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Ecological Research Series

METHYLMERCURY: FORMATION IN PLANT TISSUES

Environmental Monitoring and Support Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Las Vegas, Nevada 89114

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METHYLMERCURY: FORMATION IN PLANT TISSUES

by

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INTRODUCTION

With the continued increase in mercury pollution of the entire ecosystem through natural and man-made sources, increased study of the exposure of vegetation is warranted. Data on the effects of mercury pollution, the uptake and distribution of mercury, the transformation and identification of mercury species, and the cycling of mercury in vegetation are vitally needed to complete the environmental picture.

MICROORGANISMS

Studies on the interactions of microorganisms with mercury show that methylation of mercury occurs with cellular populations of *Clostridium* (Yamada and Tonomura, 1972) and with extracts of methanogenic bacterium (Wood, Kennedy and Rosen, 1968). Biological methylation by aquatic microorganisms has been shown to occur anaerobically (Jensen and Jernelov, 1969; McKinney, 1972; Yamada, 1972). The kinetics of aerobic and anaerobic mercury methylation have been shown by Bisogni and Lawrence (1973). The uptake, biotransformation and biodegradation of phenylmercury by microorganisms have been studied by various investigators (Fang, 1973; Matsaumura, Gotch and Bousch, 1971; Nelson, Blair and Bunkman, 1973).

The microbial conversion of mercury in cell-free systems was found to involve cytochrome c in a mercury resistant strain of *Pseudomonas* by Furukawa and Tonomura (1973) and Tonomura et al. (1971). Kasahara and Anraku (1972) have shown that mercury has an inhibitory effect in the respiratory chain of *Escherichia coli*. Landner (1971) has suggested a possible relationship between the methylation of mercury and methionine biosynthesis in *Staphylococcus*. Jensen and Jernelov (1969) suggest methylation via a complex between inorganic bivalent mercury and homocysteine. The deactivation and degradation of mercurial seed dressings by soil microorganisms have been shown to occur (Balicka et al., 1973; Balicka and Musial, 1972; Kressling, 1961; and Kimura and Miller, 1964). Rose (1968) and Wedding and Kendrich (1959) have shown the mechanisms of binding of soluble mercuric compounds by microorganisms. The volatilization of mercury by certain bacteria was shown by Magos et al. (1964).

ANIMALS

The metabolism of methyl- and dimethylmercury has been studied in animals (Ostlund, 1969). The biotransformation and biodegradation of mercury and mercury compounds were studied in animals by LeFevre and Daniel (1973) and Norseth and Clarkson (1970). The use of a liver homogenate was employed by Imura, Pan and Ukita (1972) to show methylation of inorganic mercury.

PLANTS

Most studies are concerned with the uptake of mercury by various plant species (Aarkrog and Lippert, 1971; Gilmour and Miller, 1973; John, 1972; Bache et al., 1973; Ross and Stewart, 1962; Gerdes, et al., 1974; Haney and Lipsey, 1973; Tkachuk and Kuzina, 1972; Lee et al., 1972; Smart, 1964, Fukunaga et al., 1972; Newsome, 1971; and Alvarez, 1974). In addition to the translocation studies of phenylmercury compounds in rice plants, Fukunaga et al. (1972) found traces of methylmercury present in phenylmercury treated rice plants.

Clendenning and North (1958), working with the giant kelp *Macrocystis pyrifera*, showed that 100 nanograms/gram of mercuric chloride ($HgCl_2$) in water caused a 50% inactivation of photosynthesis. Harriss et al. (1970) reported that concentrations of organomercurial fungicides as low as 0.1 nanograms/gram in water reduced photosynthesis and growth of plankton. The fresh water planktonic diatom, *Synedra ulna*, was investigated by Fujita and Hashizume (1972) for its ability to accumulate mercury. The uptake of mercury from the medium occurred rapidly during the first 7 hours. The mercury was found mainly adsorbed to the surface of the cells.

An inhibition of photophosphorylation by isolated chloroplasts was found by Bradeen et al. (1973) to be inhibited by the addition of mercuric chloride. Only one of the two functionally isolated sites of photophosphorylation coupled to electron transport, the site located between the oxidation of plastoquinone and the reduction of cytochrome f was found to be sensitive to mercuric chloride. Watling-Payne and Selwyn (1974) found that phenylmercuric acetate was a poor inhibitor of photophosphorylation. Radmer and Kok (1974) showed that mercuric chloride, when added to isolated chloroplasts, inhibited the electron flow between Photosystems I and II. This was due to the inactivation of plastocyanin, an electron carrier close to P_{700}.

Ahmed and Grant (1972) showed cytological abnormalities occurring in root tips of *Tradescantia* and *Vicia faba* following treatment of the tips with 1 to 5 micrograms/gram of Panogen 15, a mercurial fungicide. Rao et al. (1966), working with pea roots in mercuric and phenylmercuric acetate solutions showed a cellular distribution of mercury based on differential centrifugation of homogenized root tips into nuclear, mitochondrial, microsomal, and soluble fractions. Spraying of phenylmercuric acetate onto the leaves of *Coffea arabica* greatly reduced the zinc content of leaves at the distal ends of the shoots. Bock et al. (1958) measured the zinc content of leaves towards the end of the dry season and eight weeks later after a rainy period and obtained the same reduction in zinc content of leaves sprayed with phenylmercuric acetate as compared to nonsprayed control leaves. Puerner and Siegel (1972) showed that mercuric chloride alone, in admixture with fluorescein or chemically combined as mercurochrome, inhibited cucumber growth and induced disorientation of root and shoot. The inhibitory effects of mercury-ion were reduced but the disorienting (ageotropic) effects enhanced by the presence of fluorescein.

Anelli et al. (1973) investigated the influence of metallic mercury vapor on the amino acid content of tobacco leaves. Their selection of tobacco is based on the work of Zimmerman and Crocker (1934) which showed that tobacco is one of the most resistant plants to mercury poisoning since it can accumulate more than 3,000 micrograms/gram of mercury in leaves and still be viable at maturity. Anelli et al. (1973) showed that the insoluble proteic amino acid content increased throughout the experiment (after a slight decrease due to initial poisoning of mercury). The amounts of cysteine, glutamic acid and glycine appeared exceptionally high. The level of cysteine in mercury vapor treated plants was over six times as great as the level in control plants.

