

Effect of Phosphate and Other Anions on Trimethylarsine Formation by *Candida humicola*

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Phosphate inhibited the formation of trimethylarsine from arsenite, arsenate, and monomethylarsonate, but not from dimethylarsinate, by growing cultures of *Candida humicola*. Phosphite suppressed trimethylarsine production by growing cultures from monomethylarsonate but not from arsenate and dimethylarsinate, and hypophosphite caused a temporary inhibition of both proliferation and the conversion of these three arsenic sources to trimethylarsine. Resting cells of *C. humicola* derived from cultures grown in arsenic-free media generated the volatile arsenical only after a lag phase. High antimonate concentrations reduced the rate of conversion of arsenate to trimethylarsine by resting cells, but nitrate was without effect.

Arsenic compounds have been and still are widely used as pesticides. Inorganic arsenicals were applied for pest control in the past in amounts such that certain areas even now cannot be farmed, owing to the presence of significant quantities of this toxic element in the soil (4). At the present time, organic arsenicals such as cacodylic acid and monosodium methanearsonate are used as herbicides or defoliants. The microbial conversion of the latter compound to arsenate and CO₂ has been demonstrated in samples of several soils (3, 7). Despite the vast amounts of such pesticides which have entered the soil, little is known about the behavior of this element in natural ecosystems.

The formation by microorganisms of volatile arsenic compounds is of special concern because volatilization may result in human exposure to this toxic element. For example, several fungi have been reported to be able to form trimethylarsine, a volatile and highly toxic metabolite (1). A culture of *Methanobacterium*, by contrast, was recently found to generate dimethylarsine from arsenite under anaerobic conditions (6), and a volatile product with a garlic-like odor, presumably an alkylarsine, has been observed to be evolved from soil treated with dimethylarsinic acid (Woolson and Kearney, Environ. Sci. Technol., in press). *Candida humicola* is of particular interest because, as shown herein, it is capable of converting salts of

arsenic, arsenious, monomethylarsonic, or dimethylarsinic acids, substances which are either pesticides or are formed from pesticides, to trimethylarsine.

The present study was initiated to determine some of the variables affecting arsenic methylation by *C. humicola*, to provide a basis for assessing the potential influence of environmental factors on this microbial process.

MATERIALS AND METHODS

All chemicals were of reagent grade. The purity of the commercial trimethylarsine and the identity of the volatile microbial product were determined by gas chromatography with a Varian Aerograph gas chromatograph, model 1700 (Walnut Creek, Calif.), fitted with a flame ionization detector. The stainless-steel column was 78-cm long and had a 2-mm inner diameter. The column used regularly was 5% (wt/wt) FFAP-coated Chromosorb G maintained at 75 C, but sometimes Chromosorb 101 at 150 C was employed. The temperature of the injector and detector was 200 C, and the flow rate of the carrier gas, N₂, was 100 ml/min.

The identities of the authentic and biologically produced trimethylarsine were verified by mass spectrometry with a Perkin-Elmer mass spectrometer, model 270 (Norwalk, Conn.), operating with an ionization voltage of 70 eV and an electron voltage of 2,000 eV. Both compounds exhibited parent ions at m/e 120.

The organisms were grown in a medium consisting of glucose (10 g), (NH₄)₂SO₄ (2.0 g), KH₂PO₄ (0.10 g), MgSO₄·7H₂O (0.05 g), KCl (0.05 g), thiamine

hydrochloride (0.01 g), and FeSO_4 (0.001 g). Succinic acid adjusted to pH 5.0 was added to a final concentration of 0.05 M. The culture medium was supplemented in some instances with 1.0 g of sodium arsenate, arsenite, monomethylarsenate, or dimethylarsinate per liter. The extent of growth was determined by measuring the absorbancy at 525 nm in a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc.).

The growth studies were initiated by growing an inoculum of *C. humicola* overnight in a medium containing one of the arsenicals. The culture was diluted in 100 ml of the same medium to an absorbancy of 0.12 to 0.15 at 525 nm. The culture vessel, which was a 300-ml Erlenmeyer flask fitted with a side arm, was sealed with a red neoprene sleeve stopper (West Co., Phoenixville, Pa.) which was held fast with a hose clamp, and it was incubated at 30 C on a reciprocal shaker. The head space above the culture was analyzed for trimethylarsine production by removing a 1.0-ml sample with a gas-tight syringe and injecting this sample into the gas chromatograph.

