15 mg/kg body wt (40 μmoles/kg); control rats received an equal volume of the solvent (1 mg/kg) and were identical to untreated animals in all parameters studied. Phenobarbital, 3-methylcholanthrene and β-naphthoflavone were each injected i.p. in single daily doses for 3 days, the first injection immediately following the single injection of tricyclohexyltin hydroxide. Phenobarbital (80 mg/kg) was dissolved in saline. 3-Methylcholanthrene (20 mg/kg) and β-naphthoflavone (40 mg/kg) were suspended in corn oil with appropriate controls receiving corn oil alone. Animals were allowed free access to water but were deprived of food for 16 hr prior to sacrifice as indicated in the legends of the tables and figures.

Livers were exhaustively perfused *in situ* with ice-cold 0.9% NaCl and homogenized in 3 vol. of potassium phosphate buffer (0.1 M, pH 7.4) containing sucrose (0.25 M). The homogenates were centrifuged at 9000 *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 all subsequent enzyme assays.

Enzyme assays. The activity of heme oxygenase (EC 1.14.99.3) was determined in the microsomal fraction as previously described [2]. ALA-synthase (EC 2.31.37) activity was measured in the washed 9000 *g* pellet by the method of Sassa *et al.* [15]. Cytochrome P-450 content was measured by the method of Omura and Sato [16]. The total concentration of microsomal heme was determined by the pyridine-hemochromagen method as described by Porra and Jones [17], using the dithionite-reduced minus oxidized difference spectrum between 541 and 557 nm and an extinction coefficient of 20.7 mM^{-1} cm^{-1}. 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 by the method of Imai *et al.* [19]. Benzo[*a*]pyrene hydroxylase activity was determined in the microsomal fraction by the method of Nebert and Gelboin [20]. Protein content was determined by the method of Lowry *et al.* [21], using crystalline bovine serum albumin as standard.

Gel electrophoresis. Polyacrylamide slab gel electrophoresis was carried out at room temperature in the presence of SDS with a discontinuous buffer system according to the procedure of Laemmli [22]. The polyacrylamide concentrations were 5% and 10% in the stacking gel and separating gel respectively. Microsomal preparations were diluted to a concentration of 5 mg of protein/ml with 0.0625 M Tris buffer (pH 6.8) containing 2% SDS, 10% glycerol (v/v) and 5% β-mercatopethanol (v/v). The samples were heated for 2 min in a boiling water bath, and equal amounts of protein (50 μg each) were applied for electrophoresis. The gels were stained with Coomassie brilliant blue R-250 to visualize the protein bands.

Statistical analysis. The data were analyzed by Student's *t*-test, and the indicated P value was regarded as denoting significance.

RESULTS

Time course of phenobarbital effects on hepatic heme-cytochrome P-450 metabolism following tricyclohexyltin hydroxide administration. The effects of tricyclohexyltin hydroxide (15 mg/kg), administered simultaneously with the first of three daily i.p. injections of phenobarbital (80 mg/kg), on the activity of heme oxygenase and on the concentration of hepatic cytochrome P-450* are shown in Figs. 1 and 2. Following the administration of tricyclohexyltin hydroxide alone, heme oxygenase activity followed an induction pattern that is typical for this organometal [9, 10], attaining an approximately 3-fold elevation throughout the entire 4-day time period examined. The concurrent administration of phenobarbital had little or no effect on the induction of heme oxygenase elicited by tricyclohexyltin hydroxide. Phenobarbital treatment alone slightly lowered heme oxygenase activity as compared with untreated controls (Fig. 1), although this effect was not significant by statistical analysis.

Following the administration of the organotin hepatic cytochrome P-450 levels were decreased from an initial mean concentration of ~0.70 nmole/mg protein to a mean concentration at 48 hr of less than 0.30 nmole/mg (Fig. 2). The concentration of cytochrome P-450 was increased 2- to 3-fold within 24 to 48 hr with the daily administration of phenobarbital alone. However, when a single injection of tricyclohexyltin hydroxide was given simultaneously with the first of three daily injections of phenobarbital, an induction effect on cytochrome P-45

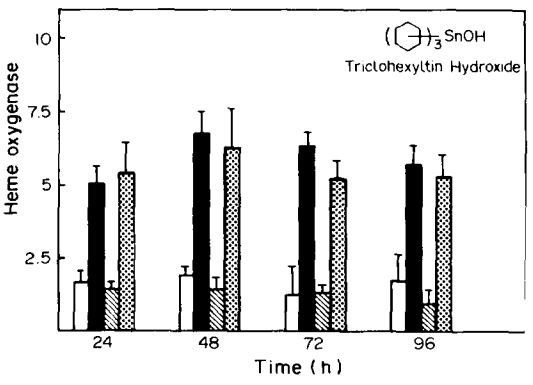

Fig. 1. Time course of tricyclohexyltin hydroxide an phenobarbital effects on hepatic heme oxygenase activit in rat. Tricyclohexyltin hydroxide (15 mg/kg, s.c.) an phenobarbital (80 mg/kg, i.p.) were administered eith alone or simultaneously to rats. Hepatic microsomal fra tions were prepared and heme oxygenase (nmoles bilirubi hr/mg protein) was measured as described under Materia and Methods. Key: (□) untreated; (■) tricyclohexylti hydroxide (▧) phenobarbital; and (▩) tricyclohexylti hydroxide plus phenobarbital. Values represent the ave age of results from at least six individual animals.

