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BIOTRANSFORMATION AND CHEMICAL FORM OF MERCURY IN PLANTS

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BIOTRANSFORMATION AND CHEMICAL FORM OF MERCURY IN PLANTS

by

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INTRODUCTION

The continuing release of mercury to the biosphere from natural as well as manmade sources warrants the study of plant exposures to mercury. Data on the effects of mercury pollution, the uptake and distribution of mercury, the transformation and identification of mercury species and the possibility of cycling of mercury in and through plants are vitally needed to complete the environmental assessment of this pollutant.

Many investigators, Aarkrog and Lippert (1971), Alvarez (1974), Barker (1972), Gerdes et al. (1974), John (1972), Lee et al. (1972), Smart (1964), Smith (1972), Tkachuk and Kuzina (1972), Van Loon (1974a and 1974b), and Yamada (1968), have reported the uptake and distribution of mercury in a variety of plant species.

Recently, the loss of metals from plants has been investigated. Siegel et al. (1974) reported on the loss of a volatile form of mercury from plants. The plants were harvested and the mercury content of fractions from the total number of plants harvested was determined. It was found that fractions of plants analyzed immediately after harvesting contained more mercury than plant fractions analyzed at later times. The suspected volatile form of mercury was found to be very soluble in hexane, less soluble in methanol and least soluble in water. This volatile mercury compound is not methylmercury or dimethylmercury.

Beauford et al. (1975) have obtained evidence that higher plants may contribute heavy metals naturally to the atmosphere. Peas, broad beans, and pine seedlings were grown in solutions of radioactive zinc, lead, or copper. A small fraction of the radioisotopes absorbed by the roots was released from the leaves. The metal release from the leaves was not found to be quantitatively associated with water loss. However, there was a requirement that the plants be continuously fed with the radioisotope. Removal of the plants from the radioactive solutions resulted in a complete cessation of radioisotope loss. All three metals were released from the plants but not in equivalent amounts per unit of tissue.

The methylation of mercury by aquatic organisms has been shown by Jensen and Jernelov (1969), Langley (1973), and Bisogni and Lawrence (1973). Wood et al. (1968) showed the methylation of mercury by methanogenic bacteria. Imura et al. (1972) showed the methylation of mercury by a liver homogenate from tuna. The role of methylmercury in these investigations has not been clearly elucidated.

Various investigators, Imura et al. (1971), Ingraham (1964), Wood et al. (1968), Wood (1974), and Bertilsson and Neujahr (1971) have used methylcobalamin as a methyl-donating compound. Wood et al. (1968) found

that the transfer of the methyl group from methylcobalamin to mercury could be accomplished non-enzymatically in mild reducing conditions.

Methylcobalamin is not the only biological compound capable of donating a methyl group in biological reactions. Cantoni (1955, 1957, and 1975) and Mudd (1973) have examined S-adenosyl-methionine as a methyl-donating compound and have found that it effectively methylates amino acids, proteins, and carbohydrates in biological reactions.

In a previous study (Gay, 1975) I reported the formation of methylmercury in *Pisum sativum* under several conditions; i.e., when peas were sprayed with a solution of mercuric nitrate, when peas were grown in soil containing mercuric nitrate or phenylmercuric acetate and when pea tissues were infiltrated and incubated in solutions of mercuric nitrate or phenylmercuric acetate. It was noted that very low concentrations of methylmercury were found and that younger peas produced more methylmercury than did older peas.

The purpose of this study was to investigate the mechanism of the methylation of mercury compounds in peas and to determine whether methylmercury is the end product in this pathway.

CONCLUSIONS

In vitro methylation of inorganic mercury has been accomplished by an enzyme system isolated from *Pisum sativum*. The methyl-donating compound was found to be S-adenosyl-methionine.

Formation of methylmercury in the peas is viewed as an intermediate step in the biochemical pathway of mercury in peas.

MATERIALS AND METHODS

The common dwarf garden pea, *Pisum sativum* cultivar Little Marvel, was used in all investigations. The seeds were planted in vermiculite and harvested 13 to 14 days after planting.

The infiltration and incubation procedure and analytical methodologies are discussed in detail elsewhere (Gay, 1975). The selected tissues are placed in a mercury solution and infiltrated under partial vacuum for 15 minutes. The tissues are then incubated at normal pressure for various periods of time in complete darkness.