To prepare cell suspensions, the inoculum prepared as above was transferred to a 2-liter Erlenmeyer flask containing 500 ml of culture medium. The culture was incubated at 30 C on a rotary shaker until an absorbancy of about 1.0 was reached, at which time the cells were collected by centrifugation, washed twice in 0.85% NaCl, and suspended in 7 to 8 ml of distilled water. A 0.5-ml portion of this suspension was placed in a 50-ml Erlenmeyer flask containing 10 ml of 0.5 M succinate buffer (pH 5.0), one of the arsenic compounds, and a compound whose effect on trimethylarsine production was to be examined. The flasks were sealed with the rubber stoppers and incubated at 30 C on a reciprocal shaker. At regular intervals, the head space was analyzed for its content of trimethylarsine.

The 10% aqueous solutions of KNO_3 , KH_2PO_4 , NaH_2PO_4 , $(\text{NH}_4)_2\text{HPO}_4$, NaH_2PO_2 , Na_2HPO_3 , and NaSbO_3 were adjusted to pH 5.0 before portions were added to representative cultures.

To determine dry weights, duplicate 0.5-ml samples of the culture were placed on tared planchets and dried overnight at 110 C. The planchets were weighed after having cooled to room temperature in a desiccator.

Trimethylarsine evolution often was detected only after several hours, and analyses were not routinely performed during this initial period. At the time of the first analysis in some experiments, therefore, some volatile arsenic was found, and the data showing this initial level are presented as the trimethylarsine concentration in the head space at zero time.

RESULTS

Trimethylarsine was produced by cultures of *C. humicola* growing in media supplemented with 0.10% of the sodium salts of arsenate, arsebute, monomethylarsenate, or dimethylarsinate. The latter three may be interme-

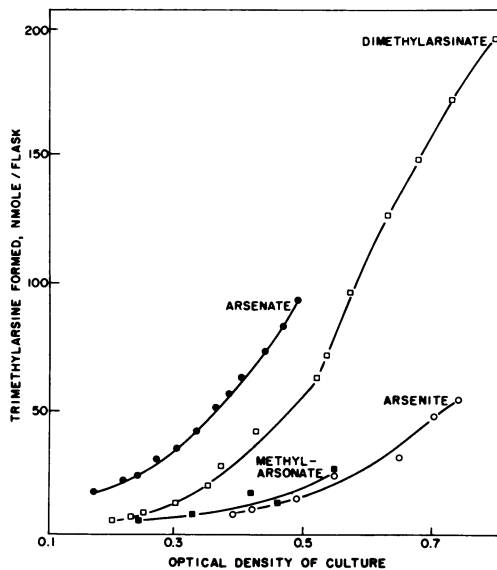


FIG. 1. Formation of trimethylarsine from arsenate (240 μg of As/ml), arsenite (544 μg of As/ml), monomethylarsenate (577 μg of As/ml), and dimethylarsinate (272 μg of As/ml) in relation to cell density in growing cultures of *Candida humicola*.

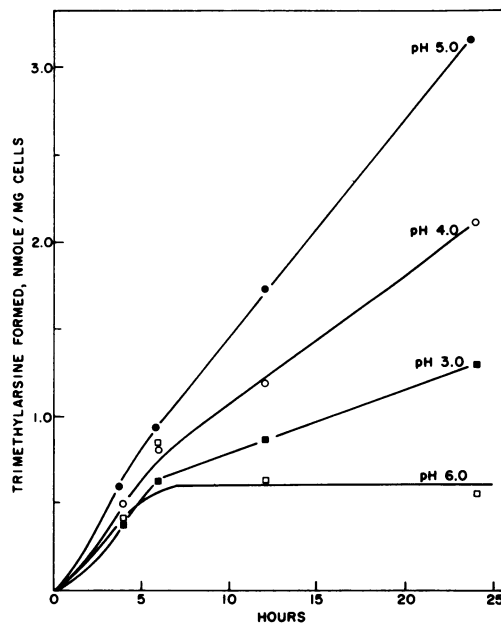


FIG. 2. Effect of pH on evolution of trimethylarsine by resting cells of *Candida humicola*.

diates in the alkylation sequence by which arsenate is converted to trimethylarsine (1). The yield of trimethylarsine was proportional to the density of cells in the growing culture, but the yield varied with the arsenic source

