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The Influence of Organometals on Heme Metabolism

In Vivo and *in Vitro* Studies with Organotins

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SUMMARY

The effects of the organometals tricyclohexyltin hydroxide (TCHH), bis(tri-*n*-butyltin) oxide (TBTO), and diethyltin dichloride (DEDC) on various parameters of heme metabolism were studied. Elevation of hepatic heme oxygenase (EC 1.14.99.3) activity and depression of cytochrome P-450 content were produced by each organified tin compound, and these effects were dose-dependent; DEDC also produced comparable changes in kidney, whereas TCHH and TBTO were inactive in this organ, despite the fact that their constituent inorganic element, tin, is an extremely potent inducer of renal heme oxygenase. Cytochrome P-450-dependent mixed-function oxidase activity in liver was also impaired in a dose-dependent manner by the organometals. These changes in heme oxygenase activity and cytochrome P-450 content occurred within 12–24 hr after administration of a single dose of DEDC, whereas with TCHH and TBTO they were not maximal until at least 48–72 hr. The changes in heme metabolism produced after organometal treatment were extremely prolonged, lasting in the case of TCHH at least 10 days and in the case of TBTO at least 8 days after single doses. δ-Aminolevulinic acid synthase (ALA-synthase: EC 2.31.37) activity underwent initial inhibition followed by rebound induction with TCHH and TBTO, whereas with DEDC initial inhibition was followed by a gradual return to normal levels. However, these perturbations of ALA-synthase activity, while qualitatively resembling those produced by inorganic metal administration (i.e., ALA-synthase depression ∿2 hr; rebound elevation ∿16 hr), were also extremely drawn out in time. Thus for TCHH and TBTO the depression in ALA-synthase activity occurred at ∿8–16 hr and the rebound increase in activity occurred at ∿48–96 hr. *In vitro* incubation of microsomes with the organotins with or without metabolic activation utilizing NADPH produced a decrease in the spectrally detectable cytochrome P-450 content that was both dose- and time-dependent, and could not be prevented by the addition of exogenous sulfhydryl groups (cysteine, glutathione), SKF-525A, or EDTA. These data describe new and striking effects of organotin compounds on heme synthesis and degradation, on the cellular content of cytochrome P-450 in liver and kidney, and on related monooxygenase activity dependent on this heme protein. The widespread distribution in the environment and continued use of these organometals makes their effects on heme metabolism of considerable importance in biological systems.

INTRODUCTION

Biochemical and toxicological interest in organometals has been stimulated by numerous reports of the biomethylation (1) of toxic trace metals by both bacterial sediments and gut flora, the large-scale production of organic compounds of lead and manganese for use in fuels as anti-knock agents, and the development and introduction into the environment of organotins as replacements for the highly persistent and carcinogenic chlorinated hydrocarbon pesticides (2). This wide use of organotins has led to the extensive study of the toxicology of these compounds. The inhibition of oxidative phosphorylation by trialkyltins (3), production of edematous lesions in the white matter of the central nervous system (4), and depression of thymus-dependent immunity (5, 6) are among the biological effects of these agents which have been examined in mammalian systems.

Organometals are of particular interest in relation to cytochrome P-450 and the enzymes of heme synthesis and degradation because they contain, in addition to the covalently bound organic substituents, a central metal atom. Certain organic compounds have the ability to

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produce significant losses of cytochrome P-450 *in vivo* and *in vitro* (7), and, as reported earlier in these (8–12) and other laboratories (13–15), many metal ions have a potent ability to alter by induction, repression, or other mechanisms key enzymes of heme degradation and synthesis.

In this study we have examined in detail the effects of three widely used organotin compounds (whose structures are shown in Fig. 1), TCHH,[1] TBTO, and DEDC on heme metabolism in liver and kidney of rats. The results show that all three organometal compounds are potent inducers of heme oxygenase, producing effects on heme metabolism which differ, qualitatively and quantitatively, from those produced by the constituent metal atom, tin, which they share in common.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (175–225 g), purchased from Holtzman (Madison, Wisc.), were used for dose-response and time-course experiments. Slightly larger rats (225–275 g), which produced larger microsomal yields, were used for *in vitro* studies. TBTO was obtained from the Ventron Corporation (Danvers, Mass.). TCHH and DEDC were a generous gift of the M&T Chemical Company (Rahway, N. J.). AIA was a gift of Hoffman-La Roche (Nutley, N. J.). Sodium phenobarbital was purchased from Mallinckrodt (St. Louis, Mo.). All other reagents were of the highest grade obtainable and were purchased from Sigma Chemical Company (St. Louis, Mo.).

Methods

Treatment of animals and preparation of microsomes. Rats received s.c. injections of the organometal compounds (in 95% ethanol) in a single dose in amounts up to 30 mg/kg of body weight. Control rats received an equal volume of the solvent ethanol (1 ml/kg), and were identical to saline-treated animals in all parameters studied. Phenobarbital was administered in the drinking water (0.1% w/v) for 4 days. Animals were allowed free access to water but were deprived of food for 16 hr prior to sacrifice.

Livers were exhaustively perfused *in situ* with 0.9% NaCl and homogenized, as were kidneys, in 3 volumes of potassium phosphate buffer (0.1 M, pH 7.4) containing sucrose (0.25 M). The homogenates were centrifuged at 9,000 × *g* for 20 min and the resultant pellet was used for determining ALA-synthase activity. The supernatant fraction was centrifuged at 105,000 × *g* for 60 min in a Beckman L5-50 ultracentrifuge. The microsomal pellet obtained was resuspended in potassium phosphate buffer (0.1 M, pH 7.4) to a protein concentration of approximately 20 mg/ml for subsequent enzyme assays, or stored in liquid nitrogen (−70°) until use. No loss of enzyme activity occurred as a result of storage. For *in vitro* studies, liver microsomes were prepared using 3 volumes of Tris-HCl buffer (0.5 M, pH 7.40) containing KCl (1.15%).