* Since evidence has accumulated that chemicals such as phenobarbital, 3-methylcholanythrene and β-naphthoflavone induce more than one distinct form of hepatic cytochrome P-450, the term P-450 (448) designates all such forms of this heme protein.

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ALTERED INDUCTION RESPONSE OF HEPATIC CYTOCHROME P-450 TO PHENOBARBITAL, 3-METHYLCHOLANTHRENE, AND β-NAPHTHOFLAVONE IN ORGANOTIN-TREATED ANIMALS

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Abstract—The effects of tricyclohexyltin hydroxide on the induction of cytochrome P-450 in liver by phenobarbital, 3-methylcholanthrene and β-naphthoflavone were studied. A single dose of the organotin (15 mg/kg body wt) prevented the full extent of phenobarbital induction of cytochrome P-450 from occurring; this was the case whether tricyclohexyltin was given 48 hr preceding a single injection of phenobarbital, or administered simultaneously with the first of three daily doses of the drug. Elevation of hepatic heme oxygenase (EC 1.14.99.3) activity accompanied these changes in cytochrome P-450, but the induction of this enzyme was not affected by phenobarbital treatment. The induction of cytochrome P-448 by 3-methylcholanthrene and β-naphthoflavone was not affected to the same extent by a single injection of tricyclohexyltin, while heme oxygenase induction was less pronounced when these cytochrome P-448 inducers were given together with the organotin. The changes in cytochrome P-450 content and in its functional activity resulting from the various treatments were further examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the microsomal fractions. The electrophoretic profiles illustrate clearly that the apoprotein moieties of the various cytochrome P-450 subspecies are affected to a considerable extent by treatment with tricyclohexyltin hydroxide alone, and staining in these bands was noticeably reduced even when phenobarbital was administered together with the organotin. In contrast, tricyclohexyltin failed to decreased the 3-methylcholanthrene- or β-naphthoflavone-induced cytochrome P-450 subspecies. These data suggest that significant metabolic interactions can occur from exposure to a combination of environmental chemicals and drugs resulting in an altered metabolism of heme and cytochrome P-450.

Heme oxygenase, the rate-limiting enzyme in heme† catabolism to bile pigment [1], is inducible in liver by various inorganic metals such as cobalt, cadmium, tin, arsenic and antimony [2–6], by the synthetic metalloporphyrin Co(cobalt)-protoporphyrin [7], as well as by its natural substrate heme [8]. Recently [9, 10], we described a potent class of chemical agents capable of producing moderate, but prolonged, elevation of heme oxygenase activity, i.e. the di- and trialkyl organotins. These organometallic compounds, in which the tin atom is covalently attached to various alkyl groups, are of considerable commercial significance, and have widespread applications in both agriculture and industry [11, 12]. Concomitant with the organotin-induced elevation of heme oxygenase activity in liver, there occurs a substantial decrease in the content and functional activities of cytochrome P-450 both *in vivo* [10, 13] and *in vitro* [10, 14]. To further examine the mechanisms by which these alterations occur and to determine whether cytochrome P-450 content is inducible in the livers of animals in which there already exists an elevated level of heme oxygenase activity, tricyclohexyltin hydroxide was administered simultaneously with either phenobarbital, 3-methylcholanthrene, or β-naphthoflavone to rats. The nature of the changes produced in hepatic heme-cytochrome P-450 metabolism was further characterized by SDS-polyacrylamide gel electrophoresis of microsomal preparations. The results of this study indicate that administration of tricyclohexyltin hydroxide to rats produces differential changes in cytochrome P-450-mediated monooxygenase activities and these are further reflected in a considerably altered induction response to chemical agents which induce the hemeprotein(s).

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† Abbreviations: heme, Fe(iron)-protoporphyrin; SDS, sodium dodecyl sulfate; and ALA, δ-aminolevulinic acid.

MATERIALS AND METHODS

Materials. Male Sprague–Dawley rats (175–225 g), purchased from Holtzman (Madison, WI), were used in all experiments. Tricyclohexyltin hydroxide was a gift of the M&T Chemical Co. (Rahway, NJ). Sodium phenobarbital was purchased from Mallinckrodt (St. Louis, MO), 3-methylcholanthrene from the Eastman Kodak Co. (Rochester, NY), and β-naphthoflavone from the Aldrich Chemical Co. (Milwaukee, WI). All other reagents were analytical grade and were purchased from either the Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburg, PA).