Through the use of x-ray crystallography, Wong et al. (1973a) determined that the binding of methylmercury to the sulphur-containing amino acid penicillamine in a 1:1 ratio occurs by the deprotonation of the sulfhydryl group. Simpson et al. (1973), using a model peptide, N-acetyl-L-cysteine, showed binding of methylmercury to the peptide via proton magnetic resonance studies.

The possibility of mercury as a trace element for plant growth and development was suggested by Dobrolyubskii (1959). In his work with grapevines sprayed twice with mercuric sulfate ($HgSO_4$), he noted an increase in the weight of 100 berries sprayed vs. control, an increased sugar content, decreased berry acidity as tartaric acid and an acceleration of grape ripening as shown by an increase in its glucoacidometric index. The chlorophyll content in treated leaves increased over the control. The carbohydrate metabolism was changed after the addition of mercuric sulfate. The sugar content increased at the expense of sucrose which was all converted to glucose and fructose. This conversion is due to invertase activity which likewise increased in the treated plants.

Siegel et al. (1974) noted analytical inconsistencies in mercury content of plant parts from a single collection of plants when assayed over a period of several hours. They have found an unknown volatile, organic-solvent soluble mercury compound that is not methyl- or dimethylmercury as determined by gas chromatography. The loss of the volatile compounds from hexane, methanol, and water follows the volatility series hexane>methanol>water.

The areas of metabolism and biotransformations of mercury in plants need further study. The purpose of this study was to determine the chemical forms of mercury in plants. Subsequent to this determination was the elucidation of the role of the plant in transforming one chemical form of mercury to another.

SUMMARY AND CONCLUSIONS

This investigation identified methylmercury (CH_3Hg) as one of the mercury species present in peas. The study also showed that methylmercury was formed by the plants (a) when grown in vermiculite and the leaves sprayed with as little as 90 milliliters of 10 micrograms/gram of mercuric nitrate ($Hg(NO_3)_2$), (b) when grown in soil with mercuric nitrate or phenylmercury added, or (c) when sections of the pea plants were surface

sterilized with 5% Chlorox or Gentamicin added to the incubation medium and incubated 20 hours in 10 micrograms/gram phenylmercury acetate. The age of the peas plays a role in the amount of methylmercury formed. Older pea tissues produce less methylmercury than young tissues when these tissues are infiltrated and incubated with 10 micrograms/gram of mercuric nitrate.

To check for the presence of methylmercury in plants grown in a natural environment but near a mercury source, samples of three species of plants were collected around an abandoned mercury mine. All three of the species show the presence of methylmercury but the influorescence of *Bromus rubens* was especially high in methylmercury. These plants were collected in early May 1975, before the summer heat had dried all aerial portions.

MATERIALS AND METHODS

PLANTS

Seeds of *Pisum sativum* L. var. Alaska and Little Marvel were purchased from Burpee Seed Company in Riverside, California. The supplier stated that the expected germination was 92%.

Plants collected from the mercury mine area were (a) *Bromus rubens*, (b) *Spharalcea ambigua*, and (c) a *Boraginaceae*.

GROWING MEDIA

Vermiculite and soil which was collected from the University of Nevada Agricultural Experiment Station at Logandale, Nevada, were used. The soil, a fine sandy loam, has the following characteristics:

(a) 54% sand
(b) 11% clay
(c) 35% silt
(d) 1.3% organic carbon
(e) pH 8.6

PLANTING METHOD

Plastic trays, 29.16 x 36.78 x 8.84 cm (11.5 x 14.5 x 3.5 inches) were filled to within 1.22 cm (½ inch) of the top with a medium of vermiculite or soil. Pea seeds were placed 2.54 to 3.76 cm (1 to 1.5 inches) apart and pushed down into the medium even with the surface. The trays were then filled to the top with medium, lightly tamped, and watered thoroughly. Additional watering was done via an automatic system twice a day at 8 a.m. and 4 p.m. If peas were grown longer than two weeks, complete nutrient solution was added weekly.

GERMINATION METHODS

Pea seeds were placed in a beaker of water or mercury solution, depending on the experiment. A stream of air was bubbled through the solution for the entire imbibing period. The swollen seeds were washed thoroughly with distilled water and placed in pans lined with moistened paper towels. The pans were covered with plastic-coated paper and the paper secured with the aid of rubber bands. The pans with seeds were then placed inside an oven set at 25° C. At the end of the germination period, the young pea plants were separated into epicotyls, roots, and cotyledons, and each weighed. The lengths of epicotyls and roots were also measured and recorded.

TISSUE EXTRACTION PROCEDURE

All tissue was washed with distilled water prior to use. The tissue was homogenized in Waring blender with 2.2N HCl (1 milliliter HCl/gram tissue) for two minutes. The resultant brei was centrifuged at 10,000 rpm for 30 to 45 minutes. The supernatant solution was decanted and filtered, and extracted with three volumes of nanograde quality benzene, each equal to the volume of supernate. All benzene fractions were passed through Whatman Phase Separating paper and combined. A freshly prepared 1% cysteine solution (Westoo 1966, 1967, 1968, 1973) was titrated to pH 8.3 to 8.4 with 5N sodium hydroxide immediately before use; 10 milliliters of the cysteine solution per 100 milliliters of benzene was added to the benzene and vigorously shaken. After separation of the aqueous and organic layers, the cysteine layer was drawn off and acidified to pH 0.5 to 0.7 with 5N HCl. This acidified solution was allowed to stand for 15 minutes after which 10 milliliters of benzene was added and vigorously shaken. After separation, the benzene layer was drawn off into a glass vial with anhydrous sodium sulfate covering the bottom.

