22. P. Wild and J. Ames, *Calif. Dep. Fish Game Mar. Resour. Tech. Rep. 20* (1974); National Fish and Wildlife Laboratory, *Selected Vertebrate Endangered Species of the Seacoast of the United States—Southern Sea Otter* (Publication FWS/OBS-80/01.18, Fish and Wildlife Service, Washington, D.C., 1980).
23. Unpublished data, Port Safety and Marine Environment Protection Branch, U.S. Coast Guard, 12th District, San Francisco.
24. U.S. Department of the Interior, *Final Environmental Impact Statement, Outer Continental Shelf Lease Sale 53* (Bureau of Land Management, Los Angeles, 1980).
25. Department of the Interior, *Final Environmental Statement, Proposed Five-Year Outer Continental Shelf Oil and Gas Lease Sale Schedule, March 1980–February 1985* (Bureau of Land Management, Los Angeles, 1980); E. Hasley, *Fed. Regist.* **45**, 84878 (1980).
26. W. Samuels and K. Lanfear, *U.S. Geol. Surv. Open-File Rep. 80-211* (1980); Bureau of Land Management, *Pacific Outer Continental Shelf Ref. Pap. 53-2* (1980).
27. We thank the staff of the U.S. Coast Guard, Monterey, Calif., for sharing with us their observations of spilled lumber, and the following people, who reported sightings (or lack thereof) of beached lumber: S. Benech, R. DeLong, E. Ebert, P. Fells, B. Keller, R. Larson, P. Lehtonin, B. Logan, M. Owings, J. Taggart, T. Tolman, J. Vandevere, D. Ven Tresca, and C. Zabel. We thank C. Benz, R. Brownell, Jr., J. Estes, E. Hooper, A. Johnson, and K. Ralls for comments on early versions of this report, J. Bodkin for help with the data analysis, and D. Bodkin and J. French for help with the final preparation of the report.

18 August 1981; revised 30 November 1981

Methylation of Tin by Estuarine Microorganisms

Abstract. *Mixed inoculums of microorganisms from Chesapeake Bay sediments transformed inorganic tin ($SnCl_4 \cdot 5H_2O$) to organotin compounds. Dimethyltin and trimethyltin species were identified as products by gas chromatography–mass spectrometry. Methylated tin species were not observed in sterile controls or in poisoned controls. Thus, estuarine microorganisms have the potential for transforming tin to toxic organotins and for mobilizing tin in the ecosystem.*

Tin was one of the first metals used by man; implements made of tin alloy date as far back as 3000 B.C. (*1*). Today, inorganic tin is used as a protective coating for steel and in solders, bearing metals, and other alloys. Organotin compounds are used as stabilizers for polyvinyl chloride plastics. They are also incorporated into biocidal preparations such as insecticides, herbicides, fungicides, and antifouling paints (*2*). Most of these compounds will eventually enter the environment where they can be leached from consumer products by chemical and biological (mainly microbiological) processes. Organotins are toxic to eukaryotes (*3*) and prokaryotes (*4*), but few studies have been done on the environmental distribution and transport of tin (*5–7*). A biological cycle for tin has been proposed (*8*) and discussed (*9*). The biotic and abiotic formation of $(CH_3)_4Sn$ from $(CH_3)_3SnOH$ in material taken from anaerobic sediments has been demonstrated recently (*10*), and methylstannanes have been found in Chesapeake Bay (*11*). The purpose of this investigation was to assess the potential of sediment microorganisms in Chesapeake Bay to biotransform inorganic tin to organotin compounds.

Sediment samples were taken in the summer of 1979 at nine sites in the northern and central Chesapeake Bay. The tin content of sediments at these sites varies with the amount of human activity at the site (*6*).