Treatment of animals and preparation of microsomes. Rats received s.c. injections of the organotin compound (in 95% ethanol) in a single dose of

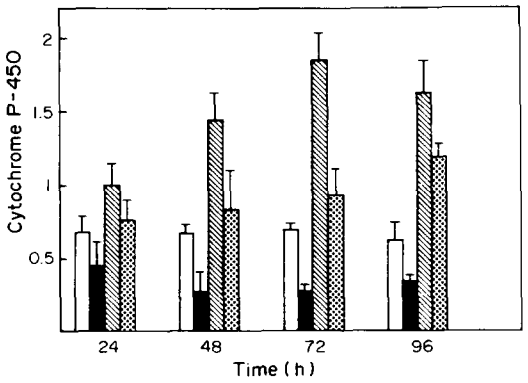

Fig. 2. Time course of tricyclohexyltin hydroxide and phenobarbital effects on cytochrome P-450 content in rat liver. Tricyclohexyltin hydroxide (15 mg/kg) and phenobarbital (80 mg/kg) were administered either alone or simultaneously to rats. Hepatic microsomal fractions were prepared, and cytochrome P-450 (nmoles/mg protein) was measured as described under Materials and Methods. Key: (□) untreated; (■) tricyclohexyltin hydroxide; (▨) phenobarbital; and (▩) tricyclohexyltin hydroxide plus phenobarbital. Values are reported as the means ± S.E.M. of six individual animals.

was produced which was intermediate between the effects produced by either tricyclohexyltin hydroxide or phenobarbital alone (Fig. 2). The concentration of microsomal heme followed a pattern of induction and inhibition similar to that displayed by cytochrome P-450 (data not shown).

ALA-synthase displayed an altered inductive response to phenobarbital following concurrent organotin treatment (Table 1). Enzyme activity was 50% greater than that of untreated animals 72 hr after tricyclohexyltin hydroxide treatment alone. Phenobarbital treatment alone produced a 200% increase in the activity of this enzyme at 72 hr. A single injection of tricyclohexyltin hydroxide concurrent with phenobarbital produced a slightly exaggerated enzyme response at this time point, in which ALA-synthase activity was 225% above controls.

Effects of tricyclohexyltin hydroxide on cytochrome P-450 content and drug-metabolizing activities at 72 hr following phenobarbital, 3-methylcholanthrene, and β-naphthoflavone administration. The altered inducibility of cytochrome P-450 to phenobarbital was also reflected in the functional activities of the hemeprotein. Administration of tricyclohexyltin hydroxide was followed immediately with the first of three daily injections of either phenobarbital, 3-methylcholanthrene or β-naphthoflavone and the effects of these various treatments on cytochrome P-450 concentration and related drug-metabolizing enzyme activities, and on heme oxygenase activity were examined (Tables 2–4).

The activity of heme oxygenase was elevated approximately 3-fold by tricyclohexyltin hydroxide, and this enzyme induction was attenuated by simultaneous treatment with phenobarbital (Table 2). The concentration of cytochrome P-450 was reduced by at least half in animals receiving the organotin compound alone and was increase 2.5- to 3-fold by phenobarbital treatment alone. However, the latter induction response of cytochrome P-450 was attenuated by prior treatment with tricyclohexyltin hydroxide. In the combined organotin–phenobarbital treatment group, the concentration of cytochrome P-450 was increased by less than 2-fold by phenobarbital, an effect that was statistically significant ($P < 0.01$) when compared with the increase produced by phenobarbital alone.

The functional activity of cytochrome P-450 in response to these treatments was monitored by the activities of aniline hydroxylase and ethylmorphine *N*-demethylase (Table 2). Aniline hydroxylase activity was lowered to less than 50% of controls by tricyclohexyltin hydroxide treatment alone, and although this enzyme activity was elevated by phenobarbital (~1.5-fold above controls), it was never fully restored to even control levels in the presence of the organotin. Ethylmorphine *N*-demethylase activity was elevated 2.5- to 3-fold by phenobarbital treatment and, even in the presence of tricyclohexyltin hydroxide, was still almost doubled compared with untreated controls. However, this induced hydroxylase activity was still significantly reduced ($P < 0.01$) when compared with that in the phenobarbital treatment group. The effects of tricyclohexyltin hydroxide on 3-methylcholanthrene induction of cytochrome P-448 are shown in Table 3; also included is the activity of aryl hydrocarbon hydroxylase. Heme oxygenase activity was increased

Table 1. Effects of tricyclohexyltin hydroxide and phenobarbital on ALA-synthase activity in rate liver*

Treatment	ALA-synthase (nmoles ALA formed/mg protein/hr)
Untreated	0.210 ± 0.029
Tricyclohexyltin hydroxide	0.296 ± 0.038
Phenobarbital	0.412 ± 0.039†
Tricyclohexyltin hydroxide + phenobarbital	0.462 ± 0.035†

* Tricyclohexyltin hydroxide was administered s.c. in a single injection (15 mg/kg) followed by three daily injections of phenobarbital (80 mg/kg). The animals were fasted for 16 hr prior to sacrifice and killed 72 hr after receiving tricyclohexyltin hydroxide. The hepatic mitochondrial fraction was prepared and the assay was performed in duplicate as described under Materials and Methods. Values are reported at the means ± S.E.M. of three separate experiments, involving a minimum of six animals per group.

† $P < 0.01$ as compared with untreated controls.

