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Phthalate Esters as Peroxisome Proliferator Carcinogens

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The phthalate ester di(2-ethylhexyl) phthalate is both a peroxisome proliferator and a hepatic carcinogen. Peroxisome proliferators as a class are hepatocarcinogenic in rodent species. However, none of the peroxisome proliferators tested to date including the phthalate esters and related alcohol and acid analogs have demonstrated mutagenic or DNA-damaging activity in the *in vitro Salmonella typhimurium*/microsomal or the lymphocyte ^{3}H-thymidine assays. A working hypothesis is proposed that peroxisome proliferation itself initiates neoplastic transformation of hepatic parenchymal cells by increasing intracellular rates of DNA-damaging reactive oxygen production. Evidence which supports such a hypothesis includes increased fatty acid β-oxidation, elevated H_2O_2 levels, accumulation of peroxidized lipofuscin, disproportionately small increase in catalase, and elevated peroxisomal uricase activity which accompany peroxisome proliferation in hepatocytes. Direct testing of this hypothesis will provide insight into mechanisms of phthalate ester carcinogenicity and cytotoxicity.

A xenobiotic which induces the proliferation of peroxisomes in liver cells is designated a peroxisome proliferator. Peroxisomes (microbodies) are single membrane-limited cytoplasmic organelles which functionally have been implicated in gluconeogenesis, lipid metabolism, and the detoxification of H_2O_2. The hypolipidemic agent clofibrate was the first xenobiotic to be identified as a peroxisome proliferator (*1*, *2*). Subsequently a large number of other xenobiotics have been discovered to be peroxisome proliferators, including structural analogs of clofibrate (nafenopin, methyl cofenapate, SaH-42, 348, and gemfibrozil), hypolipidemic drugs structurally unrelated to clofibrate (tibric acid, Wy-14,643, BR-931), and a group of miscellaneous compounds which were not developed for pharmacological utilization as hypolipidemic drugs but were incidentally discovered to express activity as peroxisome proliferators (*3*, *4*). The industrial plasticizer di(2-ethylhexyl) phthalate (DEHP) and related plasticizers, including di(2-ethylhexyl) adipate, are important members of the miscellaneous peroxisome proliferators group (Fig. 1). All peroxisome proliferators studied to date, including DEHP, have been observed to induce hepatomegaly and hypotriglyceridemia when fed to rodents (*5*, *6*).

Sustained peroxisome proliferation in rodent liver has been associated with an increased appearance of hepatocellular carcinoma. Peroxisome proliferators which have demonstrated hepatocarcinogenicity in rodents include nafenopin (*7*), Wy-14,643 (*8*), clofibrate (*9*), BR-931 and tibric acid (*10*). The development of liver tumors in animals fed these five structurally diverse hypolipidemic drugs led to the conclusion that peroxisome proliferators as a class are carcinogenic (*10*). Since the formulation of this hypothesis, the industrial plasticizer DEHP has been observed to induce hepatocellular neoplasms in both F-344 rats and B6C3F1 mice (*11*). The reported carcinogenicity of DEHP in rodents is particularly disturbing, since DEHP is a common environmental contaminant (*12*, *13*) and it is probable that human exposure to DEHP has been extensive. Our laboratories are currently investigating cellular/molecular mechanisms involved in peroxisome proliferator carcinogenesis, including DEHP and related plasticizers. In this report, possible mechanisms of peroxisome proliferator carcinogenesis based on current knowledge of the properties of these xenobiotics will be reviewed.