A Hewlett-Packard gas chromatograph equipped with a nickel-63 linearizing electron capture detector is used to detect the presence and quantities of methylmercury in the samples.

Partial purification of enzyme systems was accomplished with an acetone protein precipitation procedure. The amount of acetone added to obtain the various fractions is expressed as the percent of acetone in the supernatant solution. Spectral grade acetone was used in all cases.

After harvesting the selected tissues, all procedures up to the incubations of enzyme fractions with the substrates were done at -4° to 2° C.

The supernatant solution obtained from the centrifugation of the ground tissues was treated successively with 25%, 50%, 70%, and 92% acetone. The acetone for each fraction was added dropwise to the supernatant solution while the solution was being agitated or stirred. The stirring continued 15 minutes after the addition of acetone. The mixture was then centrifuged at 15,000 rpm for 30 minutes at -4° C. The supernatant solution was treated with the next amount of acetone, consecutively, and the pellet obtained was placed under a stream of nitrogen to drive off the acetone. The pellet was dissolved in a small volume of buffer and used as an enzyme preparation.

All reaction mixtures, consisting of the substrates and the enzyme system, were incubated at 30° C, in a constant temperature bath for the duration of the incubation period.

RESULTS AND DISCUSSION

A time course of methylmercury formation in pea stems via the infiltration and incubation procedure was carried out to determine the incubation time required for the formation of the maximum concentration of methylmercury. Ten grams of stems from the peas grown for 14 days in the greenhouse were used for each time determination. The infiltration and incubation solution contained 10 parts per million mercuric nitrate. The results obtained for the time course (Figure 1) show that the maximum formation of methylmercury occurred after 10 hours of incubation and declined to approximately 25% of the maximum after 20 hours of incubation. These findings suggest that methylmercury is either lost by the tissues or it is transformed to another form of mercury which cannot be detected with the equipment and methodologies used.

In our previous study (Gay, in press) an experiment was set up to determine whether methylmercury or ethylmercury was being liberated from the incubation mixture. A nitrogen stream was directed into the incubation solution and subsequently into a carbonate-phosphate trap as described by Kimura and Miller (1960) and into a cysteine trap. After 20 hours of incubation, the traps were analyzed. No methylmercury or ethylmercury was detected. The total mercury balance in the infiltrated and incubated tissues has not been determined at this time, so the question of the transformation of methylmercury to a volatile mercury species which is lost or the transformation of methylmercury to a species which is not detected cannot be answered at this time.

The supernatant solution obtained after the centrifugation of pea tissues ground in 0.01M phosphate buffer with a mortar and pestle was used initially as a crude enzyme preparation. Methionine was selected as the first compound to be used as a substrate for a methyl-donating compound with which to methylate mercuric nitrate. After various periods of incubation, no methylmercury was found. Several cofactors were added in various combinations and molarities in an attempt to complete the enzyme system.

Methylmercury was formed when 6 milliliters of a supernatant solution, obtained by grinding 1 gram of tissue per 5 milliliters of phosphate buffer, was incubated for 4 hours at 30° C, in the dark with magnesium, manganese, ATP, methionine and mercury in the following final concentrations: 4×10^{-4}M magnesium and manganese, 1×10^{-2}M ATP, 2.5×10^{-4}M methionine, and 5.5×10^{-5}M mercuric nitrate.