MODIFICATION OF WESTOO'S CYSTEINE EXTRACTION SOLUTION

Westoo's cysteine extraction solution consists of cysteine· HCl, sodium acetate, and anhydrous sodium sulfate. The pH of the resulting solution is ∿3.8. Using a freshly prepared methylmercury solution, only 50 to 60% recovery of the methylmercury could be obtained. Wong et al. (1973b) found that binding of methylmercury to another sulfur-containing amino acid, penicillamine, occurred via deprotonation of the sulfhydryl group. The ionization of cysteine at pH 7 is only 8%. Raising the pH to 8.3 results in 50% ionization of the sulfhydryl group of cysteine. Westoo's cysteine solution pH of 3.8 was adjusted to pH 8.3 to 8.4 with 5% sodium hydroxide and used immediately. The recovery of methylmercury increased to 86%.

INFILTRATION AND INCUBATION METHODS

Selected plant sections were placed in a flask containing the mercury solution. This flask was placed inside a plastic desiccator fitted with a stopcock. The clear plastic lid was covered with aluminum foil to make the chamber light-free. An in-line activated charcoal filter was placed in the pressure tubing between a vacuum pump and the desiccator. The tissue was placed under a vacuum for 15 minutes, the vacuum pump turned

off, and the inside of the desiccator allowed to return to an equilibrium with the ambient pressure. The stopcock was then closed and the incubation allowed to proceed inside the darkened chamber.

GAS CHROMATOGRAPHY

A Hewlett-Packard[1] Gas Chromatograph Model 5713 with a nickel-63 linearizing electron-capture detector and a 6-foot column packed with 5% HIEFF was used to separate organic mercurial halides. The carrier gas was 95% argon/5% methane obtained from Matheson[2] and used at a flow rate of 60 milliliters/minute. The oven temperature was 170° C. and the detector and injection port temperatures were 200° C. The column was on-line in the injection port.

CHELATING RESIN

To remove ionic mercury from samples before injection into the gas chromatograph, Srafion NMRR[3] chelating resin was used. Preliminary work by Law (1971) indicated the possibility of separating ionic mercury from methylmercury as a function of pH. Experiments at the Environmental Monitoring and Support Laboratory-Las Vegas confirm this separation. The resin chelates ionic mercury at low pH (pH ∿0.5) and chelates methylmercury at pH 6.4. The methodology of utilization of the resin is as follows. The resin is washed repeatedly with glass distilled water, allowed to settle for 10 minutes and the liquid decanted. After washing, 10 to 20 grams of the resin is poured into a column. A glass fiber filter is cut to fit the inside diameter of the column. With the stopcock open, the filter is gently pushed down on top of the resin bed. The pH of the solution to be placed on the column is critical for chelation of the desired mercury species.

In this case, the chelation of ionic mercury was desired. The sample (10 to 20 milliliters) was placed on the column and flow rate adjusted to 0.5 to 0.6 milliliters/minute. After the void volume of the column had passed through, the volume collected was equal to the volume of sample plus 10 milliliters of the glass distilled water. Elution of the ionic species of mercury from the column was accomplished by the addition of a 5% thiourea in 0.06N HCl. When this elution was complete, the column was flushed with 300 milliliters of glass distilled water and regenerated for future use.

MERCURY TRAP SYSTEM

To determine whether ethyl- or methylmercuric chloride was released during the incubation of tissue in mercuric nitrate solution, a modification of the Kimura and Miller (1960) mercury entrapment procedure was used.

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[1] Hewlett-Packard, 11300 Lomas Boulevard, Albuquerque, New Mexico 87123

[2] Matheson Gas Products, 8300 Utica Avenue, Cucamonga, California 91730

[3] Ayalon Water Conditioning Company, P.O. Box 586, Haifa, Israel

Nitrogen gas was bubbled through the incubation solution and through two traps. The first was a carbonate-phosphate solution followed by a 1% cysteine solution at pH 8.3. The flow rate of nitrogen was 112 milliliters/minute for 20 hours of incubation. The carbonate-phosphate trap removes volatile ethyl- and methylmercuric chloride. The cysteine trap is used as a check on the primary carbonate-phosphate trap. Figure 1 depicts the flow system.

MONITORING MERCURY FROM COLUMNS

A Gilson UV Monitor[4] was attached to the column by 1.59-centimeter (1-1/16-inch) tubing. By selecting the 254-nanometer filter, mercury compounds separated by column chromatography can be easily monitored. The small volume of the cell, 1 milliliter coupled with a reference cell, allows for the detection of mercury down to 0.5 micrograms/gram.

VERIFICATION OF METHYLMERCURY FROM GAS CHROMATOGRAPHY

To verify that mercuric nitrate and/or the concentration of mercuric nitrate used in the experiments was not producing a peak on the chromatogram at the same time as methylmercuric chloride, mercuric nitrate at the concentration of 10 micrograms/gram was dissolved in distilled water and 90 milliliters of this solution was taken through the extraction procedure. A 5-microliter aliquot of the extract was injected into the gas chromatograph. No methylmercuric chloride peak appeared.

With the formation of the chloride species of mercury in the extraction procedure and the possibility of forming mercuric chloride from the mercuric nitrate, a mercuric chloride solution at the concentration of 170 micrograms/gram was made in benzene. When 5 milliliters of this solution was injected onto the gas chromatographic column, the mercuric chloride peak appeared the same time as the reference methylmercuric chloride peak. This 170-microgram/gram mercuric chloride benzene solution was extracted via the cysteine procedure and 5 microliters of the final 10-milliliter benzene layer was injected onto the gas chromatograph column. In this case, no peak corresponding to the methylmercuric chloride peak appeared.

EXPOSURE OF MERCURY COMPOUNDS TO ULTRA-VIOLET IRRADIATION

Quartz cuvettes were filled with 50 or 100 micrograms/gram of mercuric acetate or mercuric nitrate dissolved in glass distilled water. Each was placed inside a fluorescent chamber and irradiated with ultra-violet (UV) wave lengths for 5 to 6 hours. At the end of the irradiation period, each was extracted with benzene, cysteine, and back into benzene. Gas chromatographic analysis showed the formation of methylmercury by mercuric acetate but not by mercuric nitrate. These results are in agreement with those of Akagi and Takabatake (1973). Mercuric nitrate was chosen for use in further experiments.