Two systems, flask cultures and Hungate tubes, were used to determine if the microbial flora could transform tin into volatile organometallic derivatives: 1) A 250-ml, screw-capped Erlenmeyer flask received 10 ml of solid nutrient medium (*12*) supplemented with 0.75 mg of tin as $SnCl_4 \cdot 5H_2O$. A sterile 10-ml beaker was placed in the center of the medium before it solidified. One milliliter of an 8 percent (weight to volume) solution of citric acid in 10 percent HCl was added to the beaker to trap volatile tin compounds. Each flask was then inoculated with 1.0 ml of a 1:10 (weight to volume) suspension of sediment in an estuarine salt solution (10.0 g of NaCl, 2.8 g of $MgSO_4$, and 0.3 g of KCl per liter of distilled water). The flask was closed and incubated for 14 days at 25° ± 2°C. Material in the beaker was then sampled and analyzed for tin by atomic absorption spectrophotometry (AAS).

2) Each Hungate tube (*13*), containing 5.0 ml of liquid nutrient medium (*12*) with 0.375 mg of tin as $SnCl_4 \cdot 5H_2O$ was inoculated with 0.5 ml of a 1:10 (weight to volume) suspension of sediment in estuarine salts and incubated for 14 days at 25° ± 2°C. The culture was then centrifuged to remove debris, and the supernatant medium was extracted with a dichloromethane-chloroform mixture (9:1, volume to volume). The organic phase was evaporated to dryness, and the residue was resuspended in methyl isobutyl ketone and analyzed by AAS.

Sterile controls containing $SnCl_4 \cdot 5H_2O$ gave negative results, and sterile controls in which $(CH_3)_2SnCl_2$ (75 mg of tin per liter) was substituted for $SnCl_4 \cdot 5H_2O$ yielded tin in the center well of flasks or the organic phase extracted from tube cultures. Thus, the two methods can be used to detect an organotin compound without interference by inorganic tin. For eight of nine sediment samples, the experimental flasks and tubes contained organotin compounds. For the ninth sample, taken from the Patuxent River, the culture did not produce evidence of volatile organotins. No organotin was detected in tubes or flasks containing the metabolic poison sodium azide. Therefore, the transformation of inorganic tin to organotin was the result of biological activity.

To identify the volatile tin compounds produced, we used larger volumes of culture. Flask cultures containing 500 ml of liquid medium (*12*) and 37.5 mg of tin as $SnCl_4 \cdot 5H_2O$ were inoculated with 0.1 g of sediment and incubated for 14 days. We analyzed the culture medium for organotin compounds by using a modified hydride reduction technique (*5, 7*). The medium was centrifuged, and 100 ml of supernatant medium was added to a gas scrubbing bottle. Using Tygon tubing, we attached a 0.2-m stainless steel chromatographic column (outside diameter, 6 mm; capacity, 2 ml) packed with Tenax GC (Supelco Inc., Supelco Park, Pennsylvania) to the bottle. The column was cooled to −40°C with liquid nitrogen. The spent medium was adjusted to *p*H 6.2 with 2*M* tris-HCl, and the solution was purged with N_2 for 2 minutes. Two milliliters of 1 percent (weight to volume) $NaBH_4$ was then added quickly, and N_2 was bubbled slowly through the solution. The bottle was immersed in a water bath at 90°C for 5 minutes. The column was then removed and allowed to warm slowly to approximately 0°C. A syringe was attached to the column with Tygon tubing, and the column was eluted with 10 ml of cold (4°C) heptane. The heptane solution was then stored at −10°C until it was analyzed. A gas chromatograph equipped with an electron-capture detector was used to separate and measure the organometallic species.

All three authentic methyltin hydrides [CH_3SnH_3, $(CH_3)_2SnH_2$, and $(CH_3)_3SnH$] and $(CH_3)_4Sn$ were resolved (Fig. 1). The extract from cultures inoculated with sediment contained two peaks which cochromatographed with $(CH_3)_2SnH_2$ and $(CH_3)_3SnH$ standards, respectively. A minor peak was also obtained that had a retention similar to that of CH_3SnH_3, but it was not detected in all replicate experiments. Sterile controls and cultures that received sodium azide yielded no metal hydride peaks. Thus, the organotins produced were the result of biological activity.