Table 2. Effects of tricyclohexyltin hydroxide and phenobarbital on heme oxygenase and drug-metabolizing activities in liver*

Treatment	Heme oxygenase (nmoles bilirubin formed/hr/mg protein)	Cytochrome P-450 (nmoles/mg protein)	Aniline hydroxylase (nmoles *p*-aminophenol/hr/mg protein)	Ethylmorphine *N*-demethylase (nmoles formaldehyde/hr/mg protein)
Untreated (4)†	2.73 ± 0.36	0.79 ± 0.03	106.3 ± 7.17	651.5 ± 49.6
Tricyclohexyltin (4)	7.39 ± 0.57‡	0.36 ± 0.01‡	51.5 ± 7.49‡	306.0 ± 49.4‡
Phenobarbital (4)	2.18 ± 0.31	2.02 ± 0.16	131.5 ± 15.2	1732.9 ± 152.8
Tricyclohexyltin + phenobarbital (8)	6.35 ± 0.50§	1.39 ± 0.12§	99.2 ± 8.3	1081.5 ± 60.3§

* Tricyclohexyltin hydroxide was administered s.c. in a single injection (15 mg/kg body wt) followed by three daily i.p. injections of phenobarbital (80 mg/kg body wt). The animals were fasted 16 hr prior to sacrifice and killed 72 hr after tricyclohexyltin hydroxide. Hepatic microsomal fractions were prepared and assays were performed in duplicate as described under Materials and Methods. Values reported are means ± S.E.M. of three separate experiments.

† Number in parentheses refers to number of animals in treatment group.

‡ $P < 0.01$ as compared with untreated controls.

§ $P < 0.01$ as compared with phenobarbital treated.

Table 3. Effects of triclohexyltin hydroxide and 3-methylcholanthrene on heme oxygenase activity, cytochrome P-450 content and drug-metabolizing activities in rat liver*

Treatment	Heme oxygenase (nmoles/bilirubin/hr mg/ protein)	Cytochrome P-450 (nmoles/mg protein)	Aniline hydroxylase (nmoles *p*-aminophenol/hr/mg protein)	Ethylmorphine *N*-demethylase (nmoles HCHO/hr/mg protein)	Aryl hydrocarbon hydroxylase (nmoles hydroxybenzo[*a*]pyrene/hr/mg protein)
Untreated (4)†	1.78 ± 0.20	0.70 ± 0.03	103.9 ± 6.9	568.0 ± 27.3	15.7 ± 2.3
Tricyclohexyltin (4)	7.18 ± 0.80‡	0.36 ± 0.03‡	59.9 ± 5.9‡	282.7 ± 17.9‡	14.5 ± 1.82
3-methylcholanthrene (4)	1.79 ± 0.34	1.14 ± 0.08	84.9 ± 9.8	536.8 ± 49.0	121.8 ± 6.2
Tricyclohexyltin +3-methylcholanthrene (8)	3.68 ± 0.51	1.11 ± 0.04	70.3 ± 5.4	505.6 ± 32.4	146.5 ± 7.7§

* Tricyclohexyltin hydroxide was administered s.c. in a single injection (15 mg/kg of body wt) followed by three daily i.p. injections of 3-methylcholanthrene (20 mg/kg body wt). Experimental details were the same as described in Table 2.

† Number in parentheses refers to animals per treatment group.

‡ $P < 0.01$ as compared with untreated controls.

§ $P < 0.01$ as compared with 3-methylcholanthrene treated.

Table 4. Effects of tricyclohexyltin hydroxide and β-naphthoflavone on heme oxygenase and drug-metabolizing activities in liver*

Treatment	Heme oxygenase (nmoles bilirubin/hr/mg protein)	Cytochrome P-450 (nmoles/mg protein)	Aniline hydroxylase (nmoles *p*-aminophenol/hr/mg protein)	Ethylmorphine *N*-demethylase (nmoles HCHO/hr/mg protein)	Aryl hydrocarbon hydroxylase (nmoles hydroxybenzo[*a*]pyrene/hr/mg protein)
Untreated (7)†	1.03 ± 0.09	0.76 ± 0.03	98.0 ± 5.7	586.2 ± 5.16	20.1 ± 3.5
TCHH (6)	4.82 ± 0.45‡	0.36 ± 0.02‡	48.1 ± 6.4‡	223.4 ± 46.2‡	13.8 ± 3.0
β-Naphthoflavone (6)	1.53 ± 0.23	1.07 ± 0.04	81.4 ± 4.3	359.6 ± 43.4	172.0 ± 21.0
β-Naphthoflavone + TCHH (10)	3.50 ± 0.36§	0.88 ± 0.05‖	52.2 ± 5.9§	283.6 ± 22.7	149.7 ± 13.3

* Tricyclohexyltin hydroxide was administered s.c. in a single injection (15 mg/kg of body wt) followed by three daily i.p. injections of β-naphthoflavone (40 mg/kg). Experimental details were the same as described in Table 2.
† Number in parentheses refers to number of animals in treatment groups.
‡ $P < 0.01$ as compared with untreated controls.
§ $P < 0.01$ as compared with β-naphthoflavone treated.
‖ $P < 0.02$ as compared with β-naphthoflavone treated.

by almost 4-fold in animals treated with tricyclohexyltin alone. Treatment with 3-methylcholanthrene had no effect on the activity of this enzyme. However, when 3-methylcholanthrene was administered simultaneously with tricyclohexyltin hydroxide, heme oxygenase activity was not elevated significantly when compared with the treatment group receiving 3-methylcholanthrene alone. There was no difference in the extent of induction of cytochrome P-448 concentration produced by 3-methylcholanthrene treatment alone (165% of controls), or when 3-methylcholanthrene was given simultaneously with tricyclohexyltin hydroxide (160% of controls). Similarly, the metabolism of aniline and ethylmorphine was not significantly different in these two treatment groups.