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[4] Gilson Medical Electronics, Middleton, Wisconsin

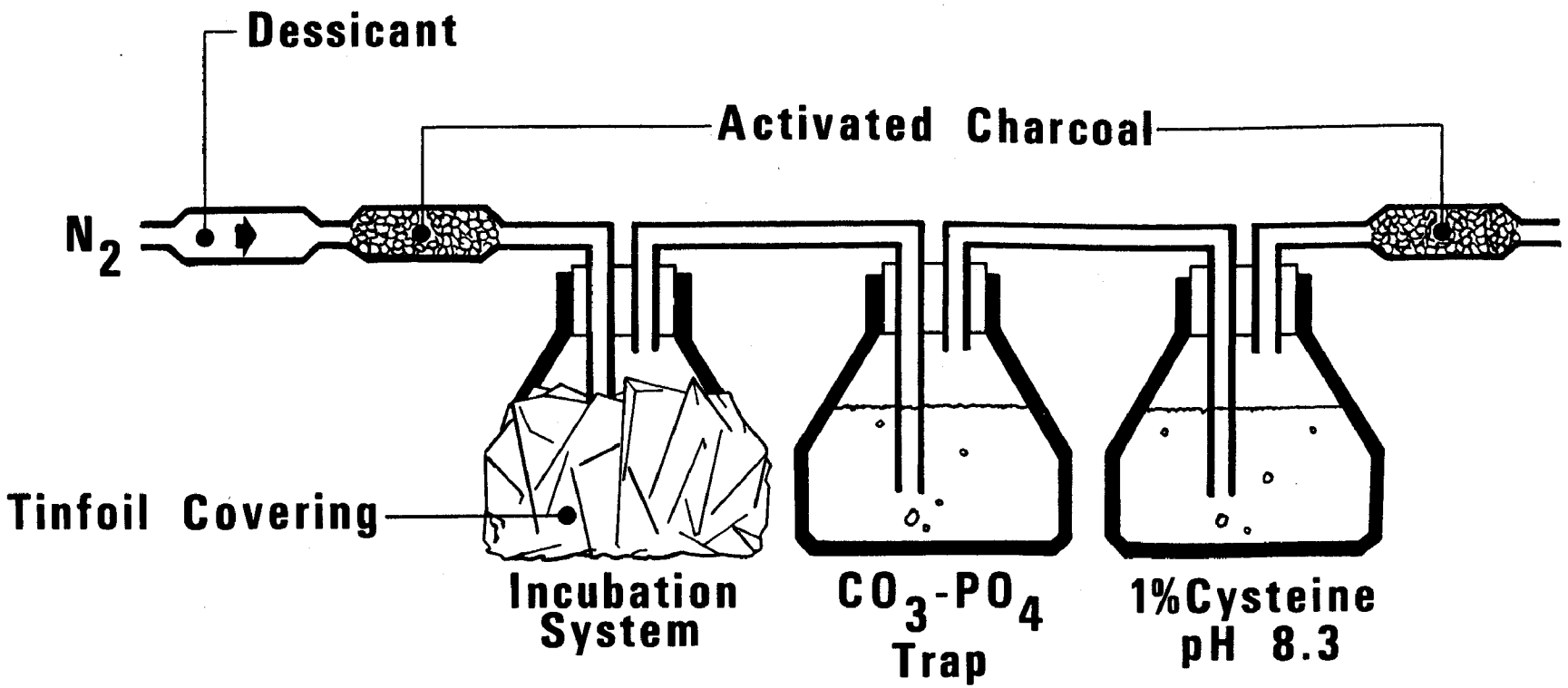

Figure 1. Mercury trapping system modification of Kimura and Miller (1960) procedure.

PURIFICATION OF REAGENT GRADE MERCURIC NITRATE

Reagent grade mercuric nitrate was found to contain mercuric chloride impurity. To remove this impurity, a small portion of the mercuric nitrate was poured into a flask and washed three times with benzene. The mixture was shaken, allowed to settle, and the benzene was carefully decanted. After three such treatments, diethylether was added and carefully swirled. After allowing to settle and decanting the ether, acetone was added and the same procedure employed except that the acetone/mercuric nitrate was in the filter. The mercury was stirred while under vacuum. The mercuric nitrate was transferred to a dark bottle and placed in the freezer for future use.

THIN LAYER CHROMATOGRAPHY OF STOCK ORGANIC MERCURY COMPOUNDS

The purities of ethyl-, methyl-, and phenylmercuric compounds were tested by thin layer chromatography with 500-microgram/gram solutions of each made in benzene. Silica gel GF-254[5] was used as the stationary phase. Glass plates 20- x 20-centimeters and 10- x 20-centimeters were prepared by mixing 22 grams of silica gel with 70 milliliters of boiling water for 2 minutes in a blender. The slurry was poured into a Brinkman-Desaga[6] adjustable spreader. The slurry was spread over 100 centimeters of glass plates at 0.25-millimeter thickness. The plates were air-dried and stored until use. Before use the plates were dried in an oven for 30 minutes at 105° C.

Twenty-five microliters of each mercury solution was spotted on the thin layer chromatography plates. The developing solvent systems used to separate the organic mercury compounds were

(a) carbon tetrachloride:methylene dichloride (2:1)
(b) hexane:acetone (93:7)
(c) pentane:ethylacetate (10:1)
(d) hexane:ether (9:1)

After developing and drying, the mercury compounds were visualized on the plate in a fluorescence chamber under short UV irradiation. Silica gel GF-254 has a fluorescein dye added that appears green under short UV light. Compounds on the thin layer chromatography plate mask this coloration leaving a dark spot. Phenyl- and methylmercury showed one spot each; ethylmercuric chloride showed a methylmercury spot as well as an ethylmercury spot.