0036-8075/82/0319-1505$01.00/0

Table 1. Retention times and mass intensity ratios of methyltin compounds and sediment unknowns obtained by gas chromatography–mass spectrometry; *m/e*, mass-to-charge ratio.

Compound (isotopic weight of molecular ion)*	Retention time† (minutes)	Ratios of peak intensity at *m/e*‡ of					
		123.9	137.9	151.9	165.9	179.9	164.9
		Standards					
$(CH_3)_2SnH_2$ (151.9)	1.1	0.974	1.000	0.219	0.018	0.020	0.181
$(CH_3)_3SnH$ (165.9)	1.9	0.864	1.000	1.210	0.038	<0.010	1.177
$(CH_3)_4Sn$ (179.9)	3.0	0.903	1.000	0.655	0.771	0.106	18.319
		Unknowns					
Peak 1	1.1	0.819	1.000	0.250	0.011	<0.010	0.225
Peak 2	1.9	0.935	1.000	1.529	0.035	<0.010	1.585

*Isotopic weights were calculated based on the most abundant isotopic mass of tin, 119, found in environmental samples (*15*). †Separated on a 10-m OV-101 glass capillary column (0.30 mm inside diameter) held at 0°C with a helium flow of 2.0 ml per minute; splitless injection. Mass spectrometer conditions were as follows: electron ionization selected ion chromatograms were obtained with an ionization voltage, 70 eV; source temperature, 150°C; electron multiplier voltage, 2200 V. ‡Six masses were monitored by mass spectrometry: 123.9, SnH_4^+; 137.9, $CH_3SnH_3^+$; 151.9, $(CH_3)_2SnH_2^+$; 165.9, $(CH_3)_3SnH^+$; 179.9, $(CH_3)_4Sn^+$; and 164.9, $(CH_3)_3Sn^+$. The area of each peak was compared to that of CH_3SnH_3.

In order to confirm the identity of peaks obtained by gas chromatography, we analyzed heptane extracts on a Hewlett-Packard 5992 gas chromatograph–mass spectrometer, using selected ion monitoring. The retention times and mass intensity ratios of the peaks obtained are presented in Table 1. The extracts from cultures inoculated with sediment contained two peaks that had the same retention times as authentic $(CH_3)_2SnH_2$ and $(CH_3)_3SnH$, respectively. Selected ion mass chromatograms were monitored for $^{119}SnH^+$, $(CH_3)_3{}^{119}Sn^+$, and the molecular ions characteristic of the three methyl ^{119}Sn hydrides and tetramethyl ^{119}Sn. We then calculated the mass intensity ratio of each peak by dividing the area of each peak in the mass chromatogram by the area of the corresponding peak for the $CH_3SnH_3^+$ mass chromatogram. The two peaks from the extract of cultures inoculated with sediment closely matched the individual mass intensity ratios, and all characteristic ions observed in the mass chromatograms of the appropriate standards were present (Table 1). Confirmation of the presence of $(CH_3)_2SnH_2$ and $(CH_3)_3SnH$ in the culture extracts was based on three criteria: (i) the identical retention times of unknowns and authentic standards, (ii) the occurrence of all characteristic ions in each unknown peak as compared to standards, and (iii) similar mass intensity ratios of each unknown peak as compared to standards.