Current models of carcinogenesis indicate both an initiation and promotion phase in the formation

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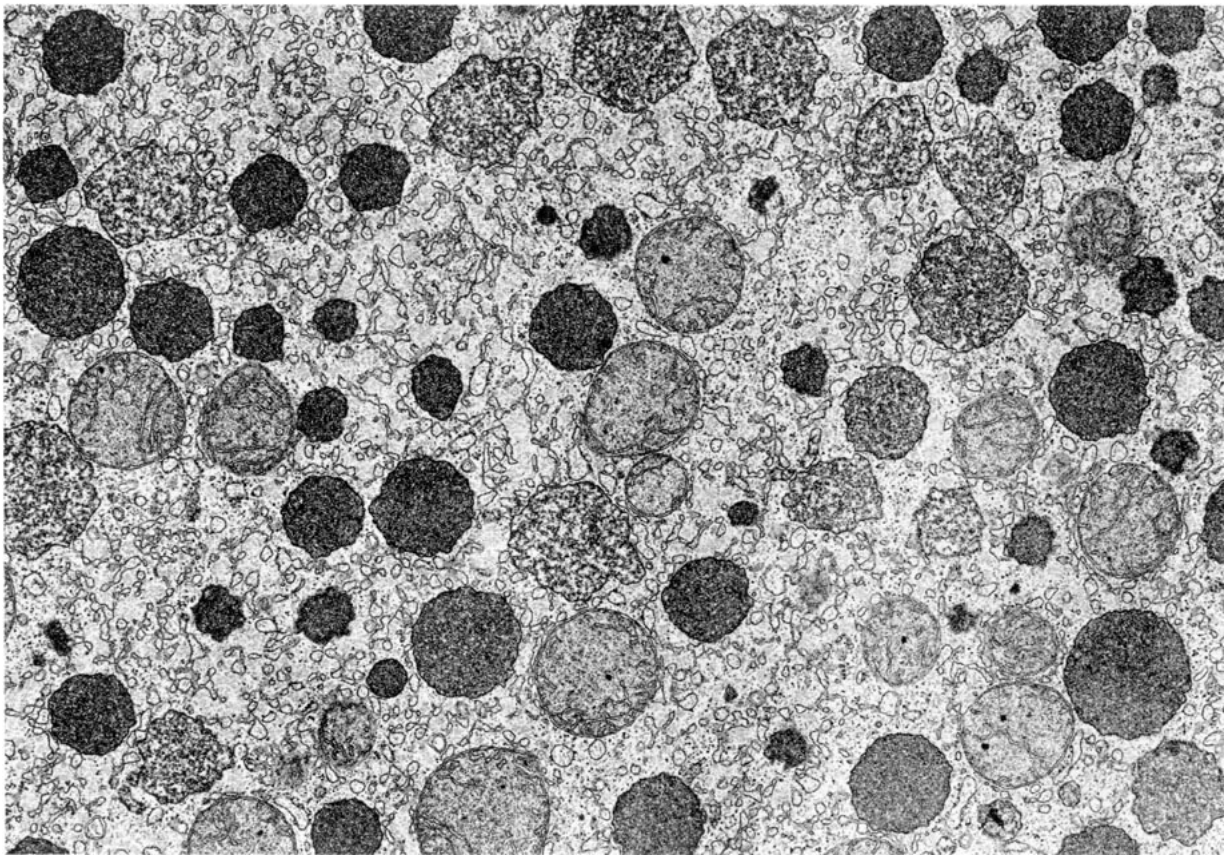

FIGURE 1. Proliferation of peroxisomes induced in F-344 rat liver cell cytoplasm by DEHP. 18,000×.