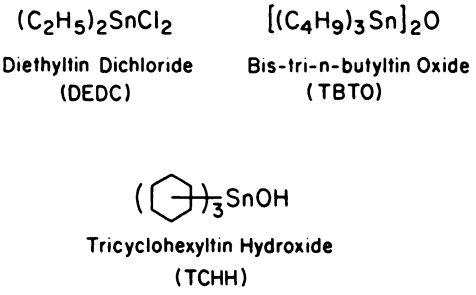

FIG. 1. *Structure of organotin compounds*

Enzyme assays. Heme oxygenase (EC 1.14.99.3) was measured in the microsomal fraction as previously described (9), with the 105,000 × *g* supernatant fraction derived from liver serving as a source of biliverdin reductase. Bilirubin formation was calculated by using an absorption coefficient of 40 $mM^{-1} \cdot cm^{-1}$ between 464 and 530 nm (8). Cytochrome P-450 and cytochrome b_5 contents were measured by the method of Omura and Sato (16). ALA-synthase (EC 2.31.37) activity was measured in the washed 9,000 × *g* pellet by the method of Sassa *et al.* (17). The *N*-demethylation of ethylmorphine was measured by the method of Nash (18), and *p*-aminophenol generated by the hydroxylation of aniline was measured colorimetrically (19). Lipid peroxidation was measured by the procedure of Buege and Aust (20), in which the MDA concentration was then quantitated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. Protein content was determined by the method of Lowry *et al.* (21), using crystalline bovine serum albumin as standard.

Incubation in vitro. KCl-washed microsomes derived from liver of phenobarbital-treated rats were incubated aerobically in the dark with constant shaking at 37° for various times up to 60 min in the presence of varying concentrations of organotins with and without NADPH (0.5 mM). The indicated organotin compound (in 95% ethanol) was injected directly into the reaction mixture in a volume of solvent no greater than 1% (v/v) to initiate the reaction. After incubation, the samples were placed in an ice-water bath, and the cytochrome P-450 content was determined immediately.

Statistical analysis. The data were analyzed by the standard *t*-test, and the indicated *p* value was regarded as denoting significance.

RESULTS

Effects of DEDC, TBTO, and TCHH on hepatic and renal heme metabolism with respect to dose and time. The effect of a single administration of increasing doses of DEDC on hepatic heme oxygenase activity measured at 16 hr is shown in Table 1. There was an increase in heme oxygenase activity of almost 2-fold with the lowest dose administered (1.9 mg/kg), and enzyme activity increased with increasing doses of the organometal; a 5-fold elevation of enzyme activity was the maximum produced at the highest dose examined (30 mg/kg). DEDC produced concomitant decreases in the content of hepatic

[1] The abbreviations used are: TCHH, tricyclohexyltin hydroxide; TBTO, bis(tri-n-butyltin)oxide; DEDC, diethyltin dichloride; ALA, δ-aminolevulinic acid; MDA, malondialdehyde; AIA, allylisopropylacetamide.

TABLE 1

Effects of DEDC on hepatic heme oxygenase and cytochrome P-450 at 16 hr

DEDC was administered in a single s.c. injection at the doses indicated to 18 rats, divided into three rats per dose concentration. The animals were fasted and killed 16 hr after treatment. Hepatic microsomal fractions were prepared and asssays performed as described under Materials and Methods. Values are reported as the means ± standard error of the mean of three separate experiments.

Dose	Heme oxygenase	Cytochrome P-450
mg/kg body wt.	*nmoles bilirubin formed/ hr/mg protein*	*nmoles/mg protein*
0	2.50 ± 0.16	0.74 ± 0.03
1.9	4.07 ± 0.13[a]	0.67 ± 0.01
3.8	5.45 ± 0.38[a]	0.57 ± 0.02[a]
7.5	5.66 ± 0.15[a]	0.52 ± 0.01[a]
15	7.51 ± 0.31[a]	0.47 ± 0.01[a]
30	12.19 ± 0.87[a]	0.43 ± 0.01[a]

[a] $p < 0.01$ as compared with controls.

TABLE 2

Effects of TBTO on hepatic heme oxygenase and cytochrome P-450 at 72 hr

TBTO was administered in a single injection at the doses indicated to 18 rats, divided into three rats per dose concentration. The animals were fasted for 16 hr prior to sacrifice and killed 72 hr after treatment. Hepatic microsomal fractions were prepared and assays performed as described under Materials and Methods. Values are reported as the means ± standard error of the mean of three separate experiments.

Dose	Heme oxygenase	Cytochrome P-450
mg/kg body wt.	*nmoles bilirubin formed/ hr/mg protein*	*nmoles/mg protein*
0	2.31 ± 0.10	0.68 ± 0.02
1.0	2.89 ± 0.17	0.61 ± 0.03
3.8	4.84 ± 0.44[a]	0.38 ± 0.01[a]
7.5	5.80 ± 0.14[a]	0.29 ± 0.01[a]
15	6.28 ± 0.15[a]	0.26 ± 0.01[a]
30	7.58 ± 0.56[a]	0.25 ± 0.02[a]

[a] $p < 0.01$ as compared with controls.

cytochrome P-450 measured at 16 hr. This decrease was dose-related, reaching a level ∿60% of control with the highest dose of DEDC tested.

A single dose (15 mg/kg) of DEDC resulted in a rapid induction of heme oxygenase activity; peak activity (4 times control levels) was reached within 12 hr and then declined; the enzyme activity returned to near-normal levels by 48 hr (Fig. 2). The induction of hepatic heme oxygenase was accompanied by a steady loss (∿50%) of cytochrome P-450 content for up to 36 hr; low levels of this heme protein persisted even when heme oxygenase activity had begun to return to normal levels (Fig. 2). ALA-synthase activity exhibited an initial decrease (∿85% below control level) which was observed at 8 hr followed by a gradual return toward control values thereafter.

TBTO produced significant dose-dependent alterations in heme oxygenase activity and cytochrome P-450 in liver (Table 2). The lowest dose of TBTO examined (1.0 mg/kg), although not producing significant induction of heme oxygenase, still lowered the contents of both cytochrome P-450 (Table 2) and cytochrome b_5 (latter results not shown) by 10–15%. The maximal alterations in heme metabolism reached a plateau at a dose of 15 mg/kg of TBTO (Table 2), with heme oxygenase activity being elevated 3- to 4-fold above control and the content of cytochromes P-450 and b_5 depressed by 60% and 50%, respectively.

