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ACTIONS OF ORALLY ADMINISTERED ORGANOTIN COMPOUNDS ON HEME METABOLISM AND CYTOCHROME P-450 CONTENT AND FUNCTION IN INTESTINAL EPITHELIUM

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Abstract—The gastrointestinal tract is a major route by which humans are exposed to environmental chemicals. We have examined in these studies the effects of oral administration of organotin compounds in the small intestinal epithelium, an organ which exhibits highly active drug and other chemical metabolism. A series of *n*-butyltin compounds was administered by gavage to male Sprague–Dawley rats (225–275 g) in single doses up to 250 μmol/kg body weight. Bis(tri-*n*-butyltin)oxide (TBTO) produced dose- and time-dependent decreases in the content and functional activity of intestinal cytochrome P-450, together with an elevation (3-fold) in the activity of microsomal heme oxygenase. The effects of di-*n*-butyltin dichloride on heme oxygenase and cytochrome P-450 were pronounced in the small intestine and extended to the liver and kidneys within 24 hr after oral exposure, whereas TBTO did not affect the liver until much later (6 days), when cytochrome P-450 content was reduced markedly (30%). Furthermore, the effects produced by tetra-*n*-butyltin on cytochrome P-450 at 24 hr were localized in the intestinal epithelium. These studies indicate important pharmacological effects of organotin compounds in the gut, and raise the possibility that concurrent oral ingestion of organotins with other environmental pollutants may alter the cytochrome P-450-dependent metabolism of xenobiotics and natural substrates of this monooxygenase system in the small intestine.

The widespread industrial and agricultural applications of organotin compounds have led, in recent years, to an extensive evaluation of the general toxicology of these compounds [1–3]. Among the biological effects of these organometallic agents which have been described in mammalian systems are a depression of thymus-dependent immunity and accompanying thymic atrophy [4, 5], production of edematous lesions in the white matter of the central nervous system [6], behavioral toxicities [7], and impairment of energy generation through inhibition of oxidative phosphorylation in isolated mitochondria [8, 9].

We have demonstrated that the administration of various organotins of differing chemical structures can also produce substantial and long-term alterations in heme metabolism, with concomitant depression of cytochrome P-450 content and related functional activities in the liver, kidneys and other tissues [10–12]. While these studies have relied on parenteral routes of administration [10, 11, 13], the gastrointestinal (GI) tract remains the most likely route by which human populations may be exposed to these agents [3]. The epithelial cells of the proximal small intestine are a potential target organ in particular since they contain highly active drug and other foreign chemical metabolizing enzyme systems [14–17] which can be subject to perturbations induced by direct exposure to environmental and other agents [18–22].

Selected n-Butyltin Compounds

$[\,(n\text{-}C_4H_9)_3Sn\,]_2O$
Bis(tri-n-butyltin)oxide
(TBTO)

$(n\text{-}C_4H_9)_XSnCl_{4-X}$

X= 1 mono-n-butyltin chloride (MBTC)
2 di-n-butyltin dichloride (DBDC)
3 tri-n-butyltin chloride (TBTC)
4 tetra-n-butyltin (TeBT)

Fig. 1. Structures of organotin compounds.

In this study, we have examined the ability of bis(tri-*n*-butyltin)oxide (TBTO), a widely used biocidal agent [1–3] and, for comparative purposes, related *n*-butyltin derivatives (the structures of which are shown in Fig. 1), to produce alterations in several critical end points of heme metabolism in the rat small intestine following oral administration. The results of these studies indicate that direct exposure

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of the GI tract to these organotin compounds resulted in substantial changes in heme metabolism that were both dose and time dependent, as well as dependent on the number of alkyl side chains covalently attached to the tin atom.

METHODS

Materials. Di-*n*-butyltin dichloride (DBDC) and tri-*n*-butyltin chloride (TBTC) were purchased from the Ventron Corp. (Danvers, MA). Mono-*n*-butyltin trichloride (MBTC), TBTO and tetra-*n*-butyltin (TeBT) were provided by the M&T Chemical Co. (Rahway, NJ). The organotins were at least 95% pure as determined by the manufacturer. All other reagents were of the highest grade commercially available and were purchased from the Sigma Chemical Co. (St. Louis, MO).