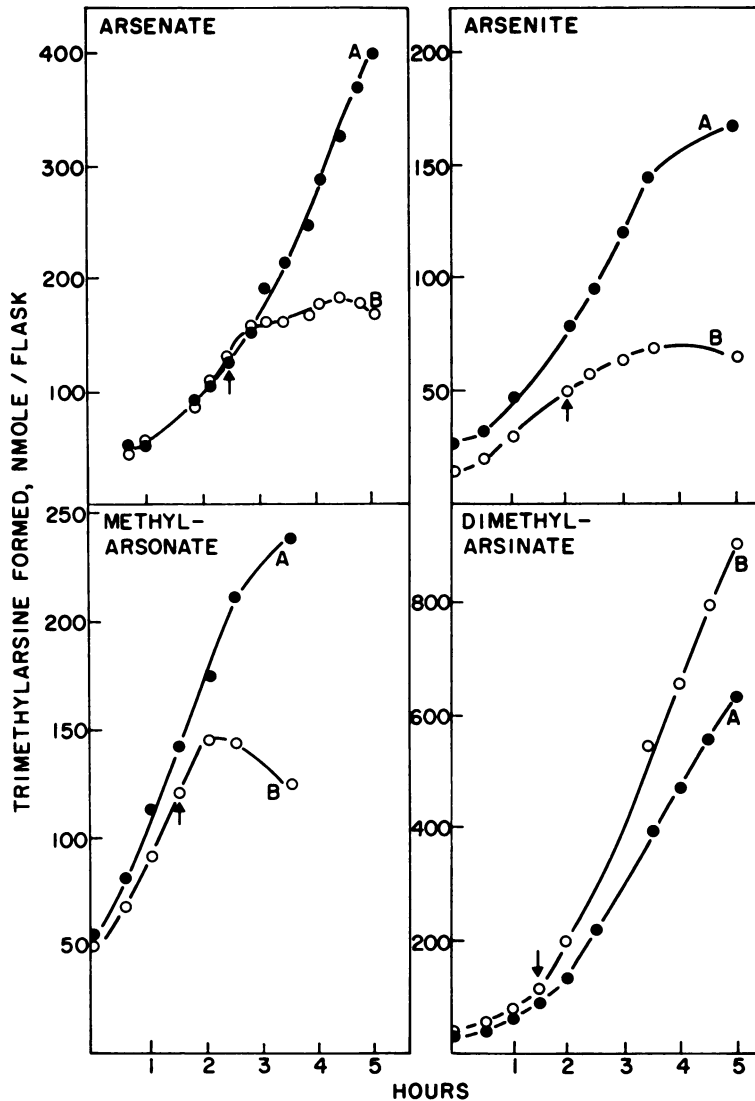


FIG. 3. Effect of high phosphate levels on the formation of trimethylarsine by *Candida humicola* grown in media with arsenate, arsenite, monomethylarsenate, and dimethylarsinate. The arrow indicates the time that the supplemental KH_2PO_4 was added. A, No additions; B, 0.1% KH_2PO_4 added.

(Fig. 1). The greatest amount of trimethylarsine relative to cell density was formed in cultures containing arsenate. Lesser quantities were generated in the presence of arsenite and monomethylarsenate even though the arsenic concentrations in these instances were higher than in flasks containing arsenate and dimethylarsinate.

The optimum pH for trimethylarsine production was determined by suspending washed cells in four different buffers, each containing 1.0 g of sodium arsenate per liter. Citrate (0.05 M) was used at pH 3.0, 4.0, and 5.0, and 0.05

maleate-tris(hydroxymethyl)aminomethane buffer was used at pH 6.0. The buffer at pH 5.0 supported the greatest activity and gave the largest product yield (Fig. 2), and it was thus used in subsequent studies. However, succinate was used at pH 5.0 in place of the citrate.

The possible effect of phosphate on the formation of this toxic gas by growing cultures was studied by comparing the product yield in media to which were added 0.01 and 0.10% KH_2PO_4 . The lower level was required to allow for adequate growth of the organism, and this concentration was present in all cultures. Addi-

tional phosphorus (0.1% KH_2PO_4) was added to half the flasks after growth was proceeding. Addition of the higher phosphate level reduced trimethylarsine production from arsenate, arsenite, and methylarsonate (Fig. 3). The effect was not the result of a pH change from the added phosphate, because the phosphate was adjusted to pH 5.0 before it was introduced. By contrast, the high-phosphate levels did not reduce arsenic volatilization in cultures containing dimethylarsinate, and an addition of even up to 0.8% KH_2PO_4 did not affect trimethylarsine evolution from this substrate. The higher KH_2PO_4 concentrations did

not affect growth of *C. humicola* in media containing any of these arsenicals.