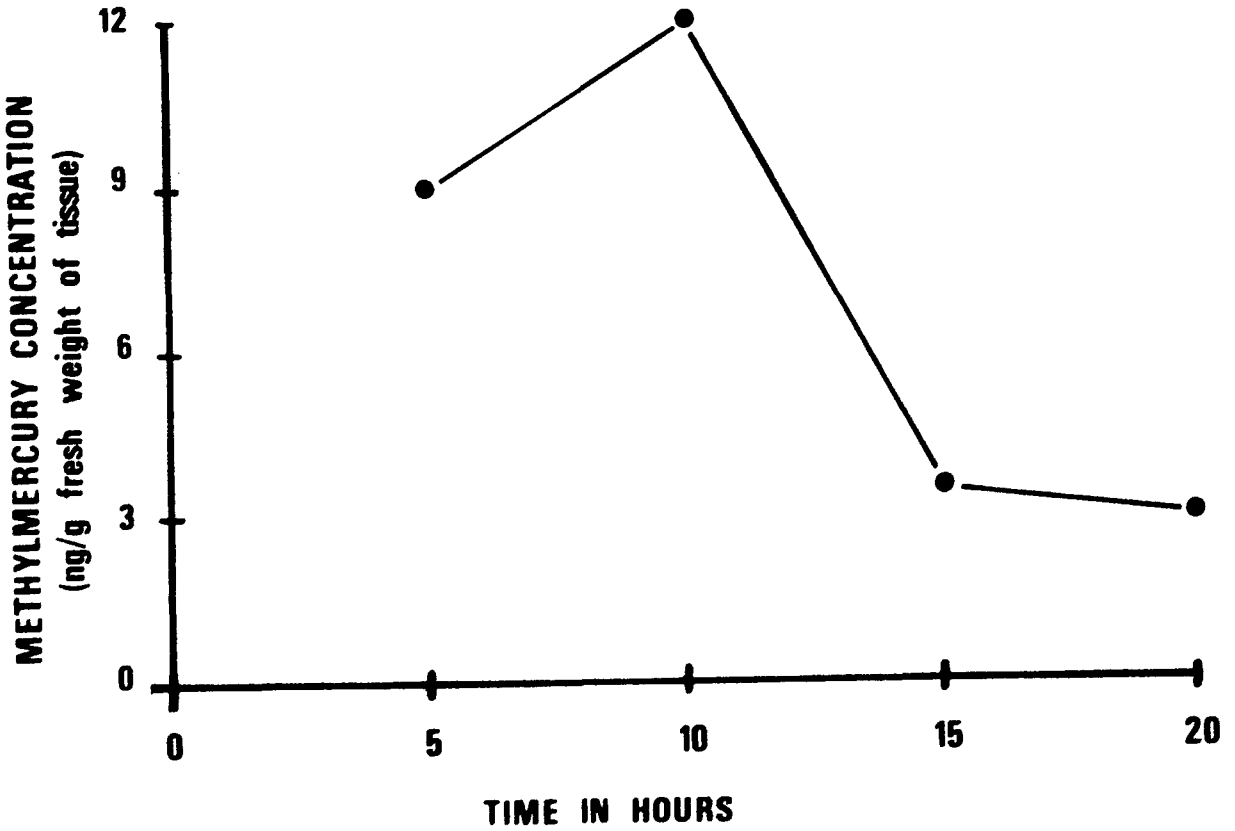

Figure 1. Time course for the formation of methylmercury in the infiltration and incubation procedure.

This same incubation mixture was used to determine the optimum pH of the enzyme system which forms methylmercury. An equal weight of tissues from peas grown for 14 days in the greenhouse was used for each of five pH values being tested. The tissues were ground in a specific pH buffer and the various cofactors were dissolved in the same buffer. After a 4-hour incubation of each at 30° C, the incubation mixture was analyzed for methylmercury. Maximum formation of methylmercury under this set of conditions occurred at pH 7.5 (Figure 2).

Two possibilities exist for the mechanism involved in the enzymatic, in vitro, methylation of mercuric nitrate with methionine and the various cofactors used. The methylation reaction may be straightforward, utilizing methionine as the methyl-donating compound but requiring the cofactors to complete the enzyme system methylating mercury. The other possibility is