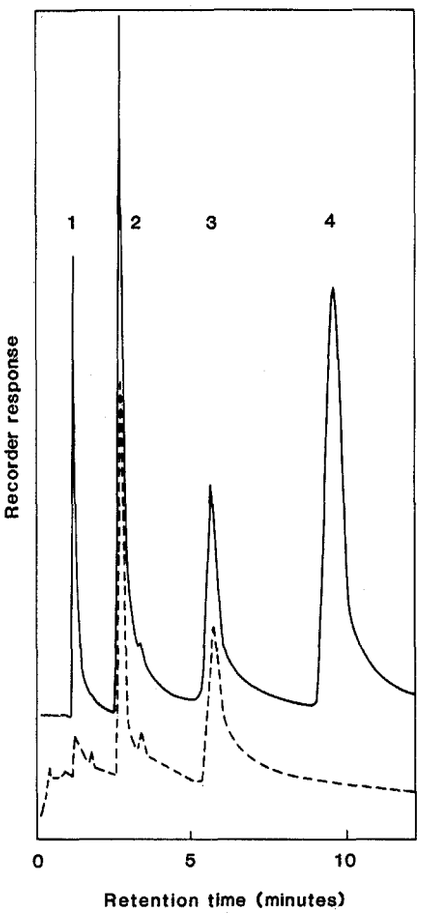

Fig. 1. Gas chromatographic tracing of organotin compounds produced in mediums inoculated with sediment. The solid line represents the standard organotin mixture: *1*, CH_3SnH_3; *2*, $(CH_3)_2SnH_2$; *3*, $(CH_3)_3SnH$; and *4*, $(CH_3)_4Sn$. The dashed line represents the extract from cultures inoculated with sediment. The 183-cm glass column was packed with 0.1 percent Carbowax 1500 on 80/100 mesh Supelcoport. The injection port was maintained at 150°C and the column at 70°C. The carrier gas (10 percent methane in argon) had a flow rate of 30 ml per minute. The methods used were not quantitative. We prepared standards by adding each known compound to 100 ml of water to yield a solution that contained approximately 10 mg of tin from each known compound. This solution was placed in a gas scrubbing bottle and treated in the same manner as the spent culture medium.

These results suggest that sediment microflora have the potential to biotransform tin in sediment. Three other groups have reported tin biotransformations. A *Pseudomonas* sp. from Chesapeake Bay biomethylated tin, and $(CH_3)_4Sn$ has been identified in the gas space of a culture containing inorganic tin (*11*). Microorganisms in Canadian lake sediments transformed Sn(II) and Sn(IV) to methyltin (*14*). Material from anaerobic sediments taken from San Francisco Bay converted $(CH_3)_3SnOH$ to $(CH_3)_4Sn$ over a period of 80 days; the conversion had both biotic and abiotic components (*10*).

The present study provides evidence of the production of dimethyltin and trimethyltin species from inorganic Sn(IV) by sediment microflora. The results are consistent with a geocycle of tin as proposed by Ridley *et al.* (*8*) and support the hypothesis that tin can be biotransformed in an estaurine environment. However, methylated tin species may result from abiotic as well as biotic events (*10, 11, 14*). As far as we know, no field studies have been performed to determine if these biotransformations play a significant role in the natural environment, but organotin compounds have been found in estuarine and coastal waterways (*5, 7, 11*). Although they may come from anthropogenic sources or may be the result of chemical and biological transformations, it is clear that estuarine microorganisms have the potential for transforming tin to organotins and for mobilizing tin in the ecosystem.

L. E. HALLAS
Chesapeake Biological Laboratory, Center for Environmental and Estuarine Studies, and *Department of Microbiology, University of Maryland, Solomons 20688-0038*
J. C. MEANS
Chesapeake Biological Laboratory, Center for Environmental and Estuarine Studies, and *Department of Chemistry, University of Maryland, Solomons*
J. J. COONEY
Chesapeake Biological Laboratory, Center for Environmental and Estuarine Studies, and *Department of Microbiology, University of Maryland, Solomons*

References and Notes

1. J. W. Price, *Fresenius Z. Anal. Chem.* **288**, 257 (1977).
2. J. J. Zuckerman, R. R. Residorf, H. V. Ellis III, R. R. Wilkerson, in *Organometals and Organometalloids*, F. E. Brinckman and J. M. Bellama, Eds. (Symposium Series 82, American Chemical Society, Washington, D.C., 1978), p. 73.
3. J. S. Thayer, *J. Organomet. Chem.* **76**, 265 (1974).
4. L. E. Hallas and J. J. Cooney, *Dev. Ind. Microbiol.* **22**, 529 (1981).