GAS CHROMATOGRAPHIC ANALYSIS OF STOCK ORGANIC MERCURY COMPOUNDS

Solutions of 50 micrograms/gram were made from the stock 500-microgram gram benzene solutions of ethyl-, methyl-, and phenylmercury chlorides. Five microliters of each was injected onto the gas chromatograph column. The contamination of ethylmercuric chloride with methylmercuric chloride was confirmed. The phenylmercury chloride showed no contamination of methyl- or ethylmercury compounds.

[5 & 6] Brinkman, 110 River Road, Des Plaines, Illinois

GAS CHROMATOGRAPH UNIFORM INJECTION PROCEDURE

To ensure consistent and known volumes injected onto a gas chromatographic column, a 0- to 10-microliter syringe was used. The procedure is as follows:

(a) Rinse the syringe twice with solution to be injected and flush.
(b) Draw 0.6 microliters of air into the syringe.
(c) Draw 5.0 microliters of solution to be injected into the syringe after the 0.6 microliters of air and then inject into the gas chromatograph.

Peak heights of standard methylmercury-benzene solutions are reproducible to within 1%.

RESULTS AND DISCUSSION

GERMINATION EXPERIMENTS

The sensitivity of Little Mervel pea seeds to concentrations of ionic mercury compounds was determined by germinating pea seeds in 1-, 10-, and 25-micrograms/gram distilled water solutions of mercuric acetate and mercuric nitrate for 6 hours with aeration. They were placed in separate germination pans after extensive washing with distilled water. The seeds were germinated at 25° C for 4 days completely in the dark. At the end of this time, the young pea plants were separated into three sections--epicotyls, roots, and cotyledons. The epicotyls and roots were measured and the combined tissues of each section were weighed. The percent germination of each lot of peas was recorded. Burpee Seeds states a 92% germination for this batch of peas. Each section for each condition was extracted according to the modified Westoo procedure to determine if any detectable organic mercury halides were present.

The results are given in Table 1. No organic mercury was detected. The data show that the nitrate and acetate forms of ionic mercury do not elicit comparable responses in the same variety of peas. Only 52% germination occurred for peas in 25 microgram/gram mercuric nitrate while 68% was noted for 25 microgram/gram mercuric acetate. The total weights of the epicotyls and roots in the 25 microgram/gram mercuric nitrate were 100% and 73% greater, respectively, than those in 25 microgram/gram mercuric acetate. The overall response of the peas to the mercuric acetate was a diminution in growth and weight with an increase in mercuric acetate concentration. The response to mercuric nitrate definitely did not follow a diminution pattern as concentration of mercuric nitrate increased. Based on these experiments, mercuric nitrate was chosen as the ionic form of mercury to be used in subsequent investigations and 10 micrograms/gram was chosen for the preliminary concentration with which to work.

TABLE 1. GERMINATION STUDIES OF LITTLE MARVEL PEAS IN VARIOUS CONCENTRATIONS OF MERCURIC ACETATE OR MERCURIC NITRATE

	HgOAc µg/g			$Hg(NO_3)_2$ µg/g		
	1	10	25	1	10	25
% Germination	76%	76%	68%	92%	88%	25%
Epicotyl length-av.	1.7 cm	1.6 cm	1.4 cm	2.2 cm	1.3 cm	1.6 cm
Root length-av.	3.4 cm	2.8 cm	2.1 cm	3.4 cm	2.6 cm	2.7 cm
Epicotyl total wt.	0.93 g	0.96 g	0.62 g	1.62 g	0.84 g	6.0 g
Root total wt.	0.96 g	0.90 g	0.52 g	1.43 g	1.7 g	7.1 g
Cotyledon total wt.	12.35 g	15.87 g	16.6 g	14.1 g	16.13 g	15.8 g
CH_3Hg present	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]

[a]Not detectable

FOLIAR APPLICATION OF MERCURY

Little Marvel peas were grown in vermiculite in exposure chambers for 34 days with weekly addition of a complete nutrient solution. On the 34th day after planting, the leaves of the peas were sprayed by an atomizer over an 8-hour period with a total of 90 milliliters of 10 micrograms/gram mercuric nitrate solution made up in distilled water. Watering was done at the vermiculite level, so little if any of the mercuric nitrate was on the vermiculite available to root absorption. One flat of peas was used and the dense canopy of leaves coupled with the small volume sprayed at any one time and the fineness of the mist eliminated any runoff to the vermiculite. The peas were grown an additional 5 days with no further addition of mercury. On the 39th day after planting, all tissue above the third node was harvested and extracted according to the modified Westoo cysteine procedure. A total of 125.53 grams of tissue fresh weight was obtained. When 5 microliters of the final benzene layer was analyzed by gas chromatography, a peak at the time of the methylmercury chloride reference appeared. Relating the peak height to standards gave a concentration of 0.16 nanograms $\pm$ 0.06 nanograms of methylmercury chloride/gram of tissue fresh weight based on a conservative 60% efficiency for the entire extraction procedure.

MERCURY ADDITION TO SOIL MEDIUM

Mercuric nitrate and phenylmercuric acetate in powder forms were each mixed with 6 kilograms of sandy-loam soil, pH 8.6, with characteristics given previously, to obtain 10 and 100 micrograms/gram soil/mercury mixture of each. This contaminated soil was placed in trays and Little Marvel seeds were planted in each 2.54 centimeters (1-inch) apart. After 14 days, the aerial portions were harvested and rinsed thoroughly. The modified cysteine extraction procedure was used to extract the tissue.

Table 2 gives the amount of methylmercury found per gram of aerial tissue from peas grown in the mercuric nitrate of phenylmercuric acetate contaminated soil. The amount of methylmercury detected in the tissue increased 100% from 10 micrograms/gram to 100 micrograms/gram mercuric nitrate in the soil. The amount of methylmercury decreased with corresponding concentrations of phenylmercuric acetate in the soil, indicating that the two forms of mercury have different effects or sites of activity within the plant.