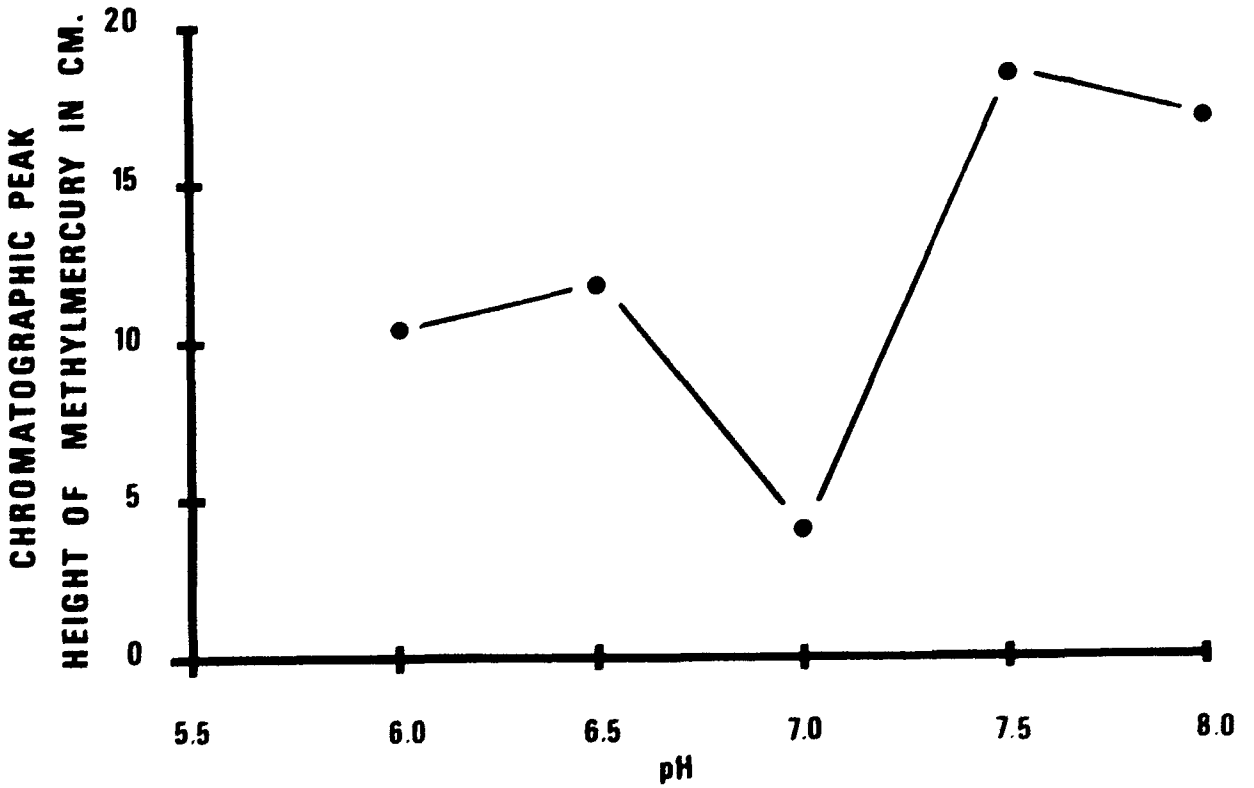

Figure 2. The in vitro methylation of mercuric nitrate as a function of pH.

that the cofactors plus methionine are involved in an enzymatic reaction forming S-adenosyl-methionine which, when formed, reacts with the mercuric nitrate enzymatically to form methylmercury. The latter possibility is the more plausible because the specific cofactors, the concentrations of the cofactors used, and the optimum pH for methylmercury formation are extremely similar to those which Cantoni (1955 and 1957) found to be required for the enzymatic synthesis of S-adenosyl-methionine.

The acetone protein precipitation procedure was used to partially purify the enzyme system forming methylmercury. Each protein fraction from the acetone precipitation procedure was incubated with methionine, mercuric nitrate and the various cofactors for 4 hours at pH 7.5. After the incubation period, each fraction was analyzed for methylmercury. The proteins precipitated between 70% and 92% acetone addition contained the enzyme system which forms methylmercury.

Because of the possibility of S-adenosyl-methionine being the methyl-donating compound, S-adenosyl-methionine (Sigma Chemical Company) was used as a substrate along with mercuric nitrate. Proteins precipitated between 70% and 92% acetone were divided into two equal volumes. One was incubated with methionine and mercuric nitrate, and the other was incubated with S-adenosyl-methionine and mercuric nitrate. The final concentrations of methionine and S-adenosyl-methionine were 1×10^{-3}M, and the final concentration of mercuric nitrate in each was 1.1×10^{-4}M. The incubation period was 4 hours at 30° C. Each incubation mixture was analyzed for methylmercury. S-adenosyl-methionine as substrate produced a relative concentration of 25 for methylmercury, while methionine as substrate produced a relative concentration of 1, barely distinguishable from background.