The activity of aryl hydrocarbon hydroxylase was differentially affected within the different treatment groups. Tricyclohexyltin hydroxide did not affect the activity of this cytochrome P-448-dependent hydroxylase system. 3-Methylcholanthrene treatment alone produced an approximate 8-fold increase in the enzyme activity; with concurrent treatment with tricyclohexyltin hydroxide there was an insignificant enhancement in the induction response (~9.5-fold).

A similar, though less pronounced pattern of enzyme induction was observed when β-naphthoflavone was administered concurrently with tricyclohexyltin hydroxide (Table 4). As was the case with 3-methylcholanthrene, β-naphthoflavone attenuated the induction response of heme oxygenase, though to a lesser extent, when administered concurrently with the organotin compound. However, β-naphthoflavone was somewhat less effective than 3-methylcholanthrene in preserving the concentration and functional activities of cytochrome P-450 when administered concurrently with the organotin. Aryl hydrocarbon hydroxylase activity was induced approximately 6.5-fold by β-naphthoflavone; simultaneous treatment with tricyclohexyltin hydroxide had little effect on this response.

Separation of microsomal proteins on SDS-polyacrylamide gel electrophoresis. Since the administration of tricyclohexyltin hydroxide to rats also receiving phenobarbital, 3-methylcholanthrene and β-naphthoflavone produced a differential effect on the concentration of microsomal cytochrome P-450 and related drug-metabolizing enzyme activities (Tables 1–4), we analyzed microsomal preparations by SDS-polyacrylamide gel electrophoresis (Figs. 3–5). The electrophoretic profile of molecular weight standards (well 1) and liver microsomal preparations from either untreated rats (well 2); or rats receiving phenobarbital (well 3); tricylcohexyltin hydroxide (well 4); or tricyclohexyltin hydroxide plus phenobarbital (well 5) are shown in Fig. 3. It has been shown by others [23, 24] that phenobarbital induces primarily the cytochrome P-450b species with a minimum molecular weight of 52,000. In our experiments microsomes from phenobarbital-treated rats (well 3) also showed that the cytochrome P-450b which was induced had the same minimum molecular weight. The intensity of this cytochrome P-450b band (showed by arrow in well 5) was reduced markedly by a single dose of the organotin administered con-

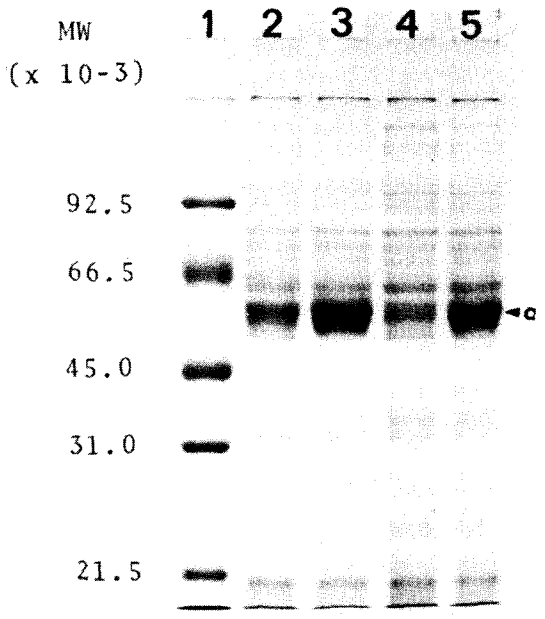

Fig. 3. SDS-polyacrylamide gel electrophoresis of rat hepatic microsomal fractions. Electrophoresis was performed as described in Materials and Methods. Well 1 contained protein standards (1 μg each) of known molecular weights: phosphorylase b (M_r = 92,500), bovine serum albumin (M_r = 66,500), ovalbumin (M_r = 45,000), carbonic anyhydrase (M_r = 31,000) and soybean trypsin inhibitor (M_r = 21,500). Wells 2–5 contained microsomal preparations (50 μg of protein) from untreated rats (well 2), phenobarbital- (well 3), tricyclohexyltin hydroxide- (well 4), and phenobarbital plus tricyclohexyltin hydroxide-treated rats (well 5).

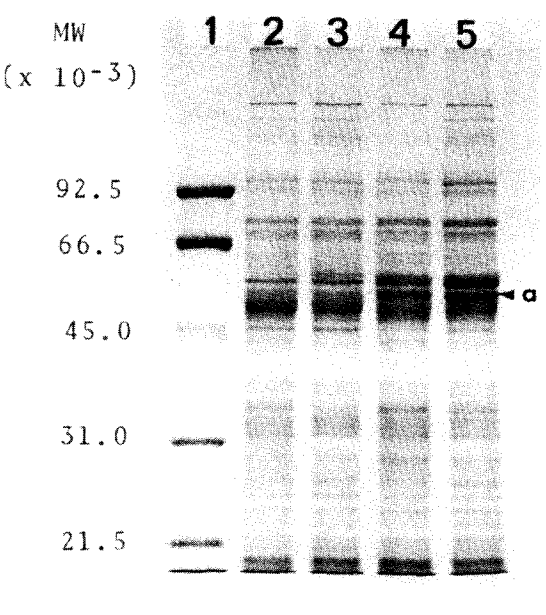

Fig. 4. SDS-polyacrylamide gel electrophoresis of rat hepatic microsomal membrane fractions: Effect of tricyclohexyltin treatment on 3-methylcholanthrene inducible proteins. Well 1 contained standards with molecular weights of 92,500, 66,500, 45,000, 31,000 and 21,500 (top to bottom). The microsomal preparations from control rats (well 2), tricyclohexyltin hydroxide- (well 3), 3-methylcholanthrene- (well 4), and 3-methylcholanthrene plus tricyclohexyltin hydroxide-treated rats (well 5) were electrophoresed as described in the text. The arrow indicates the position of cytochrome P-448 induced by 3-methylcholanthrene.

