Biochemical Pharmacology, Vol. 25, pp. 2291–2295. Pergamon Press, 1976. Printed in Great Britain.

INHIBITION OF GLUTATHIONE-*S*-ARYLTRANSFERASE FROM RAT LIVER BY ORGANOGERMANIUM, LEAD AND TIN COMPOUNDS

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(*Received* 12 *February* 1976; *accepted* 19 *March* 1976)

Abstract—Compounds having three carbon—germanium, carbon—tin or carbon—lead bonds were found to be effective inhibitors of rat liver glutathione-*S*-aryltransferase activity with K_i values in the μM range. Classical competitive inhibition was observed with 1,2-dichloro-4-nitrobenzene as the limiting substrate. However, when the second substrate, glutathione, was limiting, inhibition of the mixed type occurred. Within the metallic series IVb, triethylsilane chloride was not active, while the effectiveness as inhibitors increased through triethylgermanium chloride, triethyltin bromide and triethyllead chloride. Except in the case of sulfur, the fourth group or atom bonded to tin in a series of triphenyltin compounds had little effect on inhibitory activity. Sodium sulfide or 2,3-dimercapto-1-propanol (BAL) was capable of protecting glutathione-*S*-aryltransferase from inhibition by triphenyltin chloride. Glutathione conjugations at aromatic carbon atoms were much more sensitive to inhibition by organotins than were conjugations at alkyl carbons or epoxides.

After acute exposure, trialkyl and triaryltin compounds are moderately toxic substances which cause: characteristic central nervous system edema in humans and other mammals [1–3] depletion of brain and heart catecholamines in the rat [4, 5], and reduced glucose utilization in the rat brain [6]. *In vitro*, compounds having three carbon—tin bonds have been reported to inhibit: mitochondrial oxidative phosphorylation [3], mitochondrial ATPase [7] and pyruvate kinase [8]. In addition, the more lipid-soluble members of this group of organotins exhibited hemolytic activity [9].

Experiments with a chemical model system suggested that Et_3Sn* sulfate did not form stable compounds with GSH or BAL [10]. However, recent observations have shown that sulfide ions, dithiothreitol and BAL decreased the ability of trialkyl and triaryltins to inhibit mitochondrial ATPase [7], cause hemolysis [9], and the swelling of rat liver mitochondria [11]. In view of evidence for diminished biological activity of these organotins in the presence of sulfhydryl compounds, it became of interest to explore for interactions of the organometallic derivatives of the group IVb metals with GSH metabolism. The present investigation has established that compounds having three carbon—germanium, carbon—lead, or carbon—tin bonds are effective inhibitors of glutathione-*S*-aryltransferase (EC 2.5.1.18) activity of rat liver.

MATERIALS AND METHODS

Enzyme preparation. A supernatant fraction from rat liver, which was the source of GSH transferases, was prepared as follows. Male Sprague–Dawley rats (220–300 g) were stunned by a blow on the head and decapitated. After thorough bleeding of the animals, the livers were removed and the following operations were completed at 0–4° as quickly as possible. The livers were processed with a steel tissue press having 1-mm openings and were weighed. The samples were homogenized with 4 vol. of 0.154 M KCl–5.0 mM potassium phosphate at pH 7.4 using a Teflon–glass homogenizer at 600 rev/min until no large pieces of tissue could be seen between the glass homogenizer and the Teflon pestle. The homogenate was then centrifuged for 1 hr at 95,000 *g*. The floating fatty layer was removed by aspiration. The remaining clear supernatant fraction was used as the enzyme source for 1 day after dilution to a protein concentration of 6.0 mg/ml with 0.10 M sodium pyrophosphate–HCl at pH 8.0. Some experiments were completed with glutathione-*S*-aryltransferase purified from rat liver by the method of Booth *et al.* [12]. Protein was determined by a biuret method [13] with bovine serum albumin as the standard.

Assays of enzymic activities. The standard glutathione-*S*-aryltransferase assay, which was similar to that of Booth *et al.* [12], contained the following in a final volume of 3.0 ml: sodium pyrophosphate adjusted to pH 8.0 with HCl, 120 μmoles; GSH, 15 μmoles; DCNB, 0.90 μmole; ethanol, 2% (v/v); and 600 μg protein of the rat liver supernatant fraction. The reaction was started by the addition of the enzyme, and the rate of formation of the GSH–DCNB conjugate was followed continuously by recording absorptivity at 344 nm and using a molar absorptivity of 8500 [14]. All assays were conducted at 30°. All second substrates and inhibitors were added to the reaction mixtures dissolved in 95% ethanol.

Substrates used for the glutathione-*S*-transferases and molar absorptivities at the wavelengths used to

* Abbreviations used are as follows: ethyl, Et; glutathione, GSH, 2,3-dimercapto-1-propanol, BAL; 1,2-dichloro-4-nitrobenzene, DCNB; sulfobromophthalein, BSP; methyl, Me; phenyl, Ph; *n*-butyl, Bu; and cyclohexyl, cH.

follow the concentrations of their GSH conjugates were as follows: 4-nitropyridine-*N*-oxide, 7000 at 295 nm [15]; 4-nitrobenzyl chloride, 1900 at 310 nm [15]; 4-nitrophenethyl bromide, 1200 at 310 nm [15]; α-menapthyl sulfate, 39,000 at 298 nm [16]; and 1,2-epoxy-3-(4-nitrophenoxy)-propane, 510 at 360 nm [17].

The conjugation of BSP with GSH was assayed by the method of Goldstein and Combes [18] with the liver supernatant fraction as the source of the enzyme. Glyoxalase I (EC 4.4.1.5) was assayed by the method of Klotzsch and Bergmeyer [19]. Glyoxalase II (EC 3.1.2.6) was assayed by adding the enzyme to a glyoxalase I assay in which steady state concentrations of methylglyoxal and *S*-lactylglutathione were present. Glutathione reductase (EC 1.6.4.2) and glutathione peroxidase (EC 1.11.1.9) were assayed by methods described by Beutler [20].

In all cases, reaction rates were recorded when they were a linear function of time. Several of the assays of glutathione-*S*-transferases were corrected for nonenzymic rates; however, the nonenzymic rate with DCNB as the substrate was not significant in the standard assay.

Materials. Tricyclohexyltin hydroxide was a gift from the Dow Chemical Co. Glutathione reductase and glyoxalase I, both from yeast, and glyoxalase II from beef liver were from the Sigma Chemical Co. All other materials were used as purchased from commercial suppliers.

Calculations and statistics. Apparent K_m values were calculated from linear regression analysis of Lineweaver–Burk plots. K_i values were either calculated using the formula for the intercept on the baseline for purely competitive inhibition according to Dixon and Webb [21] or taken directly from secondary plots of inhibitor concentration versus slope of the Lineweaver–Burk regression lines [22].

The Student *t*-test was used at a significance level of 0.05 when indicated.

RESULTS

Effect of organotins on some enzymes of GSH metabolism. Initial experiments demonstrated that 0.10 mM Me_3SnCl, 0.10 mM Et_3SnBr, 0.10 mM Et_2SnBr_2 or 100 μM Ph_3SnCl did not significantly affect glyoxalase I activity from yeast or glyoxalase II activity from beef liver. Preliminary experiments also demonstrated that 0.10 mM Et_3SnBr or 10 μM Ph_3SnCl had no significant effect on the activity of glutathione reductase activity of the rat liver supernatant fraction, or the glutathione peroxidase activity of human red blood cells. Thus, it appears that compounds having two or three carbon—tin bonds are not general inhibitors of enzyme-catalyzed reactions in which new carbon—sulfur bonds are formed or the oxidation state of the sulfur of GSH is changed.

Screening of the test compounds for activity against glutathione-*S*-transferases of rat liver demonstrated that trialkyl and triaryltin compounds were very effective inhibitors of glutathione-*S*-aryltransferase activity. The activity was demonstrated both in the rat liver supernatant fraction and the purified preparation of Booth *et al.* [12] using the standard assay.

Table 1. Effect of dialysis on the inhibition of glutathione-*S*-aryltransferase by Ph_3SnOH and Me_3SnCl*

Dialysis time (hr)	Sp. act. (μmoles/mg protein/min)		
	Control (8)†	Ph_3Sn (4)	Me_3Sn (4)
0	7.7 ± 0.4	4.9 ± 0.2‡	4.9 ± 0.1‡
3	8.3 ± 0.3	5.8 ± 0.3‡	6.6 ± 0.1‡
20	8.1 ± 0.3	5.0 ± 0.5‡	7.8 ± 0.3

* Two ml of the rat liver supernatant fraction (20–30 mg of protein/ml) was diluated with 4.3 ml of 0.154 M KCl and 0.2 ml of either 1.00 mM organotin or 95% ethanol. This solution was placed in Visking 20/32 dialysis tubing which had been boiled in Na_2CO_3–EDTA [25] and dialyzed against 125 vol of distilled water with constant sitrring in the cold. The water was changed after about 6 hr of dialysis. After the indicated dialysis period, the specific activity of the dialysate was determined by using 0.1 ml of the dialysate as the source of the enzyme in the standard assay. Protein was determined in the dialysate at the time of assay to calculate specific activity.

† Numbers in parentheses indicate replicates.

‡ Significant from control at $P \leq 0.05$.

Since Et_3Sn is reported to bind strongly to rat hemoglobin [23], there was concern that hemoproteins in the enzyme preparations might affect the inhibition by organotins. However, since the pyridine hemochromogen assay [24] showed that glutathione-*S*-aryltransferase, purified by the method of Booth *et al.* [12], contained significant heme, the decision was made to use the rat liver supernatant fraction as a source of glutathione-*S*-transferases for subsequent experiments.

Nature of the inhibition. Dialysis and preincubation techniques were used to assess the reversibility of the inhibition of rat liver glutathione-*S*-aryltransferase by Me_3Sn and Ph_3Sn. The data in Table 1 show that inhibition by Me_3SnCl was reversible by dialysis; however, inhibition by Ph_3SnOH was not reversed by dialysis for 20 hr. On the other hand, the degree of inhibition caused by 1.0 μM Me_3SnCl or 1.0 μM Ph_3SnCl was not significantly changed by preincubation for 10 min with the components of the standard assay, minus that component used to start the reaction, and then starting the reaction by the addition of GSH, DCNB or the enzyme.

Kinetics of the inhibition. Figure 1 is a Lineweaver–Burk plot for the inhibition of glutathione-*S*-aryltransferase activity with DCNB as the limiting substrate. Classical competitive inhibition is indicated as was the case for all compounds tested having three carbon—germanium, carbon—lead, or carbon—tin bonds. A secondary plot of slope versus inhibitor concentration (insert Fig. 1) is linear, indicating simple enzyme—inhibitor interaction [22]. Large positive deviations occurred in the double reciprocal plots when the concentration of R_3SnCl was high enough to cause greater than 70 per cent inhibition. The kinetics of inhibition in this high concentration range were not investigated. The K_i values for the most active compounds tested are listed in Table 2. Among the organotins tested, those having three carbon—tin bonds exhibited the lowest K_i values. Those having one or four carbon—tin bonds were relatively inactive

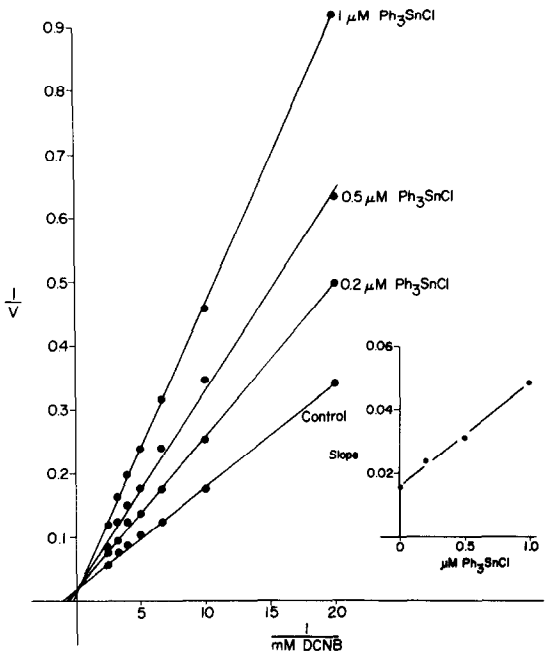

Fig. 1. Kinetics of the inhibition of glutathione-*S*-aryltransferase activity of rat liver supernatant fraction by Ph_3SnCl with DCNB as the limiting substrate. The standard assay, described in Materials and Methods, was used with the indicated DCNB concentrations. The regression lines represented were calculated from three to six replications of the experiment.