Treatment of animals. Male Sprague–Dawley rats (225–275 g) were purchased from Taconic Farms (Germantown, NY). Rats were maintained on Standard Purina Rodent Laboratory Chow (St. Louis, MO), and were allowed to acclimatize to a light-cycled room (12 hr light/dark cycle) for at least 1 week prior to study. The organotin compounds were suspended in corn oil and administered by gavage to unfasted rats in a single dose of up to 250 μmol/kg body wt (1.0 ml/kg) using a Perfektum stainless steel 18 gauge animal feeding needle (New Hyde Park, NY) at the times indicated in the legends to tables and figures. Control animals received an equivalent amount of corn oil (1.0 ml/kg). In one experiment, TBTO was dissolved in ethanol and administered subcutaneously at a dose of 50 μmol/kg (1.0 ml/kg). Control animals received an equivalent volume of ethanol. All rats were allowed free access to food and distilled water.

Preparation of subcellular fractions. Animals were decapitated and then exhaustively perfused *in situ* with ice-cold 0.9% NaCl through the left ventricle. The small intestine was cut at the pyloric junction, and the entire length of the intestine was irrigated *in situ* with 30 ml of ice-cold 0.9% NaCl to remove the intestinal contents. The first 15 cm of small intestine were then removed and irrigated once more with 30 ml of cold saline. The excised intestine was then placed onto a watch glass kept on ice and cut longitudinally to expose the mucosal surface. The mucosal cells were gently scraped off with a stainless steel scalpel and placed in ice-cold potassium phosphate buffer (0.1 M, pH 7.4) containing sucrose (0.25 M), 20% glycerin (v/v), trypsin inhibitor (5 mg/ml) and heparin (3 units/ml) as described by Stohs *et al.* [18]. Following homogenization with a tight-fitting Potter–Elvehjem Teflon–glass homogenizer, the samples were subjected to sonication at 4° using three 5-sec pulses at 30 W/min. The sonication step greatly increases the yield of microsomal material.* This procedure allowed all assays to be performed on individual preparations from a single intestinal mucosal scraping. Liver and kidney homogenates were prepared as described previously [11]. The homogenates were centrifuged at 9000 *g* for 20 min, and the resultant pellet was used for determining δ-aminolevulinate (ALA)-synthase (EC 2.31.37) activity. The supernatant fraction was centrifuged at 105,000 *g* for 60 min in a Beckman L5-50 ultracentrifuge to obtain the microsomal pellet on which all other enzyme assays were performed. The microsomal pellet was rinsed and resuspended in 2.0 ml of potassium phosphate buffer (0.1 M, pH 7.4) to a protein concentration of approximately 5–10 mg/ml in kidney and intestinal preparations, and 15–20 mg/ml in liver preparations. Microsomes were freshly prepared on a daily basis for all subsequent enzyme assays.

Enzyme assays. The activity of heme oxygenase (EC 1.14.99.3) was determined as previously described [23], using a hemin concentration of 50 μM with intestinal microsomes and 16 μM with hepatic and renal microsomes. ALA-synthase activity was measured in the washed 9000 *g* pellet as described previously [24]. Arylhydrocarbon hydroxylase (AHH) activity was measured in intestinal microsomes by the procedure of Nebert and Gelboin [25], modified for small samples as described by Proia *et al.* [26]. Cytochrome P-450 content was measured in liver microsomes by the method of Omura and Sato [27]. Renal and intestinal cytochrome P-450 was measured in microsomal suspensions containing 1–2 mg protein/ml from the dithionite-reduced difference spectrum of CO-bubbled samples (60 sec), using a molar extinction coefficient of 104 $mM^{-1} \cdot cm^{-1}$ for the absorption difference between 450 and 490 nm [28]. Protein content was determined by the method of Lowry *et al.* [29], using crystalline bovine serum albumin as a standard.

Statistical analysis. The data were analyzed by Student's *t*-test, and a P value of <0.05 was regarded as statistically significant.