The influence of other phosphorus salts on trimethylarsine production was also tested with growing cultures. The addition of Na_2HPO_3 to give a final concentration of 0.10% did not affect trimethylarsine biosynthesis in media containing 0.10% of the salts of arsenate or dimethylarsinate. However, this level of phosphite inhibited arsenic volatilization when the medium contained methylarsonate (Fig. 4). On the other hand, phosphite did not reduce the rate of growth in solutions containing methylarsonate.

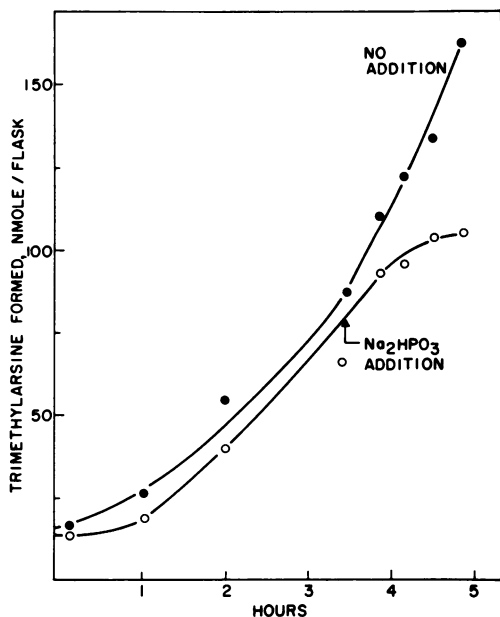


FIG. 4. Inhibition of trimethylarsine production upon addition of 0.10% Na_2HPO_3 to cultures of *Candida humicola* grown in media with monomethylarsonate. Arrow designates time of phosphite addition.

The addition of NaH_2PO_2 to growing cultures to a final concentration of 0.10% had a different effect than that of the other phosphorus compounds. Introduction of hypophosphite into *C. humicola* cultures caused an immediate reduction or even a cessation of trimethylarsine evolution from arsenate, methylarsonate, and dimethylarsinate (Fig. 5). However, this inhibition disappeared rapidly, and the rate of formation of volatile arsenic was similar or essentially the same as that in the cultures not treated with hypophosphite.

A study was then made of the influence of varying the hypophosphite concentration on trimethylarsine production from dimethylarsinate by growing cultures. A distinct inhibition was again noted (Fig. 6), but this inhibition was soon overcome and arsenic gas production recommenced. The length of the period before trimethylarsine evolution resumed was proportional to the hypophosphite concentration, and the rate after the period of suppression was essentially the same as in untreated cultures. The temporary suppression by large amounts of hypophosphite may have resulted from a short-lived inhibition of growth. Thus, measurements of the absorbancy at 525 nm demonstrated that the addition of 0.05 to 0.8%

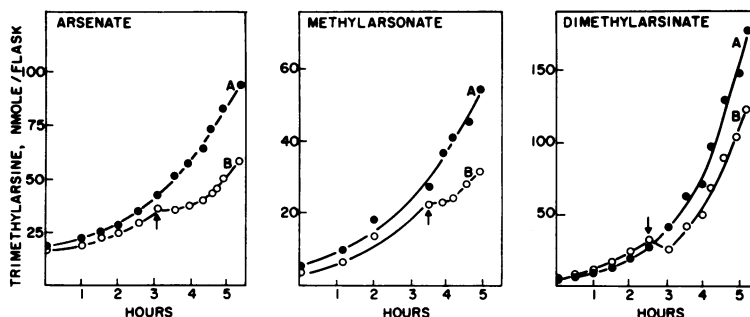


FIG. 5. Influence of hypophosphite on the formation of trimethylarsine from arsenate, methylarsonate, and dimethylarsinate. Arrow designates the time of hypophosphite addition. A, No additions; B, hypophosphite added.

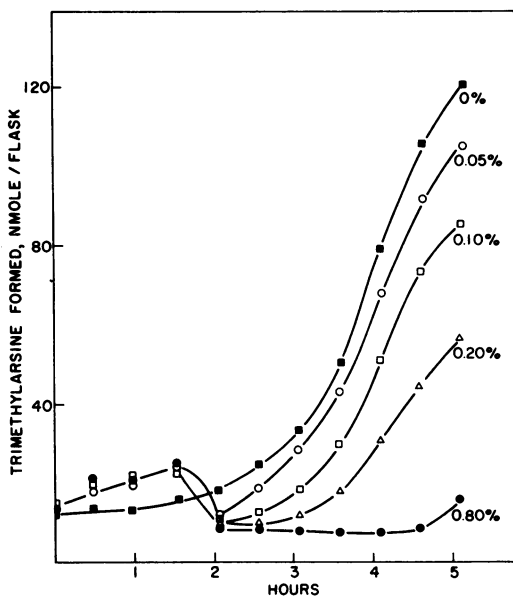


FIG. 6. Temporary inhibition of trimethylarsine formation in media containing dimethylarsinate and various hypophosphite concentrations. NaH_2PO_2 was added after 90 min. Values are the final NaH_2PO_2 concentrations.

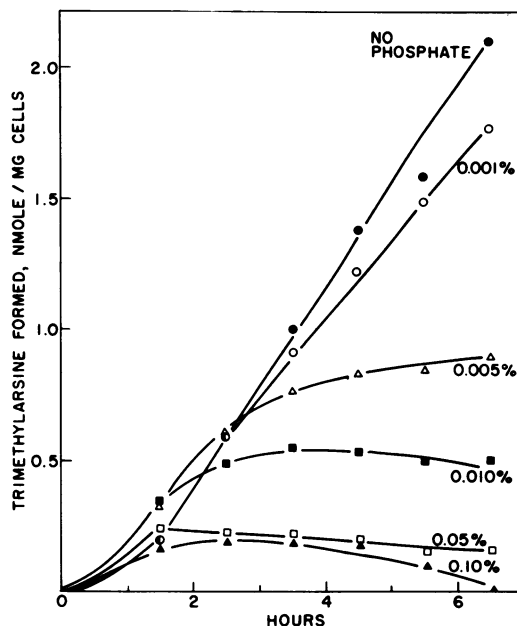


FIG. 8. Inhibition of trimethylarsine formation from arsenate ($240 \mu\text{g}$ of As/ml) by resting cells in the presence of various KH_2PO_4 concentrations.

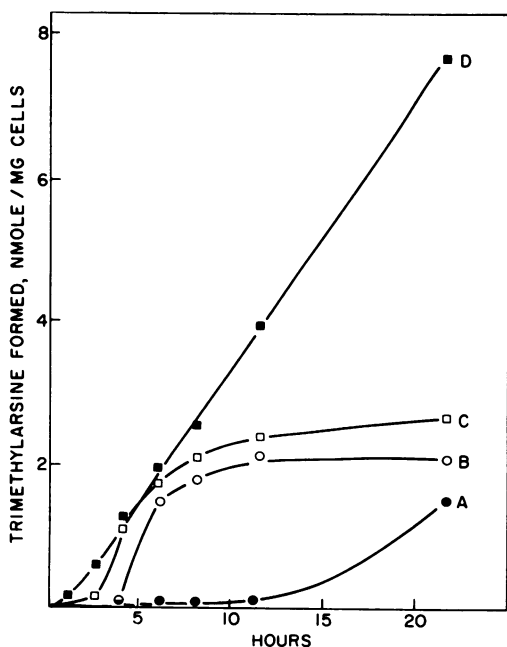


FIG. 7. Trimethylarsine formation by resting cell suspensions of *Candida humicola* grown in the presence of 0.00 (A), 0.01 (B), 0.05 (C), and 0.20% NaH_2AsO_4 (D). Suspensions were incubated with 0.1% NaH_2AsO_4 .

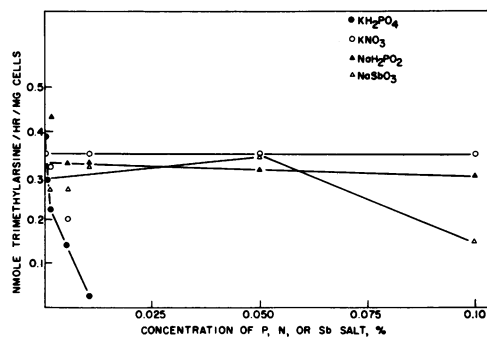


FIG. 9. Influence of orthophosphate, hypophosphite, nitrate, and antimonate on the production of trimethylarsine by resting cells.

NaH_2PO_2 led to an immediate cessation of growth, but proliferation resumed at the same rate as in the untreated flasks within periods of about 1 h or less. The inhibition of trimethylarsine production sometimes was as long as 2.5 to 3 h at 0.4 and 0.8% NaH_2PO_2 . Relief of the toxicity may be a consequence of the conversion of hypophosphite to a nontoxic product.

Subsequent studies were conducted with resting cell suspensions of *C. humicola* to eliminate possible effects of the test compounds on multiplication. An experiment was conducted to determine whether these cell

suspensions would have greater activity if the organisms were previously grown in media containing an arsenical than if grown in solutions devoid of this element. The organism was thus cultured in medium with and without arsenate, and the cells were washed and then incubated in the presence of 0.10% NaH_2AsO_4 . Cells from arsenic-free media generated the alkylarsine only after a long lag period (Fig. 7). A short lag phase was evident in cells collected from media with 0.01 and 0.05% arsenate. By contrast, cells from media with 0.20% arsenate began to synthesize trimethylarsine with essentially no lag, and the reaction was linear with time.

Phosphate reduced the extent of trimethylarsine formation by resting cells. The arsenic source in these tests was 0.1% NaH_2AsO_4 , and the cells were suspended in succinate buffer at pH 5.0. The rates of gas evolution depicted in Fig. 8 demonstrate a modest inhibition at 0.001% KH_2PO_4 and a significant depression at concentrations of 0.005% or greater. Almost no activity was observed in the presence of 0.05% of the phosphate salt. The results were similar whether the phosphate was provided as the sodium or the ammonium salt, demonstrating that the anion is probably responsible for the inhibition.

To determine whether other elements of group 5 would suppress this activity, resting cells were incubated with 0.10% NaH_2AsO_4 in the presence of various concentrations of KNO_3 , NaH_2PO_3 , NaSbO_3 , and KH_2PO_4 . Nitrate and hypophosphite were without significant effect (Fig. 9). Antimonate was not toxic at the lower concentrations, but a reduction in activity occurred in the presence of 0.10% NaSbO_3 . The solubility of the antimony salt is low, and a greater inhibition might have been evident were more of the antimonate in solution. Orthophosphate, as before, at 0.01% almost totally abolished activity.

DISCUSSION

The observation that phosphate does not inhibit the conversion of dimethylarsinate to trimethylarsine by *C. humicola* is of interest in light of the report by Da Costa (2) that phosphate failed to relieve the toxicity of dimethylarsinate to arsenic-tolerant and arsenic-sensitive fungi, although it did overcome arsenate and arsenite toxicity. Considering the present findings on the phosphate inhibition of trimethylarsine production from the test arsenicals, it may be postulated that phosphate suppresses gas evolution by blocking the conversion of the arsenicals to trimethylarsine at a

stage between the mono- and dimethylarsenic compounds. If the inhibition of fungi noted by Da Costa (2) results from the formation of a single toxicant from the various arsenicals he tested, then phosphate may overcome the inhibition of arsenate and arsenite by blocking its formation. This hypothetical inhibitor might then be a product of the sequence leading to trimethylarsine production, and the relief of arsenic inhibition of fungi by phosphate and of gas evolution by *C. humicola* may have a common basis.

The finding that *C. humicola* is able to convert widely used arsenic-containing pesticides to a volatile product, herein identified as trimethylarsine, reemphasizes the need for a reassessment of the widespread use of such pest-control agents. Whether the final product is dimethylarsine, as reported by McBride and Wolfe (6), or trimethylarsine, the potential release from treated soils of such potent toxicants should be examined to avoid possible instances of human intoxication. Nevertheless, with the exception of Epps and Sturgis (5), who reported an unidentified volatile arsenic compound to be released from soil, and the observation that a substance with a garlic-like odor is evolved after soil treatment with dimethylarsinate (Woolson and Kearney, Environ. Sci. Technol., in press), no study has been made of the possibility of gas evolution from soils naturally containing arsenic compounds or treated with arsenic-containing pesticides.

ACKNOWLEDGMENTS

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