TABLE 2. METHYLMERCURY FORMATION FROM LITTLE MARVEL PEAS GROWN 14 DAYS IN 10 AND 100 μg/g MERCURIC NITRATE AND PHENYLMERCURIC ACETATE CONTAMINATED SOIL

Mercury contaminated	Concentration in soil	Weight of aerial tissue	Methylmercury present ng/g fresh weight
$Hg(NO_3)_2$	10 μg/g	12.9 g	3.1 ± 0.9 ng/g
$Hg(NO_3)_2$	100 μg/g	23.6 g	7.6 ± 2.3 ng/g
Ph-Hg-OAc	10 μg/g	29.1 g	3.8 ± 1.1 ng/g
Ph-Hg-OAc	100 μg/g	24.1 g	2.1 ± 0.6 ng/g

Various investigators (D'Itri, 1972; Hale and Wallace, 1970; and Van Loon, 1974), working with soil types and soil pH have found the root uptake of mercury into plants to decrease with neutral or alkaline soils. The use of an alkaline soil was to determine if mercury uptake would occur and, if so, if the small amount taken up would be transformed to methylmercury in concentrations large enough to be detected with the system at hand.

INFILTRATION AND INCUBATION EXPERIMENTS WITH MERCURIC NITRATE

The detection and identification of methylmercury in pea plants after the addition of mercuric nitrate to leaves or applied to the soil could be the result of microbial conversion of the ionic to an organic mercury species with the concomitant uptake of the methylmercury compound by the plant. An experimental procedure was devised to determine if the plant tissue was capable of transforming ionic mercury to methylmercury. Alaska peas were grown in vermiculite and after 20 days from planting various internodes were harvested, weighed, thoroughly rinsed with distilled water, and placed in flasks with 100 milliliters of 10 micrograms/gram mercuric nitrate in glass distilled water. The flasks were placed under vacuum from a water aspirator for 30 minutes. The flasks were sealed and incubated for 89½ hours. The tissue in each flask was thoroughly rinsed with distilled water (a minimum of five complete rinses was used) and extracted via the modified Westoo procedure.

Gas chromatographic analysis of 5 microliters of each final benzene extract revealed the presence of methylmercury chloride from all stem sections. Table 3 gives the weight of tissue in each fraction and the amount of methylmercury chloride detected. The amount of methylmercury in internode sections up to the apical segments is three times as much as the subtending internodes.

TABLE 3. WEIGHTS OF ALASKA PEA INTERNODES USED IN THE INCUBATION EXPERIMENT WITH $Hg(NO_3)_2$ AND THE AMOUNT OF CH_3HgCl FORMED PER GRAM OF TISSUE IN 90 HOURS.

Stem internode	Total weight	CH_3HgCl ng/g tissue
0-3	14.6 g	3.4 ± 1
4	15.0 g	3.3 ± 1
5	17.8 g	2.5 ± 0.7
6	14.2 g	2.3 ± 0.7
Apical	8.1 g	10.9 ± 3.3

Little Marvel peas were harvested after 17 days growth into stems and apical region. No leaves or laterals were used. The same infiltration procedure was used. The incubation period was only 20 hours. After thoroughly rinsing the tissue, each was extracted according to the modified Westoo procedure. A comparable amount of tissue was used for a control. The stems plus apices were thoroughly washed and infiltrated the same length of time in glass distilled water in separate chambers with in-line activated charcoal to eliminate the possibility of mercury contamination. The control tissue was incubated 20 hours after which it was washed and homogenized in 2.2N HCl in a blender and centrifuged. The supernatant solution was extracted via the modified Westoo procedure. The pellet was resuspended in glass distilled water by magnetically stirring for 15 minutes. The suspension was extracted via the modified Westoo procedure.

The results of the tissue incubated in 10 micrograms/gram mercuric nitrate and the control tissue are given in Table 4. After 20 hours of incubation in mercuric nitrate, stems and apices from methylmercury with the stems forming more per gram than apical region. The control plants with no addition of mercuric nitrate show no methylmercury chloride in either the supernate or the pellet.

TABLE 4. LITTLE MARVEL STEMS AND APICES INFILTRATED AND INCUBATED 20 HOURS WITH 10 µg/g $Hg(NO_3)_2$ AND CONTROL PLANTS WITH NO ADDITION OF MERCURY TO INCUBATION SOLUTION

Tissue	Total weight	CH HgCl ng/g tissue
Stems	22.3 g	7.3 ± 2.2
Apices	3.6 g	4.1 ± 1.2
Control	27.8 g	*

* Supernatant = none detected
Pellet = none detected

The formation of methylmercury from mercuric nitrate by plant tissue within 20 hours would seem to rule out bacterial contamination as causing the transformation because the time involved is so short. However, to determine if bacteria associated with the pea stems are causing this transformation, two experiments were performed using two different sterilization procedures. Stems from Little Marvel peas grown 18 days were harvested and divided into

two 10-gram fractions. Both fractions were washed thoroughly with distilled water and one fraction was washed again for 5 minutes in 5% Chlorox solution. The other fraction was the control. The Chlorox was rinsed off thoroughly and both were infiltrated under vacuum in separate chambers for 15 minutes and incubated for 20 hours in 100 milliliters of 10 microgram/gram mercuric nitrate. The stems were thoroughly rinsed again to remove any mercury on the surfaces and extracted via the modified Westoo procedure. The second experiment involved stems from Little Marvel peas grown 38 days. Two 10-gram fractions were obtained. Both fractions were washed, one placed in 100 milliliters of 10 microgram/gram mercuric nitrate to which Gentamicin, an antibacterial agent, was added (2 milligrams/milliliter). Both were infiltrated and incubated in separate chambers for 20 hours. The stems were again rinsed and extracted via the modified Westoo procedure. Also via the modified Westoo procedure, 50 milliliters of concentrated Chlorox was extracted to determine if any methylmercury was present as a contaminant in the Chlorox. The results of both experiments are given in Table 5.

