention (Wolfe et al., 1977; Bradway and Shafik, 1977). Dimethoate has also been studied and the degradation products characterized (Hassen et al., 1969). All the studies have shown that *O,O'*-dimethyl phosphorodithioate (DMPDT) is an important degradation product. Wolfe et al. (1977) in particular have verified that at 27 °C malathion will undergo 74% elimination to give *O,O'*-dimethyl phosphorodithioic acid and ethyl hydrogen fumarate. Since this temperature is similar to those commonly found in tropical aquatic ecosystems, one might expect an elevated concentration of *O,O'*-dimethyl phosphorodithioate (DMPDT). The species as such, however, might be short lived since DMPDT is an excellent chelating agent. Studies have shown that (DMPDT) will readily complex with lead, copper, cadmium, and thallium (Kakovsky, 1957; Tulyupa, 1969). We will address ourselved in this study to the products of reaction between copper(II) and *O,O'*-dimethyl phosphorodithioate.

EXPERIMENTAL SECTION

Materials. *O,O'*-Dimethyl phosphorodithioate ammonium salt (NH_4DMPDT) was obtained from Aldrich Chemical Co. and recrystallized from ethyl acetate. Copper sulfate was obtained from Merck and used directly. Chloroform was obtained from Fisher, distilled under vacuum, degassed, and dried with magnesium sulfate prior to use. Spectral grade acetone (Fisher) was used without further purification.

Synthesis of (*O,O*-dimethyl phosphorodithioato)copper(I) reference compound (Cu_2DMPDT_2). To an excess of *O,O'*-dimethyl phosphorodithioate ammonium salt in a saturated aqueous solution of SO_2, 1 mL of sulfuric acid was added. Copper sulfate was then added slowly with continuous stirring. This produced a white precipitate which was removed, washed with acetone, and then dried in a vacuum desicator. Elemental analysis yielded % C = 10.88, % H = 2.75; % Cu = 28.8%. The theoretical values for Cu_2DMPDT_2 yield % C = 10.88; % H = 2.74; % Cu = 28.79%. The elemental analyses for carbon and hydrogen were performed by Atlantic Microlab Inc., Atlanta, GA. The percent copper was calculated by comparing the absorption of Cu_2DMPDT_2 dissolved in nitric acid with the absorption of copper standards prepared from elemental copper. All atomic absorption values were obtained on a Varian AA-5 atomic absorption spectrophotometer. A single slot burner designed for use with an air-acetylene flame was employed. A standard manganese, copper, chromium hollow cathode lamp was used as the source, and the copper resonance line at 3427 Å was used for all measurements.

In order to provide a standard of comparison the infrared spectrum of Cu_2DMPDT_2 (Figure 1) was obtained on a Perkin Elmer Model 283 spectophotometer using the micro KBr disc technique. A comparison of the spectrum with that of the homologous (*O,O*-diethyl phosphorodithioato)copper(I) complex clearly substantiated the structural assignment (Sakata and Nanjo, 1970).

Spectral Analysis. Visible spectra were obtained on a Perkin Elmer, Hitachi 200, recording spectrophotometer; 10 mm quartz cells were used for all measurements. ^{31}P NMR spectra were obtained on a JEOL FX-90Q NMR spectrometer, operating at 36.20 MHz with internal deuterium lock. Two hundred scans were accumulated using 60° pulses (18 μs), with 0.5-s delays between pulses. A spectral width of 5 KHz was employed with complete proton decoupling. Phosphoric acid was used as the external reference. Electron spin resonance spectra were obtained utilizing the X band of a Varian E-9 spectrometer equipped with dual cavity. A field of 3400 G and a scan width of 1000 G were employed. A microwave power of 1.5 mW and a modulation frequency of 1.6 G were selected for all experiments.