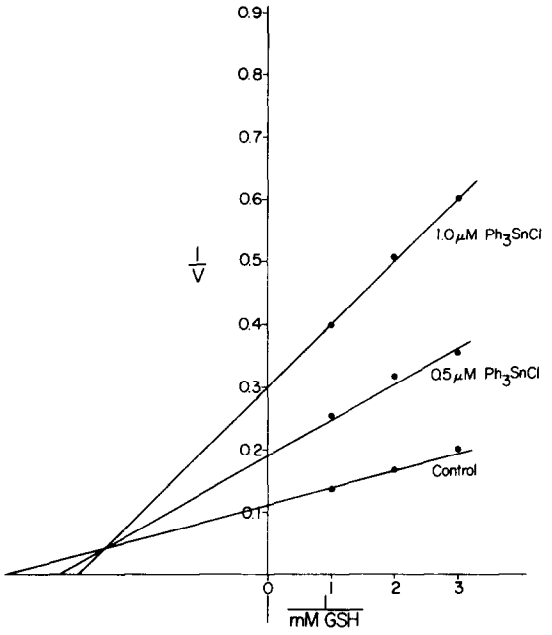

Fig. 2. Kinetics of the inhibition of glutathione-*S*-aryltransferase activity of rat liver supernatant fraction by Ph_3SnCl, with GSH as the limiting substrate. Except for DCNB and GSH concentrations the standard assay was used. The regression lines represented were calculated from seven to nine replications of the experiment.

in the standard assay. Considering the triethyl derivative of metals of group IVb, the K_i values decreased as the atomic number increased from germanium through lead. The following compounds had no activity in the standard assay: 0.01 mM Et_3SiCl, 0.05 mM triphenylmethanol and 0.05 mM triphenylchloromethane.

Because of the limited solubility of DCNB in the standard assay, it was not possible to completely saturate the enzyme with respect to this substrate. However, with low concentrations of GSH and 3.0 mM DCNB in the assay the double reciprocal plot (Fig. 2) indicates that organotin inhibition is a mixed type [21]. Under these conditions the K_m for GSH was 0.20 mM and the K_i for Ph_3SnCl was 7.0 μM. A biphasic K_m for GSH with a low value of 0.25 mM has been reported [14].

Effect of the fourth group or atom bonded to tin on activity. Unless it is sulfur, the fourth group or atom bonded to tin in several organotins has been shown to have little effect on the ability of these compounds to cause hemolysis [9] or the non-energy-dependent swelling of mitochondria in an ammonium chloride medium [11]. The data in Table 3 indicate that this generalization holds for the inhibition of glutathione-*S*-aryltransferase by Ph_3Sn derivatives. The results suggest that the various Ph_3Sn derivatives, except $(Ph_3Sn)_2S$, are rapidly converted to a mixture of Ph_3SnOH and $(Ph_3Sn)_2O$ in the assay. The bis-sulfide presumably has low intrinsic activity and is only slowly hydrolyzed in the assay.

Table 2. Inhibitor constants (K_i values) for the inhibition of glutathione-*S*-aryltransferase by organotins*

Compound	K_i (μM)
Et_3SnBr	0.21
Bu_3SnCl	0.29
Ph_3SnCl	0.48
Me_3SnCl	0.95
cH_3SnOH	4.97
Ph_2SnCl	5.87
$Bu_2Sn(Ac)_2$	19.10
Et_2SnBr_2	54.50
Et_3PbCl	0.17
Et_3GeCl	5.20

* The experiments and treatment of the data were as illustrated for Ph_3SnCl in Fig. 1.