TABLE 5. FORMATION OF METHYLMERCURY AS INFLUENCED BY SURFACE STERILIZATION AND GENTAMICIN TREATMENT

Treatment	Age of peas (days)	Weight of stems (grams)	CH HgCl formed ng/g tissue
5% Chlorox	18	10	7.6 $\pm$ 2.3
No Chlorox	18	10	5.6 $\pm$ 1.7
Gentamicin	38	10	4.0 $\pm$ 1.2
No Gentamicin	38	10	4.0 $\pm$ 1.2
Concentrated Chlorox		None present	

There are no significant differences between comparable experiments. The age of tissue seems to have an effect on the ability to transform ionic mercury into methylmercury. Stems 38 days old produced only 61% as much methylmercury as did the stems 18 days old (using the average of both treatments).

The incubation medium used so far has been glass distilled water. To determine if different media would have an effect on the transformation of ionic mercury to methylmercury, the infiltration/incubation procedure was utilized. Comparisons were made with glass distilled water, 0.01M dibasic potassium phosphate/monobasic potassium phosphate (K_2HPO_4/KH_2PO_4) at pH 7.0 and K_2HPO_4/KH_2PO_4 at pH 7.0 with 2% sucrose. Little Marvel stems 57 days old were harvested and separated into 6 fractions, 10 grams each. A control with no mercury added was used for each incubation medium because the stems were much older than those previously used. The fractions of tissue containing 10 micrograms/gram of mercuric nitrate were infiltrated and incubated in separate chambers. All the control tissue was infiltrated and incubated in one chamber. The incubation time was 20 hours. At the completion of the incubation period each fraction of tissue was rinsed and extracted via the modified Westoo procedure.

The results are given in Table 6. Incubation in glass distilled water produces the least amount of methylmercury but within an overall efficiency estimate of 70%; the formation of methylmercury is comparable in all three media.

The influence of pH on the transformation of ionic mercury to methylmercury was determined by adjusting the pH of the incubation solutions to 3.5, 4.5, 5.5, 6.5, 7.5 or 8.5. A physiologic concentration of 0.01M was used for dibasic potassium phosphate and monobasic potassium phosphate. The lowest pH (3.5) was obtained by adding phosphoric acid. Little Marvel peas, stems and apices 13 and 35 days old were used. The tissue from each age group of peas was divided into 6 10-gram fractions, infiltrated and incubated 20 hours. The results as given in Figure 2 show that the age of the tissue has some effect on the transformation of ionic mercury to methylmercury. It is interesting to note that the peak heights of methylmercury at the two extremes of pH comparing age of tissue are almost the same. Obviously, other factors that are not present or suppressed in the physiological pH range are influencing the formation of methylmercury at these extremes.

TABLE 6. LITTLE MARVEL PEA STEMS 57 DAYS OLD, 10G, WERE USED FOR COMPARISON ON INCUBATION MEDIA UPON CH_3 Hg FORMATION

	Peak Height
Control in glass distilled water	3.5 units
10 µg/g $Hg(NO_3)_2$ in glass distilled water	12 units
Control in PO_4 buffer	0 units
10 µg/g $Hg(NO_3)_2$ in PO_4	12 units
Control in PO_4 buffer + 2% sucrose	2.5 units
10 µg/g $Hg(NO_3)_2$ in PO_4 buffer + 2% sucrose	14 units

With the possibility of the loss of some volatile organic mercury compounds from the incubation flask during incubation, an experiment was performed to trap these volatile organic mercury compounds. Little Marvel pea stems 13 days old were harvested and 21.9 grams obtained. The stems were thoroughly rinsed and infiltrated in a glass distilled water incubation medium with 10 micrograms/gram mercuric nitrate. The incubation flask with stems was placed in a nitrogen gas train as described in the methods. The incubation period was 20 hours after which the tissue was rinsed thoroughly and extracted via the modified Westoo procedure. The carbonate-phosphate solution and cysteine solution were removed from the traps and also extracted via the modified Westoo procedure. Gas chromatographic analysis of the traps revealed the possibility of a very small amount of methylmercury present. If such peaks are indeed present on the chromatograms, they are almost at the level of background noise. The stems showed 8.3 $\pm$ 2.5 nanograms/gram tissue methylmercury.

INFILTRATION AND INCUBATION EXPERIMENTS WITH PHENYLMERCURIC ACETATE

Rice plants treated with phenylmercuric acetate reportedly produce small quantities of methylmercury (Fukunaga et al., 1972). To determine if phenylmercury is transformed to methylmercury, Little Marvel stems with apices and leaves were harvested and 10 grams of each tissue was infiltrated and incubated in separate chambers with phenylmercuric acetate. Two 10-microgram/gram aqueous solutions were attempted but