Toxicity Studies. In the standard laboratory procedure, snails (*A. glabratus*) were placed in glass jars with ten specimens per jar in 2 L of solution. Twelve jars were used, with a tapwater control and three different concentrations of Cu_2DMPDT_2 (1, 0.5, 0.1 ppm), each run in triplicate. The ppm solutions of Cu_2DMPDT_2 were prepared by mixing 30 mg of Cu_2DMPDT_2 with 300 mL of water and then adding aliquots of 20, 10, and 2 mL of the standard solution to the jars. *A. glabratus* snails were exposed to Cu_2DMPDT_2 for a full 72 h with observations recorded at 48 and 72 h. The test consisted of three stages: (a) applying the chemical, (b) at the end of 72 h rinsing the containers and the snails three times, and (c) placing the snails in 2 L of tapwater with lettuce or snail food for 4 days of subsequent observation. Death was defined as

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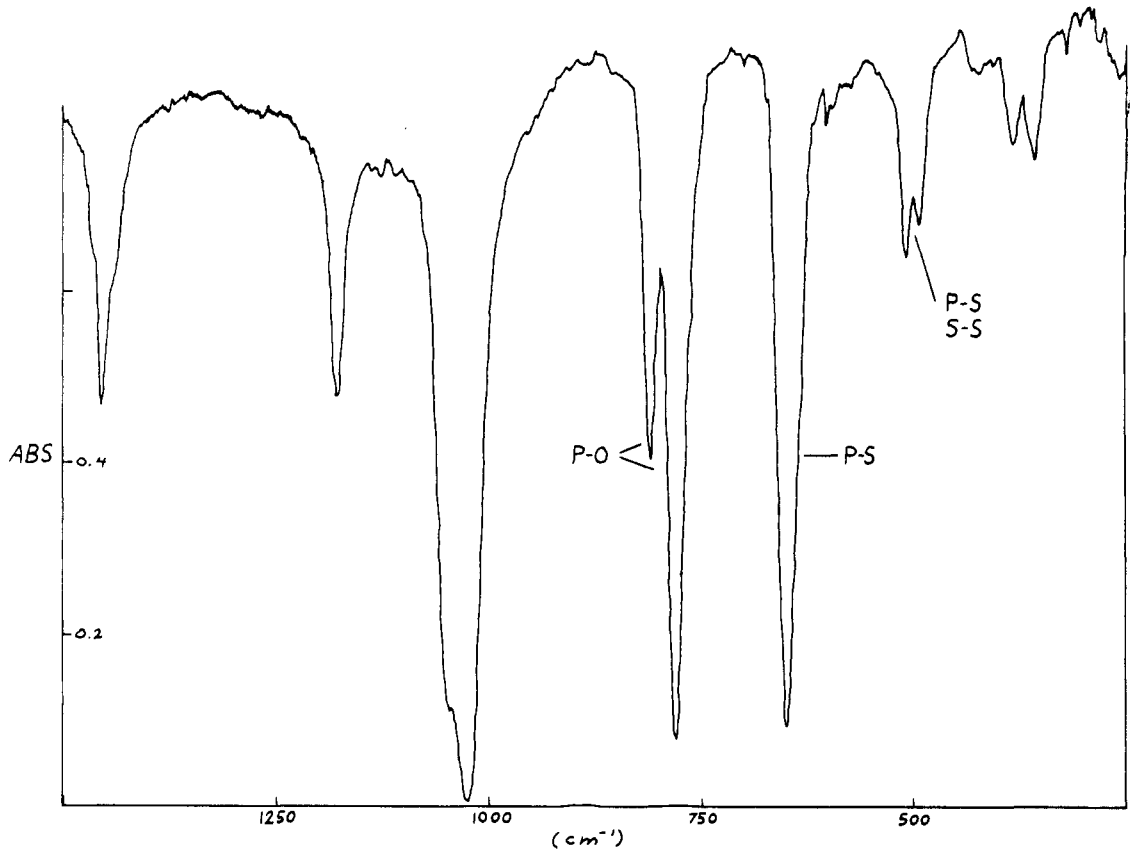

Figure 1. Infrared spectrum of *O,O'*-dimethyl phosphorodithioato) copper(I).