of neoplasms (*14*). Initiation of cells for neoplastic transformation is irreversible and requires interactions of nucleophilic centers in cellular DNA with electrophilic regions of chemical carcinogens or carcinogen metabolites (*14*). An estimate of the electrophilic reactivity of carcinogens can be obtained by mutagenicity measurements. We have tested five different hypolipidemic drugs (clofibrate, nafenopin, SaH-42-348, Wy-14,643 and BR-931), both structurally related and structurally unrelated to clofibrate, for *in vitro* mutagenic activity against each of the different *Salmonella typhimurium* test strains TA98, TA100, TA1535, TA1537 and TA1538. The hypolipidemic drugs, whether tested without or with rat liver S-9 microsomal preparation, were uniformly negative as mutagens in the Ames *S. typhimurium* assay (*15*). Our results have been extended by de la Iglesia and his co-workers (*16*), who reported that the hypolipidemic drug gemfibrozil or five *in vivo* metabolites of gemfibrozil isolated from rat urine were without mutagenic activity toward the same *S. typhimurium* tester strains utilized by us. Gemfibrozil is both a peroxisome proliferator and hepatocarcinogen in rats (*5*). Also, methyl clofenapate in very recent work was not mutagenic in the Salmonella/microsome assay with tester strains TA98 and TA100 (*17*). We have also utilized the lymphocyte ^{3}H-thymidine assay to test the five hypolipidemic drugs found by us to be negative in the Ames assay plus tibric acid for electrophilic reactivity. The hypolipidemic agents, tested either with or without rat liver S-9 microsomal preparation, resembled nonmutagenic drugs and not mutagenic carcinogens in the lymphocyte ^{3}H-thymidine assay (*15*). These data indicate, therefore, that the hypolipidemic agents fail to behave as electrophilic reactants capable of covalent interaction with cellular nucleic acid.

In preliminary work, we have tested plasticizers and related compounds for reactivity in both the Salmonella/microsome and the lymphocyte ^{3}H-thymidine assay. A dose-dependent increase in the number of His^+ revertant colonies was not observed over a wide concentration range of the plasticizers DEHP and di(2-ethylhexyl) adipate (DEHA) when tested in the absence or presence of rat liver S-9 microsomal mixture with *S. typhimurium* strains TA98 and TA100 (Table 1). The related compounds,

Table 1. *In vitro* assay of plasticizers with *Salmonella typhimurium* strains.[a]

		His$^+$ revertants/plate[b]			
		TA98		TA100	
Compounds	Concentration, *M*	–S-9	+S-9	–S-9	+S-9
DEHP	10^{-2}	27	24	105	106
	10^{-4}	28	21	95	97
	10^{-6}	33	20	111	90
DEHA	10^{-2}	32	27	125	109
	10^{-4}	28	37	110	88
	10^{-6}	20	35	93	108
Ethyl-2-hexanol	10^{-2}	22	24	98	96
	10^{-3}	26	12	105	90
	10^{-4}	23	18	109	108
Ethylhexanoic acid	10^{-2}	22	18	91	90
	10^{-4}	35	21	101	94
	10^{-6}	21	27	103	122

[a]*S. typhimurium* TA98 and TA100 were incubated in the absence or presence of S-9 microsomal mixture on histidine-deficient agar with carcinogen or plasticizers for 2 days at 37°C.

[b]As a positive control, the indirect carcinogen 2-acetylaminofluorene (10 μg/plate) added in the presence of S-9 microsomal mixture was included in each experiment. In the experiment with strain TA98, 690 revertant colonies were counted per plate and with strain TA100, 353 revertant colonies per plate.

ethyl-2-hexanol and ethylhexanoic acid, were also without mutagenic activity in the Ames assay (Table 1). Both 2-ethylhexyl alcohol and 2-ethylhexanoic acid are potent peroxisome proliferators and it has been suggested that 2-ethylhexyl alcohol is the active part of the DEHP or DEHA molecule responsible for peroxisome proliferation (*4*). Also, 2-ethylhexyl alcohol is a metabolite of DEHP. In the lymphocyte ^{3}H-thymidine assay, DEHP, DEHA and ethyl-2-hexanol induced a dose-dependent inhibition of ^{3}H-thymidine incorporation into the replicating DNA of proliferating (concanavalin A-stimulated) C57BL/6J splenic lymphoid cells (Table 2). The dose-dependent inhibition of ^{3}H-thymidine incorporation into replicating DNA was accompanied by a dose-dependent increase in the ratio of acid-soluble to DNA-incorporated ^{3}H-thymidine in the plasticizer-treated lymphocytes (Table 2). This indicates that factors other than suppressed uptake of exogenous thymidine by plasticizer-treated lymphocytes are critical for the inhibition of ^{3}H-thymidine incorporation into DNA (*15*). To test whether the inhibition of ^{3}H-thymidine incorporation could be due to plasticizer-induced damage to lymphocyte DNA, plasticizer-treated lymphocytes were incubated in fresh culture medium without plasticizer. Previous work has established that inhibition of ^{3}H-thymidine into replicating lymphocyte DNA induced by DNA-damaging carcinogens such as methyl methanesulfonate (MMS) is not reversed by 3 hr incubation of carcinogen-treated lymphocytes in fresh medium without carcinogens (*18*, *19*). However, inhibition of ^{3}H-thymidine incorporation by non-DNA-damaging drugs which act by metabolic mechanisms, such as hydroxyurea, can be reversed by 3 hr incubation of drug-treated lymphocytes in fresh medium without drug. As reported in Table 3, suppression of ^{3}H-thymidine incorporation into DNA by DEHP, DEHA and ethyl-2-hexanol was reversed by incubation of treated lymphocytes for 3 hr without the compounds. As with other peroxisome proliferators, therefore, DEHP and DEHA or related alcohol and acid analogs do not have the properties of electrophilic mutagens under standard *in vitro* conditions of testing.