currently with phenobarbital. The interpretation of electrophoretic profiles of crude microsomal preparations may be complicated because some microsomal proteins other than cytochrome P-450 also have molecular weights in the range of 45,000–60,000 [25, 26]. The inducibility of the cytochrome P-450b band by phenobarbital has been widely studied and well characterized. Therefore, these gross changes reflected in the electrophoretic pattern produced by tricyclohexyltin hydroxide in phenobarbital-treated rats are readily apparent in this molecular weight region.

It is also known that treatment of rats with either 3-methylcholanthrene or β-naphthoflavone results in the induction of specific subspecies of cytochrome (P-450 (M_r = 56,000–59,000) which preferentially catalyze certain monooxygenase activities including aryl hydrocarbon hydroxylase, biphenyl-2-hydroxylase and 2-methyl-4-aminoazobenzene *N*-demethylase [27, 28]. Figures 4 and 5 show the effect of tricyclohexyltin hydroxide treatment on 3-methylcholanthrene and β-naphthoflavone inducible proteins, respectively, as observed on SDS-polyacrylamide gel electrophoresis. The protein staining band, indicated by the arrow (well 5), remained unaltered by the combined treatments with tricyclohexyltin hydroxide plus 3-methylcholanthrene (Fig. 4) and tricyclohexyltin hydroxide plus β-naphthoflavone (Fig. 5). This differential effect of the organotin on specific cytochrome P-450 species could be due to an altered stability or impaired induction response. It is possible that the organotin might also affect some minor species of cytochrome P-450 or other microsomal proteins in addition to the major inducible protein by the specific inducers of cytochrome P-450. To quantitate the effects of the organotin on individual cytochrome P-450 species, it is important to have antibodies to these hemeproteins.

Induction of cytochrome P-450 in untreated and tricyclohexyltin hydroxide-treated animals. Rats were depleted of hepatic cytochrome P-450 by prior treatment with tricyclohexyltin hydroxide (48 hr earlier) and were subsequently administered phenobarbital in a single injection (80 mg/kg). The pattern of recovery of cytochrome P-450 and intracellular heme and the activity of heme oxygenase were then examined over the following 3 days. As shown in Fig. 6, the mean concentration of cytochrome P-450 was doubled by phenobarbital (from ~0.70 nmole/mg to ~1.5 nmoles/mg) by 48 hr in control animals. However, in animals pretreated with the organotin, although the mean levels of cytochrome P-450 were also doubled from the initial chemically-altered set point (0.30 nmole/ml up to 0.75 nmole/mg) at 48 hr, the maximum extent of induction was still only one-half of that compared with animals treated with phenobarbital alone. Furthermore, there was a reciprocal relationship between the concentration of

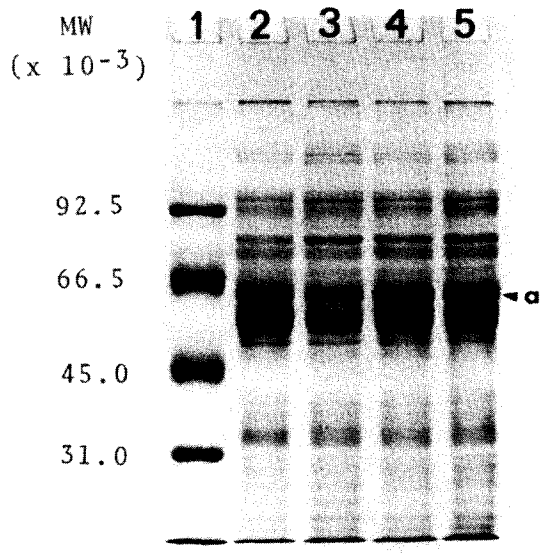

Fig. 5. SDS-polyacrylamide gel electrophoresis of rat hepatic microsomal membrane fractions from variously treated rats: Effect of tricyclohexyltin treatment on β-naphthoflavone inducible proteins. The molecular weight standards used are shown in well 1. The liver microsomal fractions were prepared and electrophoresed as described in the text. Key: control (well 2), tricyclohexyltin hydroxide treated (well 3), β-naphthoflavone treated (well 4) and β-naphthoflavone plus tricyclohexyltin hydroxide tested (well 5). The arrow indicates the position of the polypeptide induced by β-naphthoflavone.