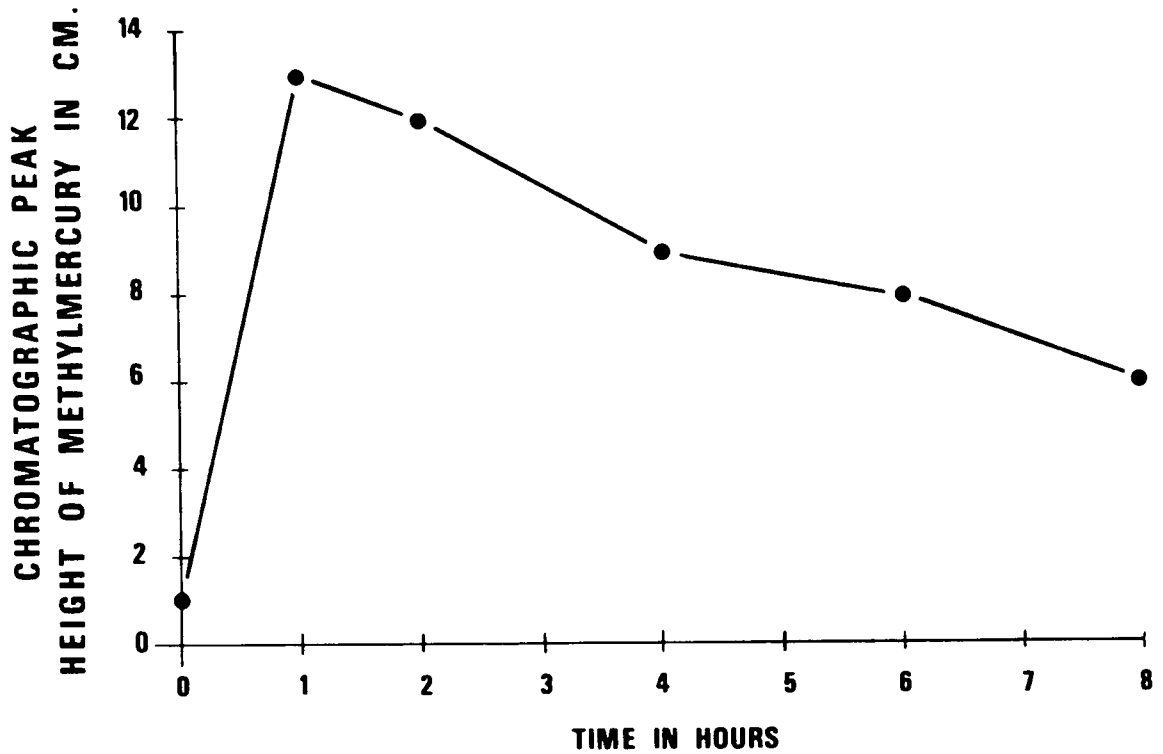

Figure 3. Time course for the *in vitro* methylation of mercuric nitrate.

A time course over an 8-hour period was done to determine when the maximum amount of methylmercury was formed with S-adenosyl-methionine as the methyl-donating substrate. Sixty grams of stems and apices from peas 14 days old were harvested. The tissues were ground in 0.01M phosphate buffer at pH 7.5 using 1.5 milliliter buffer per gram fresh weight of tissue. The 70% to 92% acetone-precipitated proteins were dissolved in 20 milliliters of phosphate buffer. S-adenosyl-methionine at a final concentration of 5×10^{-4}M and mercuric nitrate at a final concentration of 1.1×10^{-4}M were added to the protein solution. Aliquots were removed at 0, 1, 2, 4, 6, and 8 hours. Each aliquot was immediately analyzed for methylmercury. Maximum formation of methylmercury occurred after 1 hour of incubation with a steady decline in amount of methylmercury thereafter to 8 hours (Figure 3).

The results correspond to the time course obtained from the infiltration and incubation experiment as shown in Figure 1. In both *in vivo* and *in vitro* experiments methylmercury concentrations reached a peak and then declined thereafter. What happens to the methylmercury is unknown at this time, but this study suggests that methylmercury is an intermediate product in the mercury pathway in peas.

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16. ABSTRACT

The *in vitro* methylation of inorganic mercury has been demonstrated using an acetone precipitated protein fraction from *Pisum sativum* and S-adenosyl-methionine as the methyl-donating compound.

A time course of the enzymatic formation of methylmercury has shown that the maximum methylmercury concentration occurred after 1 hour of incubation of the substrates with the enzyme system. At all subsequent incubation times reduced concentrations of methylmercury in the reaction mixture were observed.

When a time course for the *in vivo* methylation of inorganic mercury was done using an infiltration and incubation procedure, the maximum concentration of methylmercury was observed after 10 hours of incubation. All subsequent incubation periods produced lesser amounts of methylmercury.

The results suggest that the methylmercury is an intermediate compound in the mercury pathway in peas (*Pisum sativum*).

17. KEY WORDS AND DOCUMENT ANALYSIS

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

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