The fundamental problem arises as to mechanisms by which peroxisome proliferators initiate cells for neoplastic transformation. A multienzyme pathway has recently been identified in rat liver peroxisomes for the β-oxidation of long-chain fatty acids (*20*), and peroxisome proliferators have been found to increase manyfold the capacity of rat liver for the β-oxidation of fatty acids (*21*). In particular, the administration of DEHP resulted in a substantial increase in peroxisomal palmitoyl-CoA oxidation in liver homogenates (*22*). A similar increase in the peroxisomal fatty acyl-CoA-oxidation system has been detected following the administration of other peroxisome proliferators, including clofibrate, nafenopin, Wy-14,643, tibric acid, gemfibrozil and methyl clofenapate (*5*). Peroxisomal fatty acyl-CoA oxidation appears to be independent of an electron transport chain and results in the production of H_2O_2 by direct transfer of electrons to O_2. A sevenfold increase in the rate of palmitoyl CoA-dependent H_2O_2 generation has been detected in the liver of rats administered nafenopin (*23*). Liver tumors as well as nontumor portions of liver in Wy-14,643-fed rats have also been found to demonstrate increased

Table 2. Amount of acid-soluble and DNA-incorporated ^{3}H-thymidine in splenic lymphoid cells treated with plasticizers.[a]

Compounds	Concentration, *M*	% of control ^{3}H-thymidine incorporation[b]		Ratio, acid-soluble ^{3}H-thymidine/ DNA-incorporated ^{3}H-thymidine[c]	
		–S–9[d]	+S–9[e]	–S–9[d]	+S–9[e]
None				0.78	0.41
DEHP	10^{-2}	55 ± 3		1.44	
	5×10^{-3}		41 ± 6		0.73
	10^{-4}	56 ± 10		1.33	
	5×10^{-5}		48 ± 7		0.66
	10^{-6}	64 ± 6		0.93	
	5×10^{-6}		71 ± 7		0.45
DEHA	10^{-2}	64 ± 9		1.40	
	5×10^{-3}		69 ± 2		0.52
	10^{-4}	89 ± 7		0.88	
	5×10^{-5}		93 ± 10		0.36
	10^{-6}	119 ± 12		0.63	
	5×10^{-6}		83 ± 4		0.42
Ethyl-2-hexanol	10^{-3}	28 ± 4		1.86	
	10^{-4}	108 ± 8		0.59	
	5×10^{-4}		59 ± 2		0.47
	5×10^{-5}		84 ± 5		0.39
	5×10^{-6}		92 ± 10		0.32

[a]Concanavalin A-stimulated C57BL/6J splenic lymphoid cells were incubated in the absence or presence of S-9 microsomal mixture in medium without or with plasticizer. Lymphoid cell DNA was labeled with 10 μCi ^{3}H-thymidine per culture during the final 30 min of incubation. Labeled cells were washed three times with phosphate-buffered saline, the labeled cells were transferred to clean glass tubes, and the cells were precipitated overnight at 4°C with 0.2*N* perchloric acid. Supernