Effects of Diethyl Phthalate and Other Plasticizers on Laurate Hydroxylation in Rat Liver Microsomes

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Diethyl phthalate (DEP) is used in pharmaceutical coatings, cosmetics, and plastic films to wrap foods. There is a health concern associated with the exposure to certain phthalate esters because they belong to a class of compounds referred to as peroxisome proliferators which have been shown to increase the incidence of liver tumors when administered to rats. In this study, we have compared DEP to four other commonly used plasticizers, 2-diethylhexyl phthalate (DEHP), dibutyl phthalate (DBP), 2-diethylhexyl adipate (DEHA), and acetyltributyl citrate (ATBC), for their ability to induce the cytochrome P450-mediated fatty acid ω-hydroxylation system, which is one of the initial cellular responses when animals are treated with peroxisome proliferators. The administration of DEHP, DBP, and DEHA to rats increased the specific activity of laurate 12-hydroxylase from 2.8 ± 1.1 in control rats to 30.3 ± 11.6, 14.5 ± 4.1, and 9.7 ± 1.9 nmol 12-hydroxylaurate formed/min/nmol P450, respectively. In contrast, laurate 12-hydroxylase activity in DEP- and ATBC-treated rats were 4.4 ± 1.2 and 4.4 ± 1.0 nmol 12-hydroxylaurate formed/min/nmol P450, respectively. In addition, whereas DEHP increased peroxisomal palmitoyl-CoA oxidation 6-fold, DEP increased this activity only 1.3-fold. Two protein bands, at 51 and 52 kDa, were found to increase 6- to 12-fold in microsomes of DEHP-, DBP-, and DEHA-treated rats, but these bands were increased only 2-fold in DEP- or ATBC-treated rats.

KEY WORDS: diethyl phthalate; plasticizers; P450; laurate hydroxylation.

INTRODUCTION

A widely used plasticizer, 2-diethylhexyl phthalate (DEHP), is a potent inducer of peroxisomes and a cytochrome P450 form which catalyzes fatty acid omega (ω)-hydroxylation (1). A health concern is associated with exposure to DEHP because long-term feeding studies were found to increase the incidence of hepatic tumors in rodents (2). A number of structurally diverse chemicals including the hypolipidemic drugs, clofibrate and ciprofibrate, also have the ability to proliferate hepatic peroxisomes, to induce the peroxisomal palmitoyl-CoA oxidase reaction, and to increase the incidence of hepatic tumors (2,3). These peroxisome proliferators also have the capacity to induce certain cytochromes P450 of the CYP4A[3] family which are unique monooxygenases that catalyze the hydroxylation of fatty acids at the ω-carbon atom (3). It has been proposed that fatty acid ω-oxidation products generated by this P450-mediated reaction may serve as the actual inducing agents of peroxisomes (4). Arachidonic acid is also ω-oxidized by this unique P450 system and recent reports have indicated that ω-oxidation products of arachidonic acid may act as physiological regulators of cells (5,6). Thus exposure to agents which may induce fatty acid ω-hydroxylase activity in cells may cause alterations of physiological processes.

A fatty acid ω-hydroxylase, designated P450 4A1, was purified from the microsomes of rats treated with clofibrate (7), its cDNA sequenced (8), and its genomic structure characterized (9). P450 4A1 represents approximately 1 to 4% of the total microsomal P450s in control rats, and following exposure to clofibrate, this P450 or related isoforms increased to 16 to 30% of the total microsomal P450s (10,11). In kidney microsomes, P450 4A forms constitute 34% of the P450s present and a two- to threefold increase may occur in these isoforms following administration of clofibrate (11). DEHP also induces fatty acid ω-hydroxylase activity in liver microsomes of rats (12) and a polyclonal antibody made to the clofibrate-inducible P450 cross-reacts with this P450 protein (8). A P450 from DEHP-treated rats has been purified and found to have similar spectral, molecular mass, and substrate properties with the P450 isolated from clofibrate-treated rats (13).

In this study, we were interested in determining whether diethyl phthalate (DEP) had the capacity to induce the fatty acid ω-hydroxylation reaction, because in addition to its presence in plastic food wrappers, DEP is contained in cosmetic and pharmaceutical preparations (14). Desiccant packages also contain DEP, and there is one report which found that DEP migrated from its package in a bottle containing the drug levothyroxine and contaminated the tablets (15). DEP also is found in a commercial coating for pharmaceutical preparations (14). In previous studies, DEP was reported not to have the toxic effects of DEHP (14) and did not proliferate peroxisomes (16). In this study, DEP was compared with other plasticizers which are known to induce the laurate hydroxylation system. In addition to DEP and DEHP, the compounds studied were di-(2-ethylhexyl)-adipate (DEHA), dibutyl phthalate (DBP), and acetyltributyl citrate (ATBC), whose structures are shown in Fig. 1.

MATERIALS AND METHODS

Materials

Male Sprague–Dawley rats (125–150 g) were purchased from the Laboratory Animal Resource Center at Washington State University. ATBC, DBP, DEHP, and DEP were purchased from Aldrich Chemicals (Milwaukee, WI). DEHA was purchased from American Tokyo Kasei (Atlanta, GA). Sodium laurate, palmitoyl-CoA, flavin adenine dinucleotide, cyanide, Triton X-100, and NAD^+ were purchased from Sigma Chemical (St. Louis, MO). Radioactive lauric acid (1-^{14}C-laurate) was purchased from Amersham Inc. (Arlington Heights, IL). NADPH was purchased from Boehringer-Mannheim (Indianapolis, IN). Hain corn or safflower oil,

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[3] The nomenclature of the cytochrome P-450 system which catalyzes fatty acid hydroxylation is taken from "The P-450 superfamily: Update on new sequences, gene mapping and recommended nomenclature," published in *DNA*, Vol. 10, pp. 1–14, 1991.

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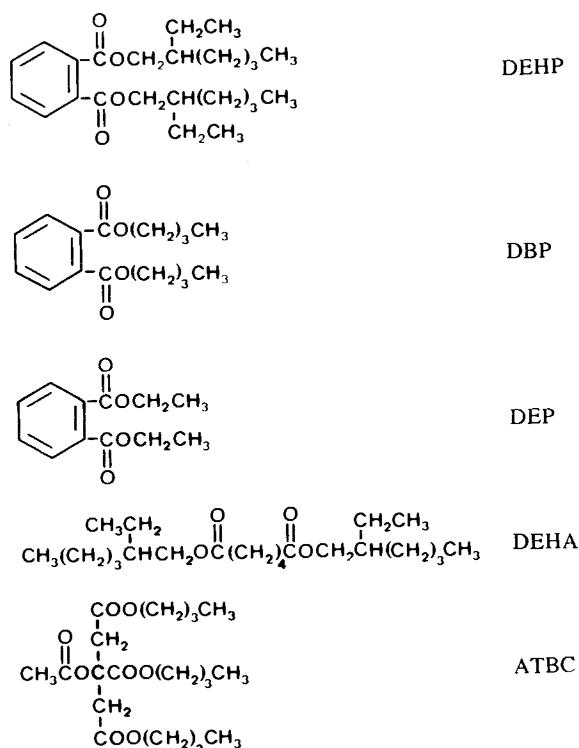

Fig. 1. Structures of the different plasticizers used to induce laurate hydroxylase activity.

which were packaged in glass containers, were used as carriers to administer the plasticizers. These oils were purchased from local grocery stores. All other chemicals were of the highest purity commercially available.

Methods

Based on studies by Barber *et al.* (1), rats were administered the plasticizers in corn oil or safflower oil by intubation using an animal feeding needle (Popper and Sons, Inc., New Hyde Park, NY) over a 3-day period at a dose of 3 mmol/kg body weight/day for DEHP, DBP, and DEHA. The doses for DEP and ATBC were 5.4 and 6.2 mmol/kg body weight/day for 3 days, respectively. Experiments with DEHP demonstrated that at a dose of 3 mmol/kg/day, induction of laurate hydroxylase activity could be detected as early as 1 day after treatment, and by the third day the extent of induction of the laurate hydroxylase activity was similar to administering the DEHP in the diet at 2% (w/w) for 2 weeks. Control rats were administered the same dose of oil (1 ml/kg body weight/day) without plasticizers. The corn oil and safflower oil were examined for the presence of peroxides and only peroxide-free oil was used to dilute the compounds. Livers of rats were worked up individually and microsomes prepared by the method of Okita *et al.* (17) and were frozen immediately after their preparation. Laurate hydroxylase activities were determined within 1 month of microsome preparation. There were no significant differences in rates of laurate hydroxylation between freshly prepared microsomes and frozen microsomes maintained at −70°C. Since it has been reported that laurate hydroxylase activity may decrease upon storage at 4°C (18), microsomes were frozen immediately after their preparation. Laurate hydroxylation reactions and detection of ω- and (ω-1)-hydroxy products were performed by the method of Okita *et al.* (19). Activities were determined in microsomes of individual rats and the values are reported as nanomoles of hydroxylated product formed per minute per nanomole of total microsomal P450. Statistical analyses were performed by Student's *t* test. Cytochrome P450 concentrations were determined by the procedure of Estabrook and Werringloer (20) and NADPH-cytochrome *c* reductase assays were performed by the procedure of Masters *et al.* (21). Protein determinations were performed by the procedure of Lowry *et al.* (22). Peroxisomal palmitoyl-CoA oxidation was performed as described by Lazarow (23). A polyclonal antibody was prepared in rabbits to the purified rat P450 isolated from DEHP-treated rats and was used in western blot analyses (13). This antibody was observed to recognize two major protein bands in microsomes of control and DEHP-treated rats similar to the reports of Hardwick *et al.* (8) and Sharma *et al.* (11). To compare the increase in immunochemically reactive P450 protein by the different plasticizers, solubilized reduced microsomal protein samples were applied to a Schleicher and Schuell (Keene, NH) microsample filtration manifold system according to the manufacturer's instruction manual using a range of protein values from 0.5 to 10 μg. The dot blots were visualized by the same procedure used for western blot analysis and staining intensities measured and quantitated by video densitometry.

RESULTS

DEP was found to have only a minor inductive effect on microsomal laurate 12-hydroxylase activity, whereas DEHP, DBP, and DEHA were found to have significant effects on the microsomal laurate 12-hydroxylase activity (Table I). DEHP demonstrated the greatest induction of laurate 12-hydroxylase activity, increasing the specific activity from 2.8 ± 1.1 nmol 12-hydroxylaurate formed/min/nmol total P450 in untreated rats to 30.3 ± 11.6 nmol/min/nmol total P450 (Table I), a 10.8-fold increase (significance, $p < 0.001$). There was considerable range in values for the laurate 12-hydroxylase activity in individual rats following DEHP treat-

Table I. Effects of Various Plasticizers on Laurate 11- and 12-Hydroxylation Activities

Treatment regimen	Hydroxy-laurate[a]	
	11-OH	12-OH
Control (n = 7)[b]	4.1 ± 1.6[c]	2.8 ± 1.1
DEHP (n = 8)	7.4 ± 2.9	30.3 ± 11.6
DBP (n = 6)	5.6 ± 1.1	14.5 ± 4.1
DEHA (n = 6)	4.7 ± 1.3	9.7 ± 1.9
DEP (n = 5)	4.6 ± 0.9	4.4 ± 1.2
ATBC (n = 5)	5.2 ± 1.9	4.4 ± 2.0

[a] Results are expressed as nanomoles of product formed per minute per nanomole of total microsomal P450.
[b] Number of rats tested.
[c] Values represent mean ± standard deviation.

ment and the recorded activities ranged between 16.5 to 46.4 nmol product/min/nmol total P450. DBP and DEHA administration induced laurate 12-hydroxylase activity 5.2- and 3.5-fold, respectively (significance levels for both compounds were $P < 0.001$), whereas DEP and ATBC increased laurate 12-hydroxylase activity only 1.6-fold (Table I). This increase in activity was not statistically significant. Laurate 11-hydroxylase was increased 1.8-fold by DEHP ($P < 0.05$), but the other compounds tested had no significant inductive effect on the 11-hydroxylase activity (Table I).

The effect of plasticizer treatment on two microsomal electron transport proteins was also examined and NADPH-cytochrome *c* reductase activities and P450 contents are shown in Table II. NADPH-cytochrome *c* reductase activity was increased 1.7-fold by DEHP treatment and 1.4-fold by DBP treatment, but the other compounds had no significant effect on reductase activity. Although none of the compounds tested was found to have a significant effect on the specific content of total P450 as determined by the absorbance spectrum of the dithionite reduced-carbon monoxide bound protein, western blot analysis using an antibody to purified P450 from DEHP-treated rats demonstrated two major protein bands at 52 and 51 kDa in liver microsomes from control and plasticizer-treated rats (Fig. 2, bands a and b, respectively). The purified P450 from DEHP-treated rats migrated with a molecular mass of 51 kDa, which corresponds to band b (data not shown). A third protein band (Fig. 2, band c) of approximately 50 kDa was also observed in western blots, but the identity of this protein has not been elucidated. To quantify the amount of immunochemically reactive P450 proteins present in the microsomes, dot blots were performed (data not shown) and the relative staining intensities quantitated by video densitometry. A comparison of the relative amounts of immunochemically reactive P450 protein with its corresponding laurate 12-hydroxylase activity is shown in Fig. 3. The microsomal P450 protein in DEHP-treated rats increased 12.5-fold compared to control rats and the laurate 12-hydroxylase activity in this microsomal preparation increased 6.5-fold. In DBP- and DEHA-treated rats, P450 contents increased 8.3- and 6.5-fold, respectively, with laurate 12-hydroxylase activity increasing 4.8-fold and 3.2-fold in DBP- and DEHA-treated rats, respectively. In contrast to the significant increases in DEHP-, DBP-, and DEHA-treated rats, twofold increases in immunochemically reactive P450 and laurate 12-hydroxylase specific activities were observed in DEP- and ATBC-treated rats.

Table II. NADPH-Cytochrome *c* Reductase Specific Activity and P450 Specific Contents of Microsomes from Rats Treated with Various Plasticizers

Treatment	NADPH-cytochrome *c* reductase (nmol/min/mg)	P450 (nmol/mg)
Control (n = 6)[a]	77.5 ± 15.2[b]	0.85 ± 0.17
DEHP (n = 5)	129.7 ± 14.4	0.87 ± 0.20
DBP (n = 6)	107.5 ± 4.5	0.73 ± 0.10
DEHA (n = 6)	87.4 ± 16.6	0.80 ± 0.16
DEP (n = 3)	101.7 ± 10.0	0.95 ± 0.11
ATBC (n = 3)	83.6 ± 12.6	0.99 ± 0.10

[a] Number of rats tested.

[b] The values are expressed as mean ± standard deviation.

Since DEP was a much weaker inducer of laurate 12-hydroxylase activity than DEHP, the effect of DEP-treatment on peroxisomal palmitoyl-CoA oxidase activity was also examined. As shown in Fig. 4, the cyanide-insensitive palmitoyl-CoA oxidation increased 6-fold in DEHP-treated rats, whereas only a 1.3-fold increase was observed following DEP treatment, demonstrating the two phthalate esters produced differential effects on this enzymatic activity.

DISCUSSION

The biological effects of plasticizers have been a concern for a number of years after reports that DEHP could leach out of blood storage bags and be transferred to the recipient of blood transfusions (24). Plasticizers also may leach out of plastic films which are used as food wrappers, and studies by Castle *et al.* (25,26) have described the contamination of foods with various plasticizers. There is very limited evidence of any adverse effects which are associated with phthalate ester contamination in humans (14); however, there is concern over the long-term exposure to these agents, since chemicals which proliferate peroxisomes are reported to increase the incidence of hepatic tumors by a nongenotoxic mechanism in rodents (2,3,27) and changes in fatty acid ω-hydroxylase activities may alter renal vascular physiology (5,6). The specific activity of fatty acid ω-hydroxylation is also increased by peroxisome proliferators and at least three P450s have been reported to increase following clofibrate induction (9). P450 4A1 has been purified from liver microsomes from clofibrate-treated rats and its properties have been characterized (7–12). A similar P450 was purified from DEHP-treated rats, but it did exhibit unique amino acid residues in a N-terminal amino acid segment compared to the clofibrate form (13).

The potential toxic effects of DEHP and other phthalates led manufacturers to substitute other plasticizers in their products. The plasticizers which are found in plastic film wraps for food include DBP and DEP as well as the nonphthalate compounds, ATBC and DEHA (14,25,26). ATBC and DEP are used as plasticizers in polyvinylidene chloride and cellulose acetate (25,26). DEP is also found in a variety of cosmetics and in the coating of pharmaceutical preparations (14).

Studies by Barber *et al.* (1) reported the increase in laurate hydroxylation and peroxisomal palmitoyl-CoA oxidation when rats were administered DEHP, DBP, or DEHA. In this study we have compared the inductive effects of five plasticizers on the P450-dependent laurate 12-hydroxylase activity. This enzyme reaction was previously reported to be induced by DEHP treatment (1,12) and in the present study a 10.8-fold increase in specific activity was observed in rats treated with DEHP. Treatment of rats with DBP and DEHA also induced laurate 12-hydroxylase activity 5.2- and 3.5-fold, respectively. Neither DEP or ATBC had a significant effect on laurate 12-hydroxylation rates. NADPH-

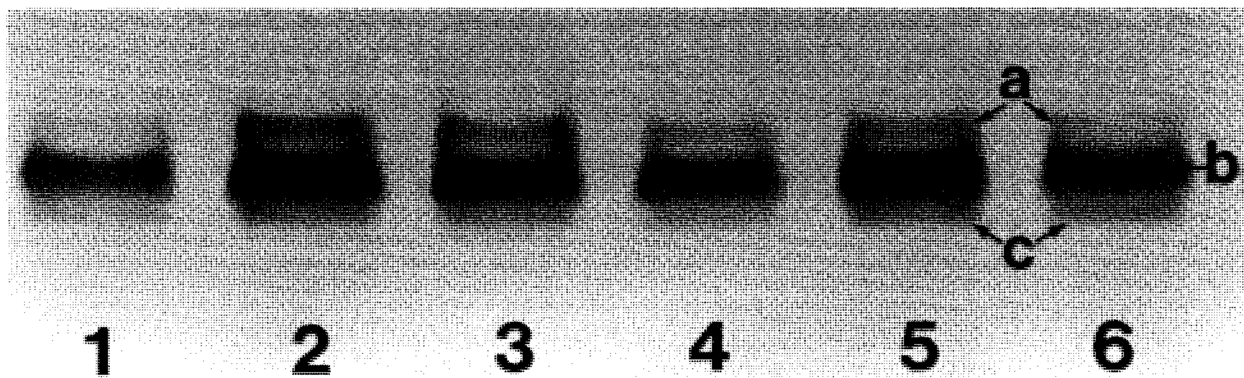

Fig. 2. Western blot of liver microsomes from rats treated with various plasticizers. Two P450 protein bands (a and b) were detected by an antibody made to the purified P450 from DEHP-treated rats. Band b corresponds to the P450 which was isolated from DEHP-treated rats. Lane 1, control; Lane 2, DEHP; Lane 3, DBP; Lane 4, DEP; Lane 5, DEHA; Lane 6, ATBC. The amount of protein applied to each lane was 5 μg.

cytochrome *c* reductase activity increased 1.7-fold with DEHP, which is in agreement with a previous study for this plasticizer (12). Although total P450 content does not increase or increases only slightly following DEHP treatment, specific P450 4A forms which catalyze fatty acid hydroxylation reactions are increased by administration of peroxisome proliferators (7,8,10). The western blot shown in Fig. 2 is similar to those of clofibrate-treated rats, which demonstrate two protein bands of greater intensity, at 51 and 52 kDa, than in control microsomes (8,11). A third immunochemically reactive protein band (Fig. 2, band c) is also visible in this western blot but this protein has not been identified. A third member of the P450 4A family (P450 4A2) has been identified in rat liver which is very similar in its sequence to the 52-kDa protein (P450 4A3), but whether protein band c is P450 4A2 is unlikely because of its lower M_r value. Administration of DEHP, DBP, and DEHA increased the immunochemically reactive P450 12.5-, 8.3-, and 6.5-fold, respectively, but only 2.3- and 2.4-fold increases were observed in DEP- and ATBC-treated rats. DEP was also found to be a poor inducer of peroxisomal palmitoyl-CoA oxidation, increasing the specific activity only 1.3-fold in comparison to the 6-fold increase following DEHP treatment.

This study demonstrates that DEP, unlike other plasticizers such as DEHP, DBP, and DEHA, has only minor inductive effects on P450 4A forms which catalyze fatty acid ω-hydroxylation reactions. Lake *et al.* (28) reported that if the monoethyl derivative of phthalate was added to freshly isolated rat hepatocytes and cultured for 70 hr, total laurate hydroxylation increased 1.6-fold, but in this study the ω- and (ω-1)-hydroxy products were not separated. DEP treatment

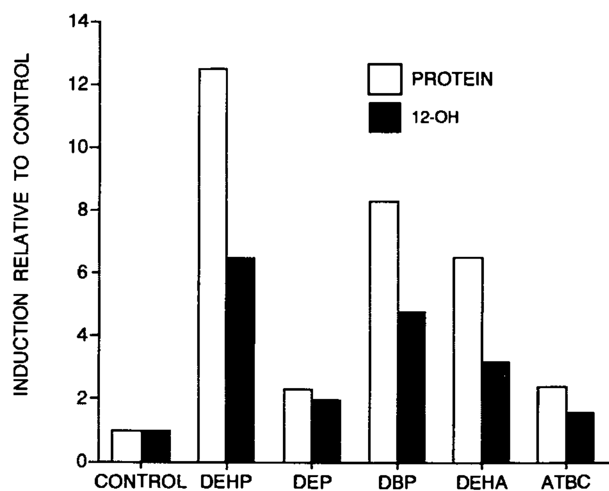

Fig. 3. Comparison of the amounts of immunochemically reactive P450 and its corresponding laurate 12-hydroxylase activity in individual rats. The P450 amounts (protein) and laurate 12-hydroxylase activities (12-OH) are expressed as fold increases of the values obtained from control rats. Laurate 12-hydroxylase activities were performed on the same microsomal preparations which were used to measure P450 amounts. The values represent the average of duplicate (protein) or triplicate (12-OH) assays.

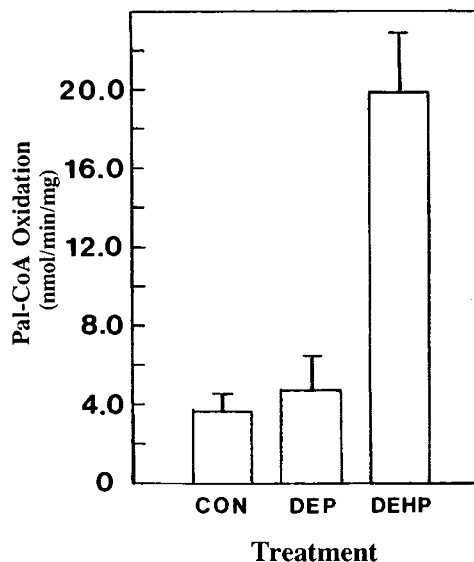

Fig. 4. The specific activities of cyanide-insensitive palmitoyl-CoA oxidation observed in liver homogenates from control, DEHP-treated, and DEP-treated rats. The specific activity of palmitoyl-CoA oxidation is reported as nmol NADH formed/min/mg protein.

also does not increase liver weight or peroxisome-associated catalase and carnitine acetyltransferase activities (16).

It is not certain why differences exist among the phthalate plasticizers to proliferate peroxisomes, induce the 4A family of P450s, or cause liver cells to undergo hyperplasia. It has been proposed that ω-oxidized fatty acids may act as the putative proliferators of peroxisomes (4) and the failure of DEP to induce fatty acid ω-hydroxylation may account for this plasticizer's inability to proliferate peroxisomes. The mechanism of action by phthalates on microsomal and peroxisomal enzymes is currently under investigation and recent studies by Isselmann and Green (29) have identified a putative receptor for compounds which serve as peroxisome proliferators. Whether peroxisome proliferators exert their hypolipidemic effects or induce tumor formation through the action of this receptor is unknown. The inability of DEP to induce laurate hydroxylation may reflect a poor interaction between this compound and the putative peroxisomal proliferator activated receptor. Whether larger concentrations of DEP or longer administration times would have induced this activity was not investigated. In the present study, conditions which were optimal to induce laurate ω-hydroxylation by DEHP (1200 mg/kg body weight/day for 3 days) were used to administer each of the various plasticizers. Based on reported values of DEP contamination of foods wrapped in plastic film or by other materials used in food packaging, the total daily exposure to DEP was calculated by Kamrin and Mayor (14) to be approximately 4 mg and they concluded the probability of human contamination with this plasticizer from food products and from cosmetics and pharmaceutical agents would be minimal. However, low concentrations of DEP have been detected in fish caught from the Pacific Ocean and the Sacramento-San Joaquin Delta in the range of 84 to 184 μg/g tissue, indicating that this compound has become widely dispersed in the environment and a contaminant of living organisms (30). The present study demonstrates that DEP has only minor inductive effects on the P450-dependent laurate ω-hydroxylation and peroxisomal palmitoyl CoA oxidation reactions and does not share similar inductive properties with other phthalate plasticizers such as DEHP or DBP.

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