Table 3. Effect of the fourth atom or group bonded to tin on effectiveness of compounds having three carbon–tin bonds as inhibitors of glutathione-*S*-aryltransferase*

Test compound	Per cent inhibition with test compound at 1.0 μM
Ph_3SnCl	47
Ph_3SnOH	41
Ph_3SnAc	41
Ph_3Sn isothiocyanate	38
$(Ph_3Sn)_2S$	29
$(Ph_3Sn)_2O$	70

* The standard assay was used with six replications for each compound.

Table 4. Effect of BAL and sodium sulfide on the inhibition of glutathione-*S*-aryltransferase activity by Ph_3SnCl*

Additions to the assay	Per cent inhibition
BAL (1 μM)	0
Ph_3SnCl (1 μM)	46
Ph_3SnCl (1 μM) and BAL (1 μM)	43
Ph_3SnCl (1 μM and BAL (10 μM)	25
Ph_3SnCl (1 μM) and BAL (100 μM)	14
Ph_3SnCl (1 μM) and BAL (1000 μM)	0
Na_2S (10 μM)	0
Ph_3SnCl (1 μM) and Na_2S (0.5 μM)	34
Ph_3SnCl (1 μM) and Na_2S (1 μM)	28
Ph_3SnCl (1 μM) and Na_2S (10 μM)	15
Ph_3SnCl (1 μM) and Na_2S (100 μM)	2

* Two to four replicates of each point were obtained using the standard assay.

Protection by sulfur compounds. As is the case for organotin-induced hemolysis [9], inhibition of mitochondrial ATPase [7], and non-energy-dependent mitochondrial swelling [11], Table 4 shows that BAL or sodium sulfide can protect glutathione-*S*-aryltransferase from inhibition by Ph_3SnCl. These results and the low activity of $(Ph_3Sn)_2S$ (Table 3) suggest that the sulfur compounds act by forming derivatives of the inhibitor having sulfur—tin bonds which exhibit low activity.

Specificity of the inhibition. Several glutathione-*S*-transferases, which differ with regard to substrate specificity, have been described [26]. The data in Table 5 show that Ph_3SnCl or Et_3SnBr is a most effective inhibitor when one of the first three compounds listed, all of which are substrates for glutathione-*S*-aryltransferase [26], is the second substrate. Except for 4-nitrobenzyl chloride, conjugations with GSH involving bond breaking and synthesis at other than aromatic carbon atoms are relatively insensitive to inhibition. Thus, the test compounds appear to be the most selective inhibitors for glutathione-*S*-aryltransferase activity reported to date.

DISCUSSION

The dialysis experiments demonstrate that the inhibition of glutathione-*S*-aryltransferase by Me_3SnCl is reversible, while inhibition by Ph_3SnOH is not. It is possible that these results reflect the relative inefficiency of dialysis as a means of removing these inhibitors rather than suggesting chemically irreversible inhibition in the case of Ph_3Sn. Thus, Me_3SnCl and Ph_3SnCl are reported to have *n*-octanol:water partition coefficients of 0.51 and 12,000 respectively [11]. The results of preincubation of these inhibitors with components of the standard assay are consistent with the views that (a) the inhibitors are not good substrates of glutathione-*S*-transferases and (b) the inhibition is of a reversible nature [21].

The general structure activity and physical property relationships for organotin inhibition of transferase activity are similar to those reported for induction of mitochondrial swelling [11], or hemolysis [9]. Protection by sulfur compounds has been reported in these systems and in the case of organotin inhibition of mitochondrial ATPase activity [7]. The mole ratios R_3Sn:sodium sulfide required for protection in the different systems tested are as follows: inhibition of glutathione-*S*-aryltransferase activity, 1:100 (Table 4); protection of mitochondrial ATPase, 1:2000 [7]; prevention of organotin-induced hemolysis, 1:0.5 [9]; prevention of mitochondrial swelling and activity in an ionophore diffusion model, 1:5 and 1:4 respectively [11].