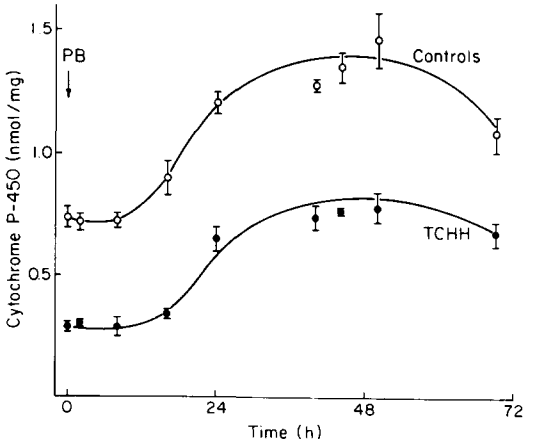

Fig. 6. Phenobarbital induction of hepatic cytochrome P-450 in untreated controls and tricyclohexyltin hydroxide (TCHH)-treated rats. Either saline or tricyclohexyltin hydroxide (15 mg/kg) was administered, s.c., 48 hr prior to a single i.p. injection of phenobarbital (80 mg/kg). Animals were killed at the times indicated, and hepatic microsomal frations were prepared and the cytochrome P-450 assay was performed as described under Materials and Methods. Key: (○) untreated controls; and (●) tricyclohexyltin hydroxide. Values are reported as the means ± S.E.M. of three to five individual animals.

intracellular hepatic heme and the activity of microsomal heme oxygenase (Table 5). At the time when phenobarbital treatment was initiated, heme oxygenase activity was already considerably elevated in animals that had earlier received tricyclohexyltin hydroxide (3- to 4-fold above controls). This enzymic activity remained elevated for ~40 hr following phenobarbital treatment, after which it gradually declined. Phenobarbital did not affect the activity of heme oxygenase in animals that had not received prior treatment with the organotin. The concentration of microsomal heme followed a pattern of change similar to that seen with cytochrome P-450 (Fig. 6). While heme content was greatly depressed in the tricyclohexyltin hydroxide treatment group prior to phenobarbital administration, the levels of heme rose quite rapidly, despite the elevated heme oxygenase activity, and reached a maximum concentration corresponding to that at the time points when cytochrome P-450 was maximally elevated (40–50 hr).

Table 5. Effects of phenobarbital on hepatic heme oxygenase activity and microsomal heme levels in control and tricyclohexyltin hydroxide-treated rats*

Time (hr)	Heme oxygenase (nmoles bilirubin formed/hr/mg protein)		Heme (nmoles/mg protein)	
	Controls	Tricyclohexyltin hydroxide	Controls	Tricyclohexyltin hydroxide
0	2.47 ± 0.16	8.51 ± 0.22	1.54 ± 0.06	0.96 ± 0.05
2	2.50 ± 0.16	7.53 ± 1.04	1.49 ± 0.05	0.90 ± 0.05
8	2.26 ± 0.10	10.29 ± 0.40	1.46 ± 0.04	0.87 ± 0.06
16	2.06 ± 0.04	8.92 ± 0.71	1.96 ± 0.17	0.96 ± 0.02
24	2.19 ± 0.16	7.81 ± 0.85	2.05 ± 0.06	1.33 ± 0.06
40	2.14 ± 0.28	6.01 ± 0.45	2.27 ± 0.18	1.47 ± 0.08
50	1.93 ± 0.13	5.79 ± 1.00	2.39 ± 0.07	1.39 ± 0.13
72	1.96 ± 0.10	5.62 ± 0.07	1.85 ± 0.01	1.31 ± 0.10

* Saline (controls) or tricyclohexyltin hydroxide (15 mg/kg) was administered s.c. in a single injection 48 hr prior to a single i.p. injection of phenobarbital (80 mg/kg). The animals were fasted 16 hr prior to sacrifice and were killed at the times indicated. Hepatic microsomal fractions were prepared and assays performed as described under Materials and Methods. Values are reported as the means ± S.E.M. of two separate experiments.

DISCUSSION

These studies describe the changes that occur in the rate-limiting enzymes of heme synthesis and degradation and cytochrome P-450 content and function in liver as a consequence of the metabolic interaction of tricyclohexyltin hydroxide, an important organotin biocidal agent, with phenobarbital, 3-methylcholanthrene, and β-naphthoflavone. The biochemical alterations that are produced in liver following simultaneous treatment with a combination of these chemicals result in induction responses of cytochrome P-450 that are intermediate between the levels that are produced by the administration of either agent alone, and these differences in response are also reflected in selected functional activities of the cytochrome P-450-dependent monooxygenase system. Our findings also raise the possibility of significant differences in the regulation of the induction response of the hemeprotein within the various subspecies of cytochrome P-450 produced by various chemicals or combinations of chemicals.

When phenobarbital was given in successive daily doses to organotin-treated rats, a single dose of tricyclohexyltin hydroxide was effective in preventing cytochrome P-450 from reaching the full extent of induction achieved in the absence of the organotin. This proved to be the case whether the organotin was administered simultaneously with phenobarbital (Fig. 1) or preceded by 48 hr the administration of a single dose of the drug (Fig. 6). The changes produced by tricyclohexyltin hydroxide are therefore long-lasting, and cannot be reversed by phenobarbital, at least through the time intervals examined.