A single dose of TBTO (30 mg/kg) produced an increase in heme oxygenase activity in liver between 8 and 12 hr (Fig. 3); however, maximal elevation of enzyme activity was not reached until 48 hr (3.5 times control levels). The cytochrome P-450 content decreased rapidly between 8 and 12 hr and reached a minimum (∿50% of control levels) between 48 and 72 hr. The time course of ALA-synthase activity was clearly biphasic: an initial loss of activity (depressed by 70% of control levels at 12–16 hr) was followed by a rebound to control levels by 48 hr, rising to maximal activity at 96 hr before returning to normal by 144–216 hr.

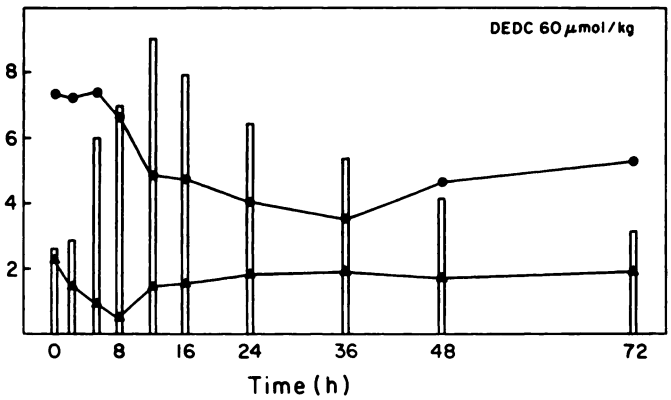

FIG. 2. *Time course of DEDC effects on hepatic heme metabolism*

DEDC (15 mg/kg body weight) was administered s.c. in a single injection to 30 rats, divided into three rats per time point. Hepatic microsomal and mitochondrial fractions were prepared and assays performed as described under Materials and Methods. ■, Heme oxygenase (nanomoles of bilirubin formed per hour per milligram of protein); ●, cytochrome P-450 content (nanomoles per milligram of protein × 0.1); ▲, ALA-synthase activity (nanomoles per hour per milligram of protein × 0.1). Values represent the average of results from at least six individual animals.

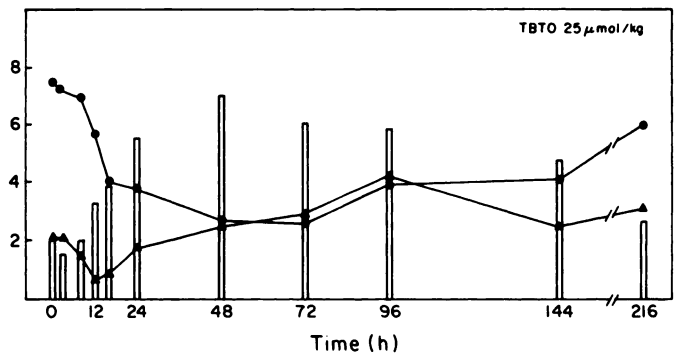

FIG. 3. *Time course of TBTO effects of hepatic heme metabolism*

TBTO (30 mg/kg) was administered s.c. in a single injection to 33 rats, divided into three rats per time point. Hepatic microsomal and mitochondrial fractions were prepared and assays performed as described under Materials and Methods. ■, Heme oxygenase (nanomoles of bilirubin formed per hour per milligram of protein); ●, cytochrome P-450 content (nanomoles per milligram of protein × 0.1); ▲, ALA-synthase activity (nanomoles per hour per milligram of protein × 0.1). Values represent the average of results from at least six individual animals.

A dose-response study of the perturbations in heme metabolism resulting from TCHH administration has already been reported (22). A single dose of TCHH (15 mg/kg) produced a substantial and extremely prolonged induction of heme oxygenase. The peak enzyme activity, which reached levels similar to TBTO, was attained by 48 hr and remained highly elevated at 96 hr; this high level of heme oxygenase activity persisted for at least 10 days (when the experiment was terminated) after the administration of a single dose of TCHH. TCHH induction of heme oxygenase was accompanied by a steady decline, up to 72 hr, in cytochrome P-450 content. The level of this heme protein remained low throughout the period of elevated heme oxygenase activity, and was still depressed (40% below control) at 10 days. As with TBTO, the response in ALA-synthase activity was biphasic, an initial inhibition at 8–12 hr followed by rebound elevation reaching maximal activity at 72 hr and returning to normal activity by 192 hr (Fig. 4).

A rapid, marked induction of heme oxygenase activity in kidney was also observed following DEDC treatment (Table 3). In contrast, no changes in renal heme oxygenase activity and cytochrome P-450 content were observed with TBTO and TCHH administration (results not shown). The induction of renal heme oxygenase by DEDC reached levels 12 times control at the highest dose examined (30 mg/kg), whereas the cytochrome P-450 content was reduced by ∼35% at the two highest doses (15 to 30 mg/kg) examined.

A single dose of DEDC (15 mg/kg) produced a rapid increase in heme oxygenase activity (8 times control levels) by 8 hr; a broad peak of increased activity (∼12 times control levels) extended from 12 through 48 hr (Fig. 5). Enzyme activity had decreased, but was still substantially above normal levels of activity at 72 hr. The levels of cytochrome P-450 in kidney rapidly declined after administration of DEDC and remained low until heme oxygenase activity had begun to decline after 48 hr. DEDC administration produced no significant lowering of renal ALA-synthase activity immediately after administration, but nevertheless produced a considerable late increase (3 times control levels) in the enzyme activity which reached a maximum at 36 hr before returning to control levels by 72 hr.

TABLE 3

Effects of DEDC on renal heme oxygenase and cytochrome P-450 at 16 hr

DEDC was administered in a single injection at the doses indicated to 18 rats, divided into three rats per dose concentration. The animals were fasted and killed 16 hr after treatment. Renal microsomal fractions were prepared and assays performed as described under Materials and Methods. Values are reported as the means ± standard error of the mean of three separate experiments.