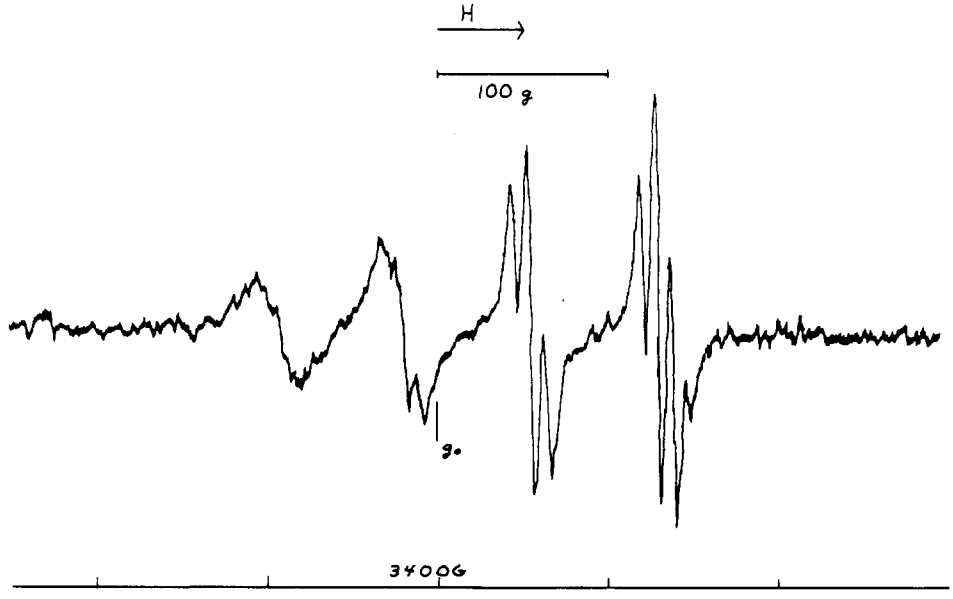

Figure 2. Electron spin resonance spectrum of bis[(*O,O'*-dimethyl phosphorodithioato)copper(II)].

continuous, submerged retraction of the snail into its shell on the third or fourth day.

Reaction of Copper with DMPDT. Copper sulfate was added to an excess of *O,O'*-dimethyl phosphorodithioate ammonium in distilled water. The reaction products were separated by centrifuging the solution and decanting the supernatant. The aqueous solution was presumed to contain unreacted species and was not subject to further study. The residue contained the products of reaction and was washed first with water and then repeatedly with acetone. The products of reaction were divided into an acetone-soluble fraction and an insoluble residue. Each was then examined using available spectroscopic techniques in order to determine the nature of the reaction products.

RESULTS AND DISCUSSION

$CuSO_4$ and NH_4DMPDT are mixed in an acidified water solution. One milliliter of H_2SO_4 ensures that copper oxide does not form and also that the amount of NH_3 present will be negligible. Furthermore, DMPDT is subject to alkaline hydrolysis at pH 8 (Wolfe et al., 1977), and the low pH will prevent the formation of *O,O'*-dimethyl phosphorothionate.

Table I. Chemical Shift (δ in ppm from H_3PO_4) for ^{31}P Nuclei, Completely Decoupled, in Acetone-d_6[a]

no.	ppm[b]	int. intensity[d]
1	-98.2	2
2	-95.1	1
3	-93.6	2
4	-93.4	5
5	-6.6[c]	1
6	-6.5	2

[a] Instrumental parameters in text. [b] The ppm shift of numbers 1-4 correlates well with that of phosphorodithioates; the ppm shift of numbers 5 and 6 correlates well with that of phosphonates (see Grayson and Griffith, 1969). [c] The NMR absorption at -6.6 ppm does not appear initially, but increases with time. [d] Integrated intensity after 15 min.

Under these conditions one observed the formation of a yellow-white precipitate. Repetitive washings of the precipitate with acetone yield a white precipitate corresponding to Cu_2DMPDT_2. The infrared spectra reveal the characteristic absorption bands due to the P-O and P=S stretching vibrations.

Cu_2DMPDT_2 exhibits limited solubility in H_2O and acetone; however, in $CHCl_3$ one observes the formation of a yellow solution, which has a maximum absorbance at approximately 418 nm. This absorbance maximum corresponds well with the maximum reported for $CuDMPDT_2$ by Hill (1969). The formation of $CuDMPDT_2$ from Cu_2DMPDT_2 in $CHCl_3$ contradicts, however, the statement of Hill that "the material Cu_2DMPDT_2 shows no absorption in the visible range of wavelengths".

In order to substantiate the presence of Cu^{2+}, we ran the ESR spectra, which did indicate the presence of a bischelate structure for the complex (see Figure 2).

A comparison of the ESR spectrum of our compound in $CHCl_3$ with bis[(*O,O'*-diethyl phosphorodithioato)copper(II)] in anisole (Wasson, 1971) and various bis[(*O,O'*-dialkyl phosphorodithioato)copper(II)] complexes in toluene (Yordanov and Shopov, 1971) confirms the structure in $CHCl_3$ as bis[(*O,O'*-dimethyl phosphorodithioato)]copper(II).

We were perplexed by the fact that Cu_2DMPDT_2, although stable to acetone and water, was forming Cu^{2+} in $CHCl_3$. In order to eliminate the possibility of photochemically or thermally induced oxidation, the extraction of Cu_2DMPDT_2 was repeated at -77 °C in the absence of light under vacuum line conditions (pressure 10^{-3} torr). $CuDMPDT_2$ was repeatedly formed as evidenced by the characteristic line pattern in the ESR spectrum. We could only assume that Cu_2DMPDT_2 underwent autoxidation or oxidation by the chlorinated solvent. The mixing of Cu_2DMPDT_2 with $CHCl_3$ appeared to be a mildly exothermic reaction.

Since Cu_2DMPDT_2 is the major reaction product formed when Cu^{2+} and *O,O'*-dimethyl phosphorodithioate are reacted, it is important to evaluate the toxicity of this compound. Since mullusks are very sensitive to even small amounts of copper(II), we have decided to evaluate the response of the snail (*A. glabratus*) to various concentrations of Cu_2DMPDT_2. The results of the toxicity studies indicate that Cu_2DMPDT_2 does not exhibit toxicological properties toward *A. glabratus*. Even at a concentration of 1 mg/L and with an exposure time of 72 h there was zero mortality among the snails. This should be compared with the toxicity of copper sulfate which will kill 99.5% *A. glabratus* after 6 h of exposure (Jobin and Unrau, 1967).

The acetone-soluble fraction can now be characterized. The ^{31}P NMR of the extract indicates that there were no less than four different phosphorodithioate species (on the basis of the ppm shift downfield from H_3PO_4) and two phosphonate species (see Table I). The absorption at -98.2 ppm relative to H_3PO_4 can be assigned to that of unreacted starting material, ammonium *O,O'*-dimethyl phosphorodithioate. NH_4DMPDT exhibits an absorption at -99 ppm relative to triethylphosphate in benzene-d_6 (Wilson, 1979). The absorptions at -93.6 and -93.4 ppm could be assigned to $CuDMPDT_2$. This assignment is based on a comparison of the spectrum with that of bis[(*O,O'*-diethyl phosphorodithioato)copper(II)] in acetone-d_6, which shows two absorptions at -88.8 ppm (relative intensity = 10) and -88.4 ppm (relative intensity = 1). Hill (1969) has suggested that an equilibrium exists in chloroform: Cu_2DMPDT_2 + bis(dimethoxyphosphorothiono) disulfide $\rightleftarrows$ $CuDMPDT_2$. Crude bis(dimethoxyphosphorothiono) disulfide absorbs at -89 ppm (Lippman, 1966). No absorption corresponding to this species is observed in the acetone extract. We have run the ^{31}P NMR of Cu_2DMPDT_2 in $CDCl_3$ at 20 °C and the compound forms a colorless solution absorbing at 105.7 ppm (relative intensity, 1) and -104.9 ppm (relative intensity, 7). The acetone extract containing $CuDMPDT_2$ shows no absorption corresponding to this species. We can only assume that $CuDMPDT_2$ does not equilibrate to form Cu_2DMPDT_2 and bis(dimethoxyphosphorothiono) disulfide in acetone. The remaining ^{31}P-NMR absorption due to a phosphorodithioate at -95.1 is unassigned.

The acetone-soluble fraction is not stable with time. The UV-visible spectrum of the acetone extract reveals two absorption maxima (414 and 418 nm) immediately after washing the reaction products. After 24 h, the absorption maximum at 414 nm does not appear, and apparently one of the initial products of reaction has decomposed. ^{31}P-NMR data also indicate compound instability in the acetone extract. After 15 min the peaks at 98.2 and 93.4 ppm diminish and a new absorption appears at 6.6 ppm. This indicates an oxidation of two phosphorodithioates to a phosphonate species. We have not tested the toxicity of the acetone extract since we could not isolate and purify the $CuDMPDT_2$ known to be present.

SUMMARY

The products of reaction between DMPDT, a degradation product of dimethyl phosphorodithioate pesticides and copper(II) have been identified. Cu_2DMPDT_2 has been isolated and characterized, and its toxicological properties have been evaluated with relation to snails. $CuDMPDT_2$ and other minor products of reaction have been qualitatively identified on the basis of ESR, ^{31}P NMR, and visible spectroscopy. $CuDMPDT_2$ cannot be tested as far as its toxicological properties since it decomposes in organic solvents, forming a mixture of products.

This work should serve as a stimulus for further investigation into the fate of pesticides and their metabolites in the presence of trace metals. There has been a paucity of data in this area to date, especially with regard to the organophosphorus pesticides. Since these pesticides are increasingly being used, there is a need to isolate and characterize all the probable products of reaction between organophosphorus pesticides and environmentally important trace metals.

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Comparison of Several Types of Cocoa Beans Relative to Fractionated Protein Components

Protein of seed cotyledons in fruit samples from the clonal collection of CATIE, Turrialba, Costa Rica, were extracted into urea buffer, purified on a column of Sephadex G-25, and separated into seven protein groups on a column of SP-25, cation-exchange resin. Among ten samples of Criollo, Trinitario, Nacional, and Forastero cocoa beans, statistically significant ($P \leq 0.01$) differences were found in total and extractable protein contents, in protein group profiles, and in amino acid patterns. Although differences were found among known types of cocoa, they were not considered great enough to suggest the procedures of this investigation as a practical approach to classifying cocoa of unknown genetic origin.

From an evolutionary viewpoint, commercial cocoa has developed from two types of beans, Criollo and Forastero of the species *Theobroma cacao*. Indigenous to the Amazon River basin, cacao has been transplanted to other tropical regions over the past century, most notably West Central Africa. Natural adaptation and breeding programs, directed toward disease resistance and increased production, now yield genetically heterogeneous commercial crops. Production has not kept pace with increasing world demand, and improved, high-yielding seed material is constantly sought. Evaluation of wild genotypes and the products of commercialization require reliable methods of characterization. Protein, which constitutes about 10% of bean dry weight, is a logical component for study.

Using a cation-exchange resin column, protein extract from Brazilian comum cocoa seed was separated into eight groups by stepwise change in pH (Timbie and Keeney, 1977). Each protein group was significantly different in amino acid composition from every other group. Thirteen amino acids were involved in this variability. Reported herein are results of protein analyses of several cocoa bean samples of known origins selected to test this approach to cacao classification.

MATERIALS AND METHODS

Samples. Cocoa fruit of authenticated genetic origin was selected by J. Soria from clonal collection of CATIE, Turrialba, Costa Rica. Varietal types were Criollo, Trinitario (hybrids of Criollo and Forastero), Nacional, and Amazon Forastero. Beans and adhering pulp material were removed and freeze dehydrated within 48 h after harvest of the fruit. The testa material was peeled away, and the dry cotyledon portion was stored in an evacuated desicator.

Fat, Total Protein, and Amino Acid Analyses. Dry cotyledons (25 g) were pulverized to a fine powder in a grinding mill, small pieces of dry ice being added to prevent melting of cocoa lipids. Soxhlet extraction with petroleum ether (18 h) yielded a defatted residue, which was ground a second time, after vacuum treatment to remove residual ether. Fat content of the cotyledons was determined by evaporating the petroleum ether fraction to constant weight. Alkaloid nitrogen [from theobromine and caffeine analysis according to Timbie and Keeney (1978)], subtracted from Kjeldahl nitrogen (Pregal and Fyleman, 1927) yielded protein nitrogen in the nonfat cocoa residue. This difference × 6.25 was crude protein. Defatted cotyledon powders and various protein fractions were hydrolyzed in 6 N hydrochloric acid and subjected to amino acid analysis (Timbie and Keeney, 1977). Amino protein values were calculated from these data.

Protein Extraction, Fractionation, and Analysis. The procedures followed were those described by Timbie and Keeney (1977). Polyphenols were removed from defatted cocoa powder by acetone extraction, before treatment with protein-solubilizing buffer. The protein extract was purified on a column of Sephadex G-25 medium (Pharmacia Fine Chemicals, Inc.). Protein was then fractionated, by stepwise increase of eluting buffer pH, on a 0.9 × 20 cm column of Sephadex SP-25 cation-exchange resin. An aliquot of each protein peak volume was used for quantitative protein determination (UV absorbance at 280 nm); the remaining portion was further purified by dialysis, before hydrolysis and amino acid analysis.

Statistical Analyses. Variances were calculated using the library program ANOVES/ANOVUM (Computation Center, The Pennsylvania State University). All mean separations were calculated for $P \leq 0.01$ using Duncan's

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