5. R. S. Braman and M. A. Thomkins, *Anal. Chem.* **51**, 12 (1979).
6. L. E. Hallas and J. J. Cooney, *Appl. Environ. Microbiol.* **41**, 466 (1981).
7. V. F. Hodge, S. L. Seidel, E. D. Goldberg, *Anal. Chem.* **51**, 1256 (1979).
8. W. P. Ridley, L. J. Dizikes, J. M. Wood, *Science* **197**, 329 (1977).
9. P. J. Craig, *Environ. Technol. Lett.* **1**, 225 (1980).
10. H. E. Guard, A. B. Cobet, W. M. Coleman III, *Science* **213**, 770 (1981).
11. J. A. Jackson, W. R. Blair, F. E. Brinckman, W. P. Iverson, *Environ. Sci. Technol.*, in press.
12. J. D. Nelson and R. R. Colwell, *Microb. Ecol.* **1**, 191 (1975).
13. R. L. Hungate, in *Methods in Microbiology*, J. R. Norris and D. W. Ribbons, Eds. (Academic Press, New York, 1969), vol. 3B, p. 117.
14. Y. K. Chau and P. T. S. Wong, in *Environmental Speciation and Monitoring Needs for Trace Metal-Containing Substances from Energy-Related Processes*, F. E. Brinckman and R. H. Fish, Eds. (Special Publication 618, National Bureau of Standards, Washington, D.C., 1981), p. 65.
15. H. Hamaguchi and R. Kuroda, in *Handbook of Geochemistry*, K. W. Wedepohl, Ed. (Springer, New York, 1969), vol. 11.
16. Contribution No. 1236, Center for Environmental and Estuarine Studies, University of Maryland.

8 July 1981; revised 24 November 1981

Inactivation of a Diol Epoxide by Dihydrodiol Dehydrogenase but Not by Two Epoxide Hydrolases

Abstract. *The mutagenicity of* r-*8*,t-*9-dihydroxy*-t-*10,11-oxy-8,9,10,11-tetrahydrobenz*[a]*anthracene (BA-8,9-diol 10,11-oxide) toward* Salmonella typhimurium *TA 100 is not decreased by the presence of large amounts of highly purified microsomal or cytosolic epoxide hydrolase. However, highly purified dihydrodiol dehydrogenase inactivates this diol epoxide, which is a major DNA-binding metabolite of benz*[a]*anthracene. The K-region epoxide, benz*[a]*anthracene 5,6-oxide (BA 5,6-oxide) is efficiently inactivated by microsomal epoxide hydrolase, is much less readily inactivated by cytosolic epoxide hydrolase, and is not inactivated by dihydrodiol dehydrogenase. This inactivation of a diol epoxide by dihydrodiol dehydrogenase points to a new significance of this enzyme and a new level of control for diol epoxides.*

Metabolic inactivation is one factor determining whether reactive intermediates formed from a polycyclic hydrocarbon will modify tissue macromolecules. In some cases, such modifications lead to mutation or to initiation of tumors. For example, K-region epoxides of polycyclic aromatic hydrocarbons are potent mutagens in microorganisms (*1, 2*) but are much less active in mammalian systems (*3*) and appear to contribute little to the total DNA binding of metabolically produced active intermediates in mammalian cells (*4*). These observations are consistent with the rapid detoxication of these epoxides by microsomal epoxide hydrolase (*5*) and by cytosolic glutathione *S*-transferases (*6, 7*), enzymes that are present in all the mammalian tissues investigated (*8*).