Dose	Heme oxygenase	Cytochrome P-450
mg/kg body wt.	*nmoles bilirubin formed/ hr/mg protein*	*nmoles/mg protein*
0	1.10 ± 0.07	0.072 ± 0.004
1.9	1.96 ± 0.01[a]	0.068 ± 0.003
3.8	2.83 ± 0.02[a]	0.072 ± 0.001
7.5	5.25 ± 0.37[a]	0.056 ± 0.002[a]
15	8.62 ± 0.40[a]	0.048 ± 0.003[a]
30	13.45 ± 0.72[a]	0.047 ± 0.003[a]

[a] $p < 0.01$ as compared with controls.

DEDC, TCHH, and TBTO inhibition of aniline hydroxylase and ethylmorphine N-demethylase activities in liver. The effects of a single administration of DEDC, TCHH, and TBTO on two cytochrome P-450-dependent drug-metabolizing enzyme activities, aniline hydroxylase and ethylmorphine *N*-demethylase, are shown in Table 4. The metabolism of these two drug substrates, which was measured when the cytochrome P-450 content was previously shown to be maximally depressed, i.e., DEDC (36 hr), TCHH, and TBTO (72 hr), was found to be significantly reduced. Depression of aniline hydroxylase activity by TCHH (42% of control) and TBTO (37% of control) closely paralleled the decreases produced in the cytochrome P-450 content by these organotins. Ethylmorphine *N*-demethylase activity was similarly lowered by TCHH (38% of control) and TBTO (34% of control).

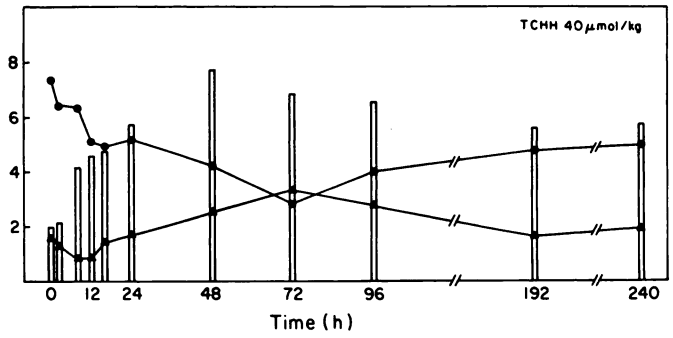

FIG. 4. *Time course of TCHH effects on hepatic heme metabolism*

TCHH (15 mg/kg) was administered subcutaneously in a single injection to 33 rats, divided into three rats per time point. Hepatic microsomal and mitochondrial fractions were prepared and assays performed as described under Materials and Methods. ■, Heme oxygenase (nanomoles of bilirubin formed per hour per milligram of protein); ●, cytochrome P-450 content (nanomoles per milligram of protein × 0.1); ▲, ALA-synthase activity (nanomoles per hour per milligram of protein × 0.1). Values represent the average of results from at least six individual animals.

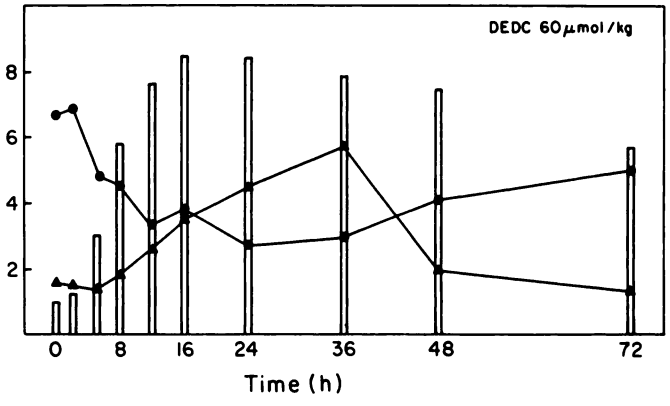

FIG. 5. *Time course of DEDC effects on renal heme metabolism*

DEDC (15 mg/kg) was administered subcutaneously in a single injection to 30 rats, divided into three rats per time point. Renal microsomal and mitochondrial fractions were prepared and assays performed as described under Materials and Methods. ■, Heme oxygenase (nanomoles of bilirubin formed per hour per milligram of protein); ●, cytochrome P-450 content (nanomoles per milligram of protein × 0.1); ▲, ALA-synthase activity (nanomoles per hour per milligram of protein × 0.1). Values represent the average of results from at least six individual animals.

TABLE 4

Effects of DEDC, TCHH, and TBTO on drug-metabolizing activities in liver

DEDC, TCHH, and TBTO were administered s.c. in a single injection (15 mg/kg of body weight). The animals were fasted 16 hr prior to sacrifice and killed at the times indicated. Hepatic microsomal fractions were prepared and assays performed in duplicate as described under Materials and Methods. Values are reported as means ± standard error of the mean of two separate experiments involving a minimum of eight animals per data point.

	Aniline hydroxylase		Ethylmorphine *N*-demethylase		Cytochrome P-450	
	nmoles p-aminophenol/hr/mg protein	*% control*	*nmoles formaldehyde/hr/mg protein*	*% control*	*nmoles/mg protein*	*% control*
Control	92.6 ± 6.17	100	465.1 ± 37	100	0.78 ± 0.03	100
DEDC[a]	91.7 ± 7.09	99	169.4 ± 43[b]	36	0.51 ± 0.02[b]	65
TCHH[c]	38.9 ± 3.73[b]	42	178.8 ± 61[b]	38	0.36 ± 0.03[b]	46
TBTO[c]	34.3 ± 2.94[b]	37	160.3 ± 10[b]	34	0.28 ± 0.02[b]	36

[a] Measured 36 hr after organometal administration.
[b] $p < 0.01$ as compared with controls.
[c] Measured 72 hr after organometal administration.

On the other hand, although ethylmorphine *N*-demethylase activity was markedly inhibited by DEDC at 36 hr (36% of control), the activity of aniline hydroxylase at this time point was unaffected (Table 4).