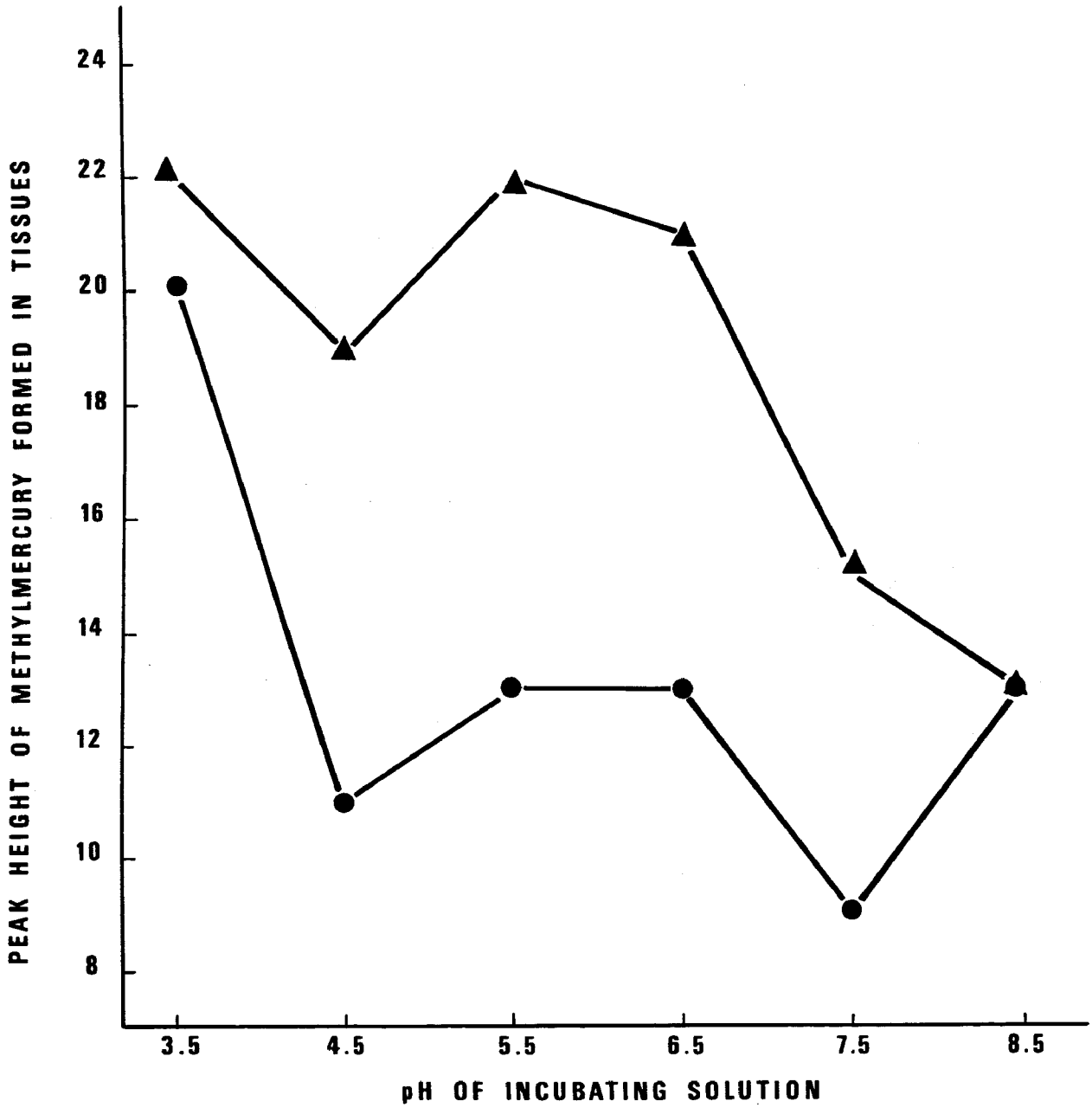

Figure 2. Effect of pH of the phosphate buffer incubation medium on the transformation of ionic mercury to methylmercury in Little Marvel Peas as determined by methylmercury peak height.

Δ = LM peas 13 days old; 0 = LM peas 35 days old.

after stirring for 48 hours an estimated 20% of the phenylmercury in each may have gone into solution. The solutions were allowed to settle and the aqueous solution decanted without carrying any of the undissolved material over into the incubation flasks. After 20 hours incubation, the tissues were thoroughly rinsed and extracted via the modified Westoo procedure. Gas chromatographic analysis of the final benzene extracts shows the presence of methylmercury in stems with apices at 10 $\pm$ 3 nanograms/gram and leaves at 8 $\pm$ 2.4 nanograms/gram. This level of transformation occurred with an estimated 2-microgram/gram solution of phenylmercuric acetate.

EXTRACTION OF PLANTS FROM A MERCURY MINE AREA

Methylmercury has been shown to be present in plant tissue from foliar application of ionic mercury, from root uptake of ionic and phenylmercury and from tissues incubated in ionic and phenylmercury solutions. Is methylmercury present in plants which are growing naturally in an area with elevated levels of mercury? To find out, several plants were collected from the mercury mine area at the Nevada Test Site in mid-May 1975. *Bromus rubens* and *Spharalcea ambigua* were two of the species collected. A *Boraginaceae* was also collected.

In plant samples collected in November-December 1974 from the Four Corners area around the coal-burning power station, no appreciable high levels of mercury were noted. The physiology of the plants during this collection period is far different from that of the same plants in the Spring. To survive the long, hot, dry summers in the desert, the plants enter a dormant state. In the Spring after a few showers, the physiology changes to an active growth period. Mid-May was selected for collection to obtain plants still actively growing and also so that small leafy plants could be obtained.

The plants were extracted in 2.2N HCl which was extracted with benzene. The modified Westoo procedure was used for the extraction and concentration of organic mercury compounds from the benzene. A 5-microliter aliquot of the final 10-milliliter benzene layer was injected onto the gas chromatographic column. All three plants showed a methylmercury peak, but that from *Bromus rubens* was exceptionally large. To determine that the peak was, in fact, methylmercury, 6 milliliters of the final benzene layer was extracted with 5 milliliters of the 1% cysteine solution at pH 8.4. The cysteine/mercury complex was broken by the addition of 5N HCl to a pH 0.7. This acidified fraction was placed on a column of Srafion NMRR resin. The eluate was extracted into benzene (6 milliliters) and a 5-microliter sample injected on the gas chromatographic column. Again the methylmercury peak showed up almost as large as before. The amount of methylmercury present in *Bromus rubens* was 10 $\pm$ 3.3 nanograms/gram, for the *Boraginaceae*, 7.5 $\pm$ 2.3 nanograms/gram, and for *Spharalcea ambigua*, 2.5 $\pm$ 0.8 nanograms/gram.

Since the purple influorescence of *Bromus rubens* accounted for 80% of the fresh weight of tissue extracted, methylmercury had concentrated in the developing seeds.

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16. ABSTRACT

Methylmercury was found in the tissue of the pea plant *(Pisum sativum)* after spraying mercuric nitrate onto the leaves, after planting in mercuric nitrate or phenylmercuric acetate contaminated soil and after infiltration and incubation of stems, leaves, and apices in mercuric nitrate or phenylmercuric acetate solutions. The concentration of mercury added in each experiment was 10 micrograms/gram. Younger pea tissue formed more methylmercury than older tissue. Methylmercury was also found in three different species of plant growing near an abandoned mercury mine.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Plant physiology	Methylmercury	17C
Plant chemistry	Mercurial transformation	06C
Mercury	Mercury uptake	06F
Trace elements	Biotransformation	07B
Legumes	Phenylmercury	07C
Absorption	Pisum sativum	

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