Although ALA-synthase activity was elevated following simultaneous treatment with tricyclohexyltin and phenobarbital (Table 1), heme oxygenase activity was increased as well (Fig. 1). This enhanced rate of degradation of cellular heme could, in part, underlie the compromised induction of cytochrome P-450 by limiting the availability of newly synthesized heme for combination with apocytochrome P-450 to form the functional hemeprotein. However, a presumed limited availability of heme alone does not fully explain the inability of the liver to attain the expected inductive response to phenobarbital in the presence of the organotin since incubation of liver homogenates with heme did not result in the reconstitution of a CO-binding hemeprotein (data not shown). Furthermore, the results of SDS-polyacrylamide gel electrophoresis illustrate clearly that the apoprotein moieties of the various cytochrome P-450 subspecies were also affected to a considerable extent by treatment with tricyclohexyltin alone, and that staining in the relevant molecular weight region (50,000–52,000) was noticeably reduced even when phenobarbital was administered concurrently with the organotin. In contrast, tricyclohexyltin failed to decrease the 3-methylcholanthrene- or β-naphthoflavone-induced cytochrome P-450 subspecies (Figs. 3–5). Further studies to quantitate the specific changes in the isozymic profile of these crude microsomal preparations necessitate the use of immunoprecipitation techniques with specific antibodies.

The role of intracellular heme levels in the regulation of cytochrome P-450 apoprotein synthesis has been a subject of considerable investigation [29–34]. Induced levels of hepatic cytochrome P-450 can be produced and sustained in both normal and phenobarbital-treated rats with only a transient, albeit pronounced, elevation of ALA-synthase activity [30]. However, a direct relationship between decreased cytochrome P-450 synthesis and reduced intracellular heme levels resulting from elevated heme oxygenase activity has not been clearly shown. It is possible that newly synthesized heme that would normally be destined for assembly with apocytochrome P-450 in the endoplasmic reticulum is instead subject to degradation by heme oxygenase in these same membranes. In this circumstance, even though heme is produced at an accelerated rate as a result of enhanced ALA-synthease activity, a competition between heme oxygenase and apocytochrome P-450 for the newly synthesized heme could prevent the full extent of hemeprotein induction from occurring. Granick *et al.* [35] have proposed a heme metabolic scheme suggesting the relative affinities of the various cellular constituents and hemeproteins for the postulated "free heme" pool. Although the binding constant for microsomal heme oxygenase is $\sim 5 \times 10^{-6}$ M, while apocytochrome P-450 has a binding constant of $\sim 10^{-13}$ to 10^{-9} M for heme, it is possible that induced heme oxygenase might exist in a topographical configuration within the membrane such as to afford a higher degree of competition for the available heme than these figures might imply. Furthermore, what effects, if any, this perturbed heme pool might have on the synthesis and degradation of the cytochrome P-450 apoprotein are presently not known. However, it has been reported recently by Muller-Eberhard and coworkers [36] that destruction of the cytochrome P-450 heme can lead to an accelerated turnover of the apoprotein moiety *in vivo*. Therefore, the enhanced degradation of intracellular heme caused by organotin-induced heme oxygenase activity could indirectly modulate the levels of cytochrome P-450 apoprotein by limiting heme availability through this mechanism.

Although 3-methylcholanthrene and β-naphthoflavone both reportedly induce identical subspecies of cytochrome P-450 in rat liver [37], 3-methylcholanthrene was a more effective inducing agent in the presence of tricyclohexyltin. Heme oxygenase induction by tricyclohexyltin was considerably lower in the presence of 3-methylcholanthrene, and to some extent with β-naphthoflavone, suggesting that these inducers of cytochrome P-448 either affect heme oxygenase directly or produce a utilization of heme such that the full extent of induction of this enzyme is not realized. These possibilities are currently under investigation. The activities of aniline hydroxylase and ethylmorphine *N*-demethylase were not induced by 3-methylcholanthrene and β-naphthoflavone; however, the activity of cytochrome P-448-dependent aryl hydrocarbon hydroxylase was substantially elevated, even in the presence of tricyclohexyltin, suggesting that the induction of this form of the hemeprotein is less influenced by the organotin than is the phenobarbital-induced form.

There are many other examples of metabolic interactions that occur among environmental chemicals and drugs (see review, Conney and Burns [38]). Studies on the effects of various inorganic metals, such as lead [39], cadmium [40] and cobalt [41–43] on the induction of cytochrome P-450 by phenobarbital and 3-methylcholanthrene have revealed a type of response similar to that described in the present report. In fact, inorganic cobalt has been used in conjuction with phenobarbital and 3-methylcholanthrene as a probe to dissociate the levels of apoprotein from heme synthesis in order to greatly elevate the levels of the apoprotein moiety [32].

The potential of tricyclohexyltin to modify the inducibility of cytochrome P-450 (P-448) by phenobarbital, 3-methylcholanthrene, and β-naphthoflavone is of considerable toxicological importance. The widespread agricultural applications of trialkyltin biocidal agents have greatly increased the relative exposure risks associated with worker handling of these materials as well as the risks of incidental exposure of the general population as a result of their ubiquitous environmental presence. Given such potential for exposure, the subsequent alterations in the response of the hepatic monooxygenase system to drug and chemical inducers necessitates a clearer understanding of how the heme metabolic pathway responds to such complex chemical challenges.

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