DEDC, TCHH, and TBTO inhibition of ALA-synthase activity in vitro. The early inhibition of ALA-synthase observed *in vivo* following organotin administration was examined *in vitro* in washed mitochondrial pellets obtained from both saline and AIA (400 mg/kg)-treated rats. Administration of AIA is a useful method by which to increase greatly the levels of ALA-synthase activity in rat liver (23). The *in vitro* inhibitory effects of DEDC, TCHH, and TBTO on the activity of this enzyme are shown in Table 5. All three compounds greatly inhibited the activity of ALA-synthase isolated from livers of saline- and AIA-treated rats in a dose-dependent manner (Table 5). The most striking inhibition (80–90% of control) was produced at the highest concentration of TCHH and TBTO (500 μM) studied, although marked inhibition (~50% of control) was evident at organometal concentrations of 200 μM. While renal ALA-synthase activity was also greatly inhibited by TCHH and TBTO, DEDC at concentrations up to 500 μM did not inhibit this renal enzyme activity, consistent with the *in vivo* findings (Fig. 5).

TCHH-, TBTO-, and DEDC-mediated losses of cytochrome P-450 in vitro. TCHH, TBTO, and DEDC produced concentration-dependent losses of cytochrome P-450 when incubated with liver microsomes isolated from phenobarbital treated rats (results not shown). The effect of incubation time on the loss of cytochrome P-450 in the presence of the three organotins (0.2 mM) was examined and the disappearance of the spectrally detectable heme protein was found to be time-dependent for at least 60 min (Fig. 6). TCHH and TBTO were somewhat more potent than DEDC in producing decreases in cytochrome P-450 content, with maximal losses of 40% (TCHH) and 50% (TBTO) at 60 min, compared with only 20% for DEDC.

The effects of preincubation of microsomal suspensions with various additions (dissolved in 1.15% KCl) prior to adding TCHH (similar *in vitro* interactions were found

TABLE 5

Organotin inhibition of ALA-synthase activity in vitro

Animals were treated with either saline or AIA and fasted for 16 hr prior to sacrifice. The mitochondrial fraction prepared as described under Materials and Methods was used as the source of ALA-synthase. Organotins (dissolved in 95% EtOH) were added directly to the incubation mixture. Values represent the means of three separate experiments performed in duplicate.

	Organotin	Liver ALA-synthase				Kidney ALA-synthase, saline-treated	
		Saline-treated		AIA[a]-induced			
	μM	*nmoles/hr/mg protein*	*% control*	*nmoles/hr/mg protein*	*% control*	*nmoles/hr/mg protein*	*% control*
Controls		0.265 ± 0.045	100	1.06 ± 0.045	100	0.308 ± 0.072	100
TCHH	50	0.218 ± 0.011	82	0.930 ± 0.020	88	0.279 ± 0.127	90
	200	0.159 ± 0.069	60	0.527 ± 0.105	50	0.183 ± 0.073	59
	500	0.074 ± 0.045	28	0.215 ± 0.075	20	0.074 ± 0.033	24
TBTO	50	0.240 ± 0.030	91	0.887 ± 0.039	84	0.235 ± 0.107	76
	200	0.123 ± 0.039	46	0.407 ± 0.096	38	0.131 ± 0.019	43
	500	0.056 ± 0.030	21	0.095 ± 0.016	9	0.059 ± 0.015	19
DEDC	50	0.220 ± 0.038	83	0.777 ± 0.083	73	0.289 ± 0.063	94
	200	0.167 ± 0.028	63	0.590 ± 0.065	56	0.298 ± 0.060	97
	500	0.137 ± 0.037	51	0.445 ± 0.010	42	0.310 ± 0.090	99

[a] AIA was administered s.c. to rats in a single dose (400/kg) 12 hr prior to sacrifice.

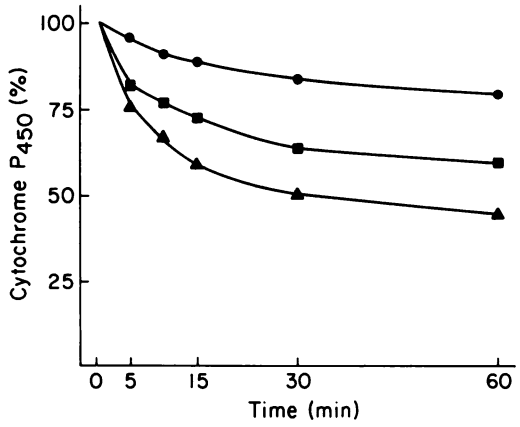

FIG. 6. *In vitro time-dependent loss of cytochrome P-450 in the presence of DEDC, TCHH, and TBTO*

Microsomes were prepared from livers of phenobarbital-treated rats. The microsomal suspensions (2 mg of protein per milliliter) in a total volume of 25 ml were incubated at 37° in the presence of 0.2 mM DEDC (●—●), TCHH (■—■), and TBTO (▲—▲). At the times indicated, 2-ml aliquots were removed and the cytochrome P-450 content was determined immediately (16). The values are reported as the percentage remaining compared with similarly incubated control microsomes minus the organotin. Values represent duplicate determinations for at least three separate preparations.

with TBTO), in the presence and absence of NADPH, are shown in Table 6. NADPH accounted for only a small increase (10%) in the disappearance of cytochrome P-450 above that produced directly by the organotin alone. TCHH stimulated lipid peroxidation both with and without metabolic activation, but there did not appear to be any correlation between this process and the disappearance of cytochrome P-450. Agents capable of blocking lipid peroxidation, such as $MnCl_2$ and EDTA (24), afforded only partial protection against the disappearance of spectrally detectable cytochrome P-450 (10–15%) in the absence of NADPH but had variable protective effects against TCHH-stimulated lipid peroxidation (Table 6). For example, $MnCl_2$, while completely abolishing the formation of malondialdehyde, could afford only nominal protection against disappearance of the heme protein. Cysteine and glutathione (oxidized and reduced) were ineffective in protecting against either heme protein loss or lipid peroxidation (results not shown).