RESULTS

Effects of TBTO on intestinal, hepatic and renal heme metabolism with respect to route of administration, dose and time. The effects of TBTO administered (50 μmol/kg body wt) either subcutaneously (s.c.) or by gavage (100 μmol/kg) on heme oxygenase activity and cytochrome P-450 content in the liver and small intestinal epithelium were examined at 48 hr after a single dose of the organotin (Table 1). This time point and dose level were chosen on the basis of earlier studies [11] which demonstrated that maximum perturbations in hepatic heme metabolism occur at 48 hr after such treatment.

Following s.c. treatment with TBTO, heme oxygenase activity in liver was elevated 2- to 3-fold with a concomitant 50–60% reduction in cytochrome P-450 levels. When administered orally, however, TBTO did not affect heme oxygenase and cytochrome P-450 in liver (Table 1) over the time period studied. Although s.c. treatment with TBTO produced a slight elevation in heme oxygenase activity in the small intestine, oral treatment with the organotin produced a greater (2.6-fold) induction of this intes-

* Lindeskog P, Haaparanta T, Glaumann H, Hansson T and Gustafsson JA, Characterization of microsomal preparation from the small intestine and immunochemical detection of intestinal cytochrome P-450 (abstract). *Sixth International Symposium on Microsomes and Drug Oxidations.* Brighton, England, 1984.

Table 1. Effects of TBTO administered either subcutaneously or by gavage on heme oxygenase activity and cytochrome P-450 content in the liver and small intestinal epithelium at 48 hr

Tissue	Treatment	Heme oxygenase (nmol bilirubin/ mg protein·hr)	Cytochrome P-450 (nmol/mg protein)
Liver	Controls	4.35 ± 0.04	0.60 ± 0.05
	TBTO, s.c.	11.31 ± 1.29*	0.27 ± 0.06†
	TBTO, oral	3.85 ± 0.09	0.67 ± 0.06
Small intestine	Controls	5.62 ± 0.38	0.035 ± 0.003
	TBTO, s.c.	8.03 ± 0.08*	0.034 ± 0.001
	TBTO, oral	11.67 ± 2.68*	0.027 ± 0.003

TBTO was dissolved either in ethanol and given s.c. (50 μmol/kg body wt), or in corn oil and given by gavage (100 μmol/kg) in a single dose. The animals were allowed free access to food and water and were killed 48 hr later. Heme oxygenase and cytochrome P-450 were determined as described under Methods. Values are the means ± SEM of at least three individual animals per group.

* Significantly different from the controls treated with corn oil or ethanol alone ($P < 0.01$, Student's t-test).

† $P < 0.02$, versus controls treated with corn oil or ethanol alone (Student's t-test).

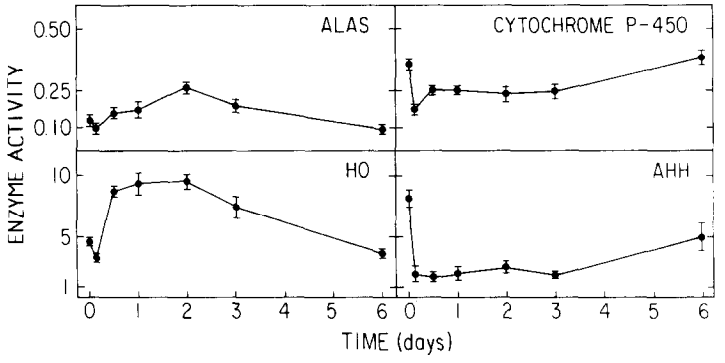

Fig. 2. Time course of TBTO effects on intestinal heme metabolism. TBTO was dissolved in corn oil and administered by gavage in a single dose (100 μmol/kg body wt). The animals were killed at the time points indicated, and intestinal microsomal and mitochondrial fractions were prepared and assays performed as described under Methods. The units shown are as follows: ALAS, nmol ALA/mg protein per hr; heme oxygenase, nmol bilirubin/mg protein per hr; cytochrome P-450, nmol/mg protein; and AHH, nmol 8-hydroxybenzo[*a*]pyrene/mg protein per hr. Values are the means ± SEM of two separate experiments, involving a minimum of six animals per time point.

tinal enzyme. In this latter treatment group, cytochrome P-450 content in the intestinal epithelium was reduced (~25%) compared with controls (Table 1).

The effects of TBTO (100 μmol/kg), administered by gavage in a single dose, on the activities of intestinal ALA-synthase and heme oxygenase were examined with respect to time (Fig. 2). Also shown in Fig. 2 are the effects of the organotin on cytochrome P-450 concentration and on the activity of AHH. Following the oral administration of TBTO, intestinal heme oxygenase activity exhibited an induction pattern that was similar, although lesser in magnitude, to that which is produced in the liver by this compound after parenteral administration [11]. Heme oxygenase activity was elevated approximately 2- to 3-fold by 24 hr. The activity remained at this level throughout the following 3 days and gradually returned to normal at 6 days. ALA-synthase activity was decreased slightly at 4 hr (~25%), followed by a return to control levels by 24 hr. The activity of ALA-synthase further increased (2-fold) at 48 hr before returning to normal levels by approximately 6 days.

Levels of cytochrome P-450 in the intestinal epithelium decreased rapidly from an initial mean concentration of 0.036 ± 0.002 nmol/mg protein to a mean concentration within 4 hr of less than 0.015 ± 0.001 nmol/mg protein after administration of TBTO (fig. 2). The concentration of cytochrome P-450 remained markedly (<60%) below controls for up to 3 days before returning to normal by 6 days after treatment. The activity of AHH was also reduced markedly following organotin treatment. TBTO produced a 75% reduction in this enzymatic activity within 4 hr, and activity remained at these

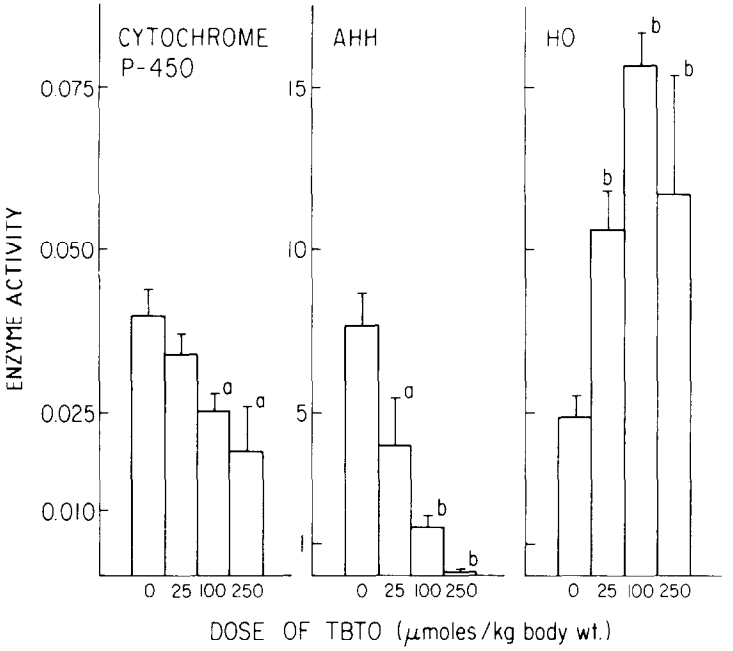

Fig. 3. Effects of TBTO on heme metabolism in small intestinal mucosa at 24 hr. TBTO was dissolved in corn oil and administered by gavage in a single dose of 25, 100 or 250 μmol/kg body weight. The animals were killed 24 hr later, and microsomal fractions were prepared as described under Methods. The units shown are as follows: cytochrome P-450, nmol/mg protein; aryl hydrocarbon hydroxylase (AHH), nmol 8-hydroxybenzo[*a*]pyrene/mg protein per hr, and heme oxygenase (HO) nmol bilirubin/mg protein per hr. Values are the means ± SEM of at least three individual animals. Key: (a) Significantly different from the controls treated with corn oil alone ($P < 0.05$, Student's *t*-test). (b) $P < 0.01$ versus control (Student's *t*-test).

greatly reduced levels for up to 3 days before returning towards normal by 6 days (Fig. 2).

Administration of TBTO produced significant dose-dependent alterations at 24 hr in the concentration of cytochrome P-450 and AHH activity in the intestinal epithelium (Fig. 3). The lowest dose of TBTO (25 μmol/kg) produced a 2-fold increase in heme oxygenase activity, while cytochrome P-450 content was lowered by ~10% and AHH activity was reduced by almost 50% compared with controls. At a dose of 100 μmol/kg of TBTO, heme oxygenase activity increased ~3-fold above controls and cytochrome P-450 content was lowered by ~35% (Fig. 3). AHH activity was lowered at this dose of TBTO (100 μmol/kg) to levels less than 20% of controls, while at the maximum dose of TBTO administered (250 μmol/kg), this enzymatic activity was barely detectable (<5% of controls).

In contrast to the rapid onset (within hours) of the effects produced by TBTO in the gut, heme metabolism in the liver was largely unaffected by oral treatment with the organotin until much later. Hepatic heme oxygenase activity remained at or near mean control levels (4.01 ± 0.05 nmol bilirubin/mg protein per hr) throughout the entire experimental period. Cytochrome P-450 content, however, was lowered ~30% in the liver (from a mean control of 0.61 ± 0.03 nmol/mg to a mean of 0.44 ± 0.03) at 6 days after oral treatment with TBTO. Both end points returned to control levels by 2 weeks after treatment. In the kidney, heme oxygenase activity and cytochrome P-450 content were unaffected at each time point examined up to 6 days after a single dose (data not shown).

Effects of varying numbers of alkyl groups on cytochrome P-450 and heme oxygenase. Varying the number of *n*-butyl groups covalently attached to the tin atom resulted in structure-dependent effects following oral administration of a single dose (100 μmol/kg) of either the mono-, di-, tri- or tetra-*n*-butyltin derivatives. The effects of these compounds on heme oxygenase activity and cytochrome P-450 content in the small intestine, liver and kidney at 24 hr are shown in Table 2. Three of the organotin derivatives, i.e. DBDC, TBTC and TeBT, produced substantial (~30–45%) decreases in the content of cytochrome P-450 at 24 hr in the intestinal epithelium, when given orally. However, only DBDC produced a significant elevation (2.5-fold) in the activity of intestinal heme oxygenase.

In the liver, only DBDC significantly lowered (~42%) the concentration of cytochrome P-450 at 24 hr. This decreased cytochrome P-450 content was accompanied by an elevation (~2-fold) in the activity of hepatic heme oxygenase (Table 2). In addition, only DBDC significantly affected renal cytochrome P-450 levels, lowering the concentration of this hemoprotein by almost 20%. Concomitant with this effect, a ~2.5-fold induction in the activity of heme oxygenase was produced (Table 2).

Changes in intestinal AHH activity at 24 hr after oral administration of the organotin compounds are

Table 2. Effects of varying numbers of *n*-butyl groups on heme oxygenase and cytochrome P-450 in small intestine, liver and kidney at 24 hr

Tissue	Organotin compounds	Cytochrome P-450 (nmol/mg protein)	Heme oxygenase (nmol bilirubin/mg protein·hr)
Small intestine	Controls	0.037 ± 0.005	4.96 ± 0.58
	MBTC	0.036 ± 0.001	6.57 ± 0.72
	DBDC	0.026 ± 0.001*	12.41 ± 0.10†
	TBTC	0.021 ± 0.004*	6.17 ± 0.26
	TeBT	0.025 ± 0.001*	5.41 ± 0.22
Liver	Controls	0.67 ± 0.04	4.45 ± 0.09
	MBTC	0.66 ± 0.02	4.87 ± 0.16
	DBDC	0.35 ± 0.03†	7.99 ± 0.96†
	TBTC	0.58 ± 0.05	5.05 ± 0.69
	TeBT	0.58 ± 0.04	4.54 ± 0.44
Kidney	Controls	0.066 ± 0.003	3.02 ± 0.30
	MBTC	0.058 ± 0.002	3.65 ± 0.27
	DBDC	0.055 ± 0.002*	8.09 ± 1.27†
	TBTC	0.069 ± 0.009	2.82 ± 0.50
	TeBT	0.063 ± 0.001	2.34 ± 0.09

MBTC, DBDC, TBTC and TeBT were dissolved in corn oil and administered by gavage in a single dose (100 μmol/kg body wt). The animals were killed 24 hr later, and cytochrome P-450 and heme oxygenase were determined as described under Methods. Values are the means ± SEM of two separate experiments, involving a minimum of four to six animals per group.

* Significantly different from the controls treated with corn oil alone ($P < 0.05$, Student's *t*-test).

† $P < 0.01$, versus control (Student's *t*-test).

shown in Fig. 4. DBDC produced the greatest reduction in the activity of this enzyme, with levels lowered by approximately 80% compared with controls. Both TBTC and TeBT resulted in smaller, although significant (35–45%), reductions in AHH activity as well (Fig. 4).

DISCUSSION

The present studies demonstrate that organotin compounds of differing structural and chemical characteristics produced significant alterations in the activities of ALA-synthase and heme oxygenase, the rate-limiting enzymes of heme synthesis and degradation, respectively, and affected cytochrome P-450 content and functional activity in the intestinal epithelium as a result of oral treatment. A critical determinant of the site of action, as well as the extent to which a toxic response is elicited by organometals, is the number of alkyl substituents covalently attached to the central metal atom [30]. Therefore, the effects of varying the number of *n*-butyl groups attached to the tin atom were examined with respect to heme metabolism, since perturbations of heme metabolism represent a sensitive index of intracellular metal-related toxicity [31]. The di- and trialkyl substituted tin compounds were most effective in perturbing intestinal heme metabolism. In the case of DBDC, alterations in heme metabolism extended beyond the small intestine to the liver and kidney.

The dose–response effects of TBTO on intestinal heme oxygenase activity and cytochrome P-450 content were examined at doses ranging from 25 to 250 μmol/kg (Fig. 3). Comparable data for this compound in the liver following parenteral administration have been reported previously [11]. TBTO produce dose-dependent decreases in intestinal cytochrome P-450 content and AHH activity at 24 hr. The activity of this cytochrome P-450-dependent monooxygenase system was barely detectable (<5% of controls) following treatment with the highest dose of the organotin. Although intestinal heme oxygenase and cytochrome P-450 were altered to a significant extent by TBTO at 24 hr, these effects did not extend to the kidney. In fact, renal heme metabolism was not affected by oral TBTO treatment (100 μmol/kg) at earlier (4 and 12 hr) or at later (3, 6 and 13 days) time points (data not shown). Furthermore, TBTO did not affect cytochrome P-450 content in the liver until 6 days after treatment (~30% reduction) and produced only minimal changes in hepatic heme oxygenase activity throughout the entire experimental period. This contrasts with the liver effects produced by TBTO between 24 and 72 hr following parenteral treatment [10, 11]. This delayed effect of the compound in the liver after its oral administration may reflect the poor absorption and/or entero-hepatic circulation of this organotin until a critical threshold level of the compound or of a metabolite is achieved in hepatic cells.

The effects on heme metabolism in the small intestine produced by a single oral dose of TBTO were qualitatively similar to the effects produced by trialkyltins in the liver following parenteral administration [11]. The slight inhibition of ALA synthase in the intestine at 4 hr, followed by rebound induction (~2-fold) between 24 and 48 hr, was similar with respect to time, although lesser in magnitude, to that