Much less is known of the enzymic inactivation of vicinal diol epoxides, the metabolites of polycyclic hydrocarbons that seem to be responsible for most of the carcinogenic and mutagenic effects induced by polycyclic hydrocarbons (*3, 4, 9, 10*). Mutagenicity and DNA-binding experiments with *trans*-7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene indicate that some inactivation is caused by the presence of glutathione (*7, 11, 12*) but not by microsomal epoxide hydrolase (*12–14*). Consistent with the latter observation is the inability of microsomal epoxide hydrolase to inactivate the *anti* isomer of the corresponding "bay-region" diol epoxide and the very weak effect on the activity of the *syn* isomer (*14*). These negative findings may result from the short half-life of the diol epoxide in an aqueous environment, although this may not necessarily be the same in a biological membrane. Low stability is not, however, a property of all vicinal diol epoxides. *r*-8,*t*-9-Dihydroxy-*t*-10,11-oxy-8,9,10,11-tetrahydrobenz[*a*]anthracene (BA-8,9-diol 10,11-oxide), which is the *anti* isomer of a non-bay-region diol epoxide, has a half-life of many hours (*15*) and is therefore useful for metabolic studies. In addition to serving as a model for less stable diol epoxides, its own metabolism is of interest, since it is mutagenic (*16, 17*) and is often the major DNA-binding species formed from benz[*a*]anthracene in vivo and in vitro (*10*). Using BA-8,9-diol 10,11-oxide, we show here that a diol epoxide can be metabolically inactivated. Inactivation did not occur with either microsomal or cytosolic epoxide hydrolase but was obtained with dihydrodiol dehydrogenase. If one assumes that the activities of the investigated enzymes in vivo are comparable to those that are present in our experiments in vitro, inactivation of the diol epoxide by dihydrodiol dehydrogenase would be slower than the rate of inactivation of the K-region oxide by microsomal epoxide hydrolase, but might still be sufficiently rapid to substantially affect diol epoxide concentrations in mammalian systems.

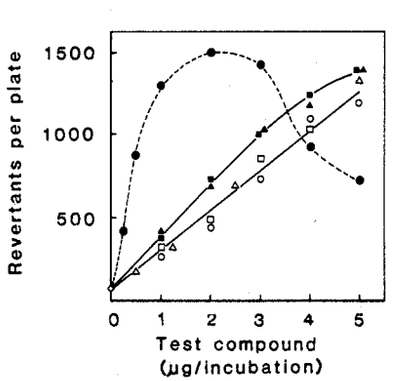

Fig. 1. Dose-dependent mutagenicity, in the absence of pure enzymes, of BA 5,6-oxide in phosphate-buffered KCl (●) and of BA-8,9-diol 10,11-oxide in phosphate-buffered KCl (○, □, and △; experiments performed on different days) or in glycine buffer with (■) or without (▲) NADP. Values are means of duplicate determinations, which differed by less than 10 percent.

To study their role in metabolic inactivation, we purified the three enzymes and used bacterial mutagenicity as an indication of their effects on the mutagenicity of BA-8,9-diol 10,11-oxide and, for comparison, benz[*a*]anthracene 5,6-oxide (BA 5,6-oxide), the K-region epoxide. The synthesis of the epoxides used here has been described (*18*). Microsomal epoxide hydrolase was purified from rat liver to apparent homogeneity (*19*). This enzyme converts a variety of alkene and arene oxides to dihydrodiols (*5, 20*). Cytosolic epoxide hydrolase was purified sufficiently (*21*) from rabbit liver to yield a preparation that contained less than 20 percent impurities. Although to date only a few animal species have been investigated, pertinent differences between cytosolic and microsomal epoxide hydrolase have been observed in all situations that have been studied. The cytosolic enzyme differs from the microsomal epoxide hydrolase immunologically (*22*) and in substrate specificity in that the cytosolic enzyme is relatively inactive toward K-region arene oxides (*23*), but is efficient in the hydrolysis of other epoxides—for example, trisubstituted oxiranes (*24*)—that are not or are only poor substrates for the microsomal enzyme (*25*). Dihydrodiol dehydrogenase was purified to apparent homogeneity from rat liver cytosol (*26*). The enzyme converts dihydrodiols such as *trans*-1,2-dihydro-1,2-dihydroxybenzene to catechols using nicotinamide adenine dinucleotide phosphate (NADP) as a cosubstrate. With carcinogenic polycyclic hydrocarbons such as benzo[*a*]pyrene and dimethylbenz[*a*]anthracene it reduces the mutagenicity in the Ames test (*27*). However, the mechanisms and metabolites involved in these effects are unknown.

0036-8075/82/0319-1507$01.00/0