TABLE 6

Effects of preincubation of various protective agents on TCHH-mediated destruction of cytochrome P-450 in vitro and stimulation of MDA formation

Microsomal suspensions (2 mg of protein per milliliter) were prepared from rats pretreated with phenobarbital as described under Materials and Methods. Samples were preincubated for 5 min with various protective agents prior to the addition of TCHH (0.2 mM) in the presence and absence of metabolic activation with NADPH (0.5 mM). All samples were then incubated in the dark at 37° with constant shaking for 20 min. Tubes were then placed in an ice-water bath, and cytochrome P-450 content (16) and MDA formation (20) were determined immediately. The content of cytochrome P-450 in treated microsomes was compared with similarly incubated controls without the indicated additions (100% cytochrome P-450 content ≈2.75 nmoles/mg of protein).

Addition	NADPH	P-450	MDA formation
		%	*nmoles/mg protein*
Controls	−	100	0.72
	+	58	4.72
TCHH (0.20 mM)	−	62	4.62
	+	54	5.51
TCHH + EDTA (1.0 mM)	−	63	4.15
	+	59	1.87
TCHH + SKF-525A	−	74	0.66
(0.5 mM)	+	66	1.09
TCHH + $MnCl_2$ (1.0 mM)	−	72	0.65
	+	59	0.77
TCHH + GSH (1.0 mM)	−	66	0.75
	+	58	2.74

DISCUSSION

The present studies demonstrate that organotin compounds of differing structural and chemical characteristics produce substantial alterations in the regulation of cellular heme metabolism, and that these alterations differ both qualitatively and quantitatively from those produced by the inorganic element, tin, which each organometal shares in common. In addition, among the three organotin compounds studied, somewhat differing effects on the several parameters of heme metabolism examined were noted.

The dose-response effects on hepatic heme oxygenase activity and cytochrome P-450 content are shown for DEDC and TBTO in Tables 1 and 2; comparable data for TCHH have been previously reported (22). With all three compounds a dose-dependent induction of hepatic heme oxygenase was observed, reaching a level at 72 hr with TBTO (15 mg/kg) of ∿3.5 times control levels and with TCHH (7.5 mg/kg) of ∿4 times control levels; with DEDC (30 mg/kg), enzyme activity at 16 hr was almost 5-fold elevated.

At the maximal dose level studied, although both TBTO and TCHH significantly induced heme oxygenase activity in liver, neither compound was able to induce the enzyme in kidney (results not shown). Inorganic tin, on the other hand, is a powerful inducer of the enzyme in kidney but not in liver (12, 25). Thus the organification of tin, in the structural forms represented by TBTO and TCHH, shifted the enzyme induction site from renal to hepatic tissue, an effect which is striking and could have considerable toxicological significance in light of the critical role of liver in the biotransformation of drugs, carcinogens, and other chemicals. The most water-soluble of the three organotins, DEDC, differed from both TBTO and TCHH in that it was a somewhat more potent inducer of heme oxygenase in liver and also significantly induced the enzyme in kidney. In this latter respect, DEDC mimicked the inducing action of inorganic tin in kidney (25), although it did not induce the enzyme to the same extent in this organ as the inorganic metal; however, at a dose calculated in relation to its actual content of tin, DEDC was substantially more potent than inorganic tin as an inducer of heme oxygenase in kidney. Thus in the case of TBTO and TCHH, organification of the metal shifted the site of action of the organometal from kidney to liver; and in the case of DEDC, led not only to a

second site of enzyme induction in liver, but enhanced the potency (based on metal content) in kidney as well.

Clear differences in the time course of induction of hepatic heme oxygenase were observed between TBTO, TCHH, and DEDC (Figs. 2–4). The induction responses evoked in liver by TBTO and TCHH were characterized by a delayed increase in heme oxygenase activity when compared with the increase in enzyme activity produced by inorganic elements (8–12) (initial increases were observed between ∿8 and 12 hr compared with ∿2 and 4 hr, respectively), and by an exceptionally protracted enzyme induction response. As Figs. 3 and 4 show, the increase in heme oxygenase produced by single doses of TBTO and TCHH reached peak levels at ∿48 hr, and persisted at high levels for at least 216 hr with TBTO, and at even higher levels for a minimum of 240 hr with TCHH. With both compounds, concomitant marked and prolonged declines in cellular content of cytochrome P-450 were produced.

It is also of considerable interest that the transient decrease in ALA-synthase activity and its "rebound" induction (at ∿2 hr and between ∿12 and 16 hr, respectively, observed after inorganic metal administration (8–12), was mimicked following organometal treatment. A notable difference, however, was that the entire process of inhibition and induction was greatly drawn out in time. Thus the early decline in ALA-synthase produced by both TBTO and TCHH reached a minimum at ∿8–12 hr and the rebound increase was maximal between 72 and 96 hr. Concurrent with the latter rebound elevation in ALA-synthase activity, the cytochrome P-450 content in the liver slowly increased, but neither at 216 hr for TBTO nor at 240 hr for TCHH had the heme protein returned to normal levels. Thus TBTO and TCHH perturbed heme metabolism in liver in a manner that, at least superficially, resembles the alterations produced by inorganic metals, yet differs markedly in two major respects: (a) all of the effects of organotins on heme oxygenase activity and cytochrome P-450 content were extraordinarily prolonged in duration (i.e., ∿200–240 versus ∿24–48 hr), and (b) the characteristic rapid perturbations in ALA-synthase produced by inorganic metals such as cobalt were long drawn out in time as a result of oganotin treatment—occurring over a period of days instead of hours.

The DEDC effects on the parameters of hepatic heme metabolism studied resembled more closely the effects produced by tin and other inorganic elements rather than those produced by TBTO and TCHH. However, even with this relatively water-soluble organotin, the decrease produced in cytochrome P-450 content was more prolonged in duration than that produced, for example, by cobalt or nickel in liver (8, 9); similarly, in the kidney (where TBTO and TCHH had no effects), DEDC induction of heme oxygenase, although of lesser degree than that produced by inorganic tin, was of longer duration. The decrease in cytochrome P-450 content produced by DEDC in kidney was also more protracted in time than that produced by inorganic tin, and low levels of this heme protein persisted, even when heme oxygenase activity was returning toward control levels. This continued decrease in cytochrome P-450 content probably resulted from the combined effect of elevated levels of heme oxygenase and the continued inhibition of hepatic ALA-synthase activity; maximal inhibition of ALA-synthase activity (∿85% below control levels) was observed at 8 hr, followed by a gradual return towards control values.