These mole ratios may correlate inversely with the affinity of biological binding sites for the R_3Sn moiety. Thus, beef heart submitochondrial particle ATPase activity would be expected to have the highest affinity binding site(s), with glutathione-*S*-aryltransferase having lower affinity binding sites, etc. This view is consistent with a K_i of 0.028 μM reported for the inhibition of mitochondrial ATPase by Ph_3SnCl [7] and a corresponding K_i of 0.48 μM reported here. It is also of interest that effects of organotins postulated to be due to the activity of these compounds as anion specific ionophores [11] are more subject to modulation by sulfide and sulfur compounds than is the inhibition of mitochondrial ATPase and glutathione-*S*-aryltransferase activity. This suggests that the sulfur compounds interact to yield substances having sulfur—tin bonds which do not rapidly undergo the anion exchanges required for ionophore activity but are capable of interacting with sites having very high affinity for R_3Sn.

The activity of the triethyl halides of Si, Ge, Sn and Pb as inhibitors correlates well with increasing

Table 5. Effect of triphenyltin chloride on the conjugation of glutathione with several substances*

Substrate	Concn (mM)	Product (μM/mg protein/min $\pm$ S.E.M.) Control	Ph_3SnCl (1.0 μM)	Ph_3SnCl (10.0 μM)
1,2-Dichloro-4-nitrobenzene	0.25	9.5 $\pm$ 0.1	4.6 $\pm$ 0.03	0.8 $\pm$ 0.03
4-Nitropyridine-*N*-oxide	0.20	10.1 $\pm$ 0.4	6.3 $\pm$ 0.3	3.0 $\pm$ 0.4
Sulfobromophthalein†	0.33	3.7 $\pm$ 0.1	2.7 $\pm$ 0.2	0.2 $\pm$ 0.01
p-Nitrophenethyl bromide	0.20	26.5 $\pm$ 2.0	26.5 $\pm$ 3.4	24.2 $\pm$ 2.1
Nitrobenzyl chloride	0.10	86.3 $\pm$ 3.4	83.0 $\pm$ 5.5	46.8 $\pm$ 0.6
α-Menaphthyl sulfate	6.70	0.4 $\pm$ 0.1		0.34 $\pm$ 0.8
1,2-Epoxy-3-(*p*-nitrophenoxy)-propane	0.50	47.0 $\pm$ 3.0	47.0 $\pm$ 1.0	40.0 $\pm$ 1.0

* The standard assay with the indicated substrate replacing DCNB was used, except in the case of BSP. N = 3 for each point.

† Inhibitor was triethyltin bromide.

metallic properties in the series as the atomic number increases. The same correlation holds when the dipole moments of the trimethyl chlorides of these elements are considered [27]. Thus, in this series of compounds, inhibitor activity appears to increase with ability of the metal atom to interact with electron-rich sites. Such interactions may include salt-like interactions between anionic sites and the metal atom, coordination between the metal atom and nitrogen, oxygen and/or sulfur atoms, and hydrophobic interactions with the organic moiety of the inhibitor.

Rat liver has been found to contain at least six enzymes with broad and overlapping substrate specificities capable of catalyzing GSH conjugations [26, 28]. The specificity of the inhibitors reported here for glutathione-S-aryltransferase may be taken as additional evidence that this activity is distinct from the other glutathione-S-transferases of rat liver. Furthermore, evidence has been presented suggesting that glutathione-S-aryltransferase and ligandin, an anion-binding protein from liver, are identical [29]. The specific inhibition of glutathione-S-aryltransferase taken with the observation of a soluble protein from rat liver with high affinity binding sites for Et_3Sn [30] suggests that glutathione-S-aryltransferase activity, anion-binding capacity, and high affinity organotin binding may all be properties of a single, soluble liver protein.

Acknowledgement—This investigation was supported by U.S. Public Health Service Grant ES 00498. The results will be included in a Ph.D. thesis by R. A. H. and some of the data were presented in preliminary form elsewhere [31].

ADDENDUM

Note of added proof. One of us, R. A. H., recently visited Dr. W. Jakoby's laboratory to investigate the effects of Ph_3SnCl on glutathione-S-transferases A and B recently purified by that group [28]. With 1-chloro-2,4-dinotrobenzene as the limiting substrate (DCNB is a very poor substrate for transferase B [28]). Ph_3SnCl was shown to be a competitive inhibitor with K_i values of 0.08 and 0.27 μM for transferase A and B respectively.

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