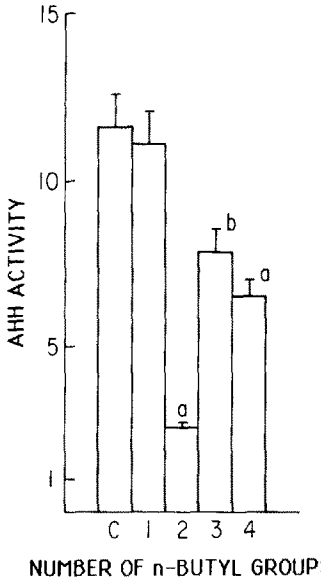

Fig. 4. Effects of oral administration of mono-, di-, tri- and tetra-*n*-butyltin on aryl hydrocarbon hydroxylase activity in small intestinal mucosa at 24 hr after a single dose. MBTC, DBDC, TBTC and TeBT were dissolved in corn oil and administered by gavage in a single dose (100 μmol/kg body wt). The animals were killed 24 hr later, and aryl hydrocarbon hydroxylase (AHH) activity (nmol 8-hydroxybenzo[*a*]pyrene/mg protein per hr) was determined as described under Methods. Values are the means ± SEM of two separate experiments, involving a minimum of four to six animals per group. Key: (a) Significantly different from the controls treated with corn oil alone ($P < 0.01$, Student's *t*-test). (b) $P < 0.05$, versus control Student's *t*-test).

observed in the liver following parenteral organotin treatment. In addition, although heme oxygenase activity was elevated for several days after the single dose of TBTO, the extent (2-fold) to which this occurred was considerably less in the intestinal epithelium than in the liver. It is likely that "control" levels of heme oxygenase in the intestinal epithelium are already somewhat elevated by exposure to various dietary influences and, as such, the enzyme may be functioning at or near maximal activity for these cells. In this case, the actual extent to which the organotins induce this enzymic activity in the intestine would be somewhat masked.

Intestinal cytochrome P-450 levels were reduced by approximately 40% between 1 and 3 days after oral treatment with TBTO (Fig. 2), although AHH activity was lowered to an even greater extent (~15% of controls). It is possible that the cytochrome P-450 subspecies responsible for AHH activity in the intestine exhibits a differential sensitivity to TBTO. Earlier *in vitro* experiments have shown that cytochrome P-448 induced by 3-methylcholanthrene is much more sensitive to TBTO than is the control or phenobarbital inducible form of the hemeprotein in liver microsomes [32]. This suggests that direct exposure of the intestinal epithelium to foreign chemicals may mimic to some extent the responses observed in the *in vitro* microsomal system.

There was some structure-dependence with respect to organotin effects on cytochrome P-450 content and function and heme oxygenase activity in the small intestine, liver and kidney. Of the four organotin compounds examined, MBTC was least effective overall at 24 hr. This might be predicted on the basis of the lower systemic toxicity that has been associated previously with the mono-alkyl substituted tin compounds [30]. The effects produced by TBTC and TeBT on heme oxygenase activity and cytochrome P-450 content and function were similar in all three tissues examined. This is consistent with substantial metabolic evidence that tetraalkyltins are converted into trialkytins *in vivo* in rabbit intestinal mucosa [33], and *in vitro* in liver microsomes from rabbits and rats [34]. Of the various organotins examined, only DBDC produced significant effects on heme oxygenase that extended beyond the small intestine to the liver and kidney. The tissue specificity observed in these and other studies [11] is probably attributable to the metabolic disposition of the organotins, and to the similarity of the divalent tin in DBDC to stannous chloride, which was shown earlier to produce substantial alterations in renal heme metabolism [35]. Additional metabolism and pharmacokinetic studies may provide further insight into the biological basis for these route- and structure-dependent effects of *n*-butyltin compounds on heme metabolism.

The results of these studies further define important actions of organotin compounds on the regulation of cytochrome P-450 content and functional activities in intestinal epithelium as a consequence of single dose oral exposure to these agents. The metabolic interactions that may occur among xenobiotics within the mucosal cells of the intestinal epithelium, which serve as a primary barrier to systemic exposure of many ingested foreign chemicals, are of considerable toxicological significance. Considering the potential for oral exposure to many of these organotin compounds, the agricultural and industrial uses of which have greatly increased in recent years [1–3], it is reasonable to suggest that these newly defined actions of organotins may have important biological implications with respect to the metabolism and subsequent disposition of endogenously derived compounds as well as concurrently ingested foreign chemicals or orally administered therapeutic agents.

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