The profound decreases in cytochrome P-450 produced by all three organotins were associated, as anticipated, by a parallel loss in the functional activity of the mixed-function oxidase system (Table 4), monitored by the activities of aniline hydroxylase and ethylmorphine *N*-demethylase.

The early and transient depression of ALA-synthase activity produced in liver and kidney by many inorganic metals has been attributed to rapid repression of enzyme synthesis. The mechanism responsible for early inhibition of ALA-synthase by organometals may have an additional and perhaps major basis since incubations *in vitro* of all three organometals with hepatic ALA-synthase preparations produced marked inhibition of enzyme activity (Table 5). Such direct inhibition *in vitro* is not observed with inorganic metals. Similar concentrations *in vitro* of, for example, inorganic tin (12), nickel, or platinum (26), were without effect on this enzyme system. TBTO and TCHH also inhibited renal ALA-synthase activity substantially, whereas DEDC, even at concentrations of 500 μM, had no effect on the activity of this renal enzyme; this latter finding conforms with the absence of significant early ALA-synthase inhibition by DEDC *in vivo* in the kidney (Fig. 5).

It is of interest that inorganic metals (11, 12), metal chelates such as natural (27, 28) or synthetic metalloporphyrins (9, 29), and organometals of the types described in this study all have a potent ability to induce heme oxygenase and to elicit the concomitant perturbations of heme metabolism associated with the induction of this enzyme. It is not clear whether the mechanisms of enzyme induction elicited by metals in these structurally varied forms are similar. However, there is strong inferential evidence that inorganic metals may act directly as inducers of heme oxygenase without the necessity to form heme chelates *in vivo* (12), a capacity which certain powerful inducers of heme oxygenase, such as platinum and nickel (26), lack. Moreover, it is evident that the organometals described in this study could not be endogenously incorporated into the protoporphyrin molecule, and thus could not act through a mechanism involving *in vivo* formation of heme chelates.

Many organic compounds have been shown to produce decreases in the content of cytochrome P-450 directly and also through indirect mechanisms (7). As shown in this study, organotins also produce losses of cytochrome P-450 *in vitro* and impair the related monooxygenase activity. Addition of NADPH was not essential for heme protein loss *in vitro*, suggesting a direct effect of the organometals rather than catalytic participation of the monooxygenase system being prerequisite for cytochrome P-450 loss.

Previous reports from other laboratories (30) have raised the possibility that a free radical process is involved in the dealkylation of organometals by liver microsomes. Thus the possibility of lipid peroxidation as a contributing factor in the losses of cytochrome P-450 was

examined. Preincubation of microsomal suspensions with various compounds that are capable of blocking lipid peroxidation and/or competitively inhibiting cytochrome P-450-dependent oxidation reactions (Table 6) afforded only partial protection (10%) of the cytochrome P-450 content upon addition of the organotin compound (TCHH). EDTA could not prevent the direct stimulation of lipid peroxidation by TCHH (in the absence of metabolic activation with NADPH); however, when NADPH was present, the formation of MDA was reduced by 50%. In either case, the content of cytochrome P-450 was not protected by EDTA. SKF-525A and $MnCl_2$ blocked TCHH-stimulated lipid peroxidation, yet only partially protected the heme protein. Therefore, the process of lipid peroxidation (as measured by MDA formation) must only account for a small fraction of the TCHH-mediated loss of cytochrome P-450. The inability of GSH to prevent loss of cytochrome P-450 produced by TCHH might be predicted on the basis of its ineffectiveness in preventing trialkyltin-induced loss of glutathione *S*-aryltransferase activity in rat liver supernatant, an effect that was attributed to the inability of the tripeptide to form a complex with the organometal (31). The failure of SKF-525A to protect against cytochrome P-450 loss *in vitro* might be explained in part by the ability of the organotin to prevent the formation of a metabolite of SKF-525A which is ultimately responsible for its protective activity (32).

The results of this study demonstrate that organotins represent a potent class of chemical agents capable of producing profound and protracted alterations in several key aspects of heme metabolism—specifically, the activities of heme oxygenase and ALA-synthase, the rate-limiting enzymes of heme degradation and synthesis, respectively, and the content and functional activity of cytochrome P-450. These alterations, for two (TCHH and TBTO) of the three organotins studied were of particular interest in that the perturbations produced by the organified metal were produced in liver rather than in kidney, which is the principal site of action of the inorganic element, tin, that both compounds contain. With respect to the third compound studied, DEDC, not only was the site of biological action extended to liver, but it was also retained in kidney; indeed, on a dose-response basis (calculated by metal content), organification considerably enhanced the biological potency of the metal in kidney. These findings are important in relation to problems of metal biochemistry and toxicology since they clearly demonstrate that organification of metals, either synthetically or through biological means, can markedly alter their site and duration of action as well as the extent of their potential detrimental effects in tissues. The wide use of organometals in agricultural, industrial, and other enterprises coupled with the powerful effects which they exert on heme metabolism as shown in these studies indicate that further study of the biochemical properties of these compounds may be of substantial biological and medical interest.

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REFERENCES

1. Wood, J. M., A Cheh, L. J. Dizikes, W. P. Ridley, S. Rakow, and J. Lakowicz. Mechanisms for the biomethylation of metals and metalloids. *Fed. Proc.* **37:** 16–21 (1978).
2. Zuckerman, J. J. (ed.). *Organotin Compounds: New Chemistry and Applications.* American Chemical Society, Washington, D. C. (1976).
3. Aldridge, W. N., B. W. Street, and D. N. Skilleter. Oxidative phosphorylation: halide-dependent and halide-independent effects of triorganotin and triorganolead compounds on mitochondrial functions. *Biochem. J.* **168:**353–364 (1977).
4. Magee, P. N., H. R. Stoner, and J. B. Barnes. The experimental production of oedema in the central nervous system of the rat by triethyltin compounds. *J. Pathol. Bacteriol.* **73:**107–124 (1957).
5. Seinen, W., J. G. Vos, R. Brands, and H. Hooykaas. Lymphocytotoxicity and immunosuppression by organotin compounds: suppression of graft-versus-host reactivity, blast transformation, and E-rosette formation by di-*n*-butyltin dichloride and di-*n*-octyltin dichloride. *Immunopharmacology* **1:**343–355 (1979).
6. Miller, R. R., R. Hartung, and H. H. Cornish. Effects of diethyltin dichloride on aminoacid and nucleoside transport in suspended rat thymocytes. *Toxicol. Appl. Pharmacol.* **55:**564–571 (1980).
7. DeMatteis, F. Loss of microsomal components in drug-induced liver damage, in cholestasis and after administration of chemicals which stimulate heme catabolism. *Pharmacol. Ther. Part A* **2:**693–725 (1978).
8. Maines, M. D., and A. Kappas. Cobalt induction of hepatic heme oxygenase; with evidence that cytochrome P-450 is not essential for this enzyme activity. *Proc Natl. Acad. U. S. A.* **71:**4293–4297 (1974).
9. Maines, M. D., and A. Kappas. Cobalt stimulation of heme degradation in the liver. *J. Biol. Chem.* **250:**4171–4177 (1975).
10. Maines, M. D., and A. Kappas. Studies on the mechanism of induction of haem oxygenase by cobalt and other metal ions. *Biochem. J.* **154:**125–131 (1976).
11. Maines, M. D., and A. Kappas. Metals as regulators of heme metabolism. *Science (Wash. D. C.)* **198:**1215–1221 (1977).
12. Drummond, G. S., and A. Kappas. Metal ion interactions in the control of haem oxygenase induction in liver and kidney. *Biochem. J.* **192:**637–648 (1980).
13. Chow, C. P., and H. H. Cornish, Effects of lead on the induction of hepatic microsomal enzymes by phenobarbital and 3,4-benzpyrene. *Toxicol. Appl. Pharmacol.* **43:**219–228 (1978).
14. Woods, J. S., G. T. Carver, and B. A. Fowler. Altered regulation of hepatic heme metabolism by indium chloride. *Toxicol. Appl. Pharmacol.* **49:**455–461 (1979).
15. DeMatteis, F., and A. Unseld. Increased liver heme degradation caused by foreign chemicals: a comparison of the effects of 2-allyl-2-isopropylacetamide and cobaltous chloride. *Biochem. Soc. Trans.* **4:**205–209 (1976).
16. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol. Chem.* **239:**2370–2378 (1964).
17. Sassa, S., A. Kappas, S. E. Bernstein, and A. P. Alvares. Heme biosynthesis and drug metabolism in mice with hereditary hemolytic anemia. *J. Biol. Chem.* **254:**729–735 (1979).
18. Nash, T. The colorimetric estimation of formaldehyde by means of the Hantzch reaction. *Biochem. J.* **55:**416–421 (1953).
19. Imai, Y., A. Ito, and R. Sato. Evidence for biochemically different types of vesicles in the hepatic microsomal fraction. *J. Biochem. (Tokyo)* **60:**417–423 (1966).
20. Buege, J. A., and S. D. Aust. Microsomal lipid peroxidation. *Methods Enzymol.* **52:**302–310 (1978).
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275 (1951).
22. Rosenberg, D. W., G. S. Drummond, H. H. Cornish, and A. Kappas. Prolonged induction of hepatic haem oxygenase and decreases in cytochrome P-450 content by organotin compounds. *Biochem. J.* **190:**465–468 (1980).
23. Marver, H. S., D. P. Tschudy, M. G. Perlroth, and A. Collins. δ-Aminolevulinic acid synthetase. I. Studies in liver homogenates. *J. Biol. Chem.* **241:**2803–2809 (1966).
24. Levin, W., A. Y. H. Lu, M. Jacobson, R. Kuntzman, J. L. Poyer, and P. B. McCay. Lipid peroxidation and the degradation of cytochrome P-450 heme. *Arch. Biochem. Biophys.* **158:**842–852 (1973).
25. Kappas, A., and M. D. Maines. Tin: a potent inducer of heme oxygenase in kidney. *Science (Wash. D. C.)* **192:**60–62 (1976).
26. Maines, M. D., and A. Kappas. Enzymes of heme metabolism in the kidney. *J. Exp. Med.* **146:**1286–1293 (1977).
27. Tenhunen, R., H. S. Marver, and R. Schmid. The enzymatic catabolism of hemoglobin: Stimulation of microsomal heme oxygenase by hemin. *J. Lab. Clin. Med.* **75:**410–421 (1970).
28. Pimstone, N. R., P. Engel, R. Tenhunen, P. T. Seitz, H. S. Marver, and R.

Schmid. Inducible heme oxygenase in the kidney: a model for the homeostatic control of hemoglobin catabolism. *J. Clin. Invest.* **50**:2042–2050 (1971).
29. Drummond, G. S., and A. Kappas. Metal regulation of heme metabolism: further observations with manganese, zinc, cobalt and cobalt heme, in *Biochemistry, Biophysics and Regulation of Cytochrome P-450* (J. Gustafsson, J. Carlstedt-Duke, A. Mode, and J. Rafter eds.), Elsevier/North-Holland Biomedical Press, New York, 203–206 (1980).
30. Fish, R. H., E. C. Kimmel, and J. E. Casida. Bioorganotin chemistry: reactions of tributyltin derivatives with a cytochrome P-450 dependent monooxygenase enzyme system. *J. Organometallic Chem.* **118**:41–54 (1976).
31. Henry, R. A., and K. H. Byington. Inhibition of glutathione-*S*-aryl-transferase from rat liver by organogermanium, lead and tin compounds. *Biochem. Pharmacol.* **25**:2291–2295 (1976).
32. Schenkman, J. B., B. J. Wilson, and D. L. Cinti. Diethylaminoethyl-2,2-diphenylvalerate HCl (SKF 525-A): *in vivo* and *in vitro* effects of metabolism by rat liver microsomes—formation of an oxygenated complex. *Biochem. Pharmacol.* **21**:2373–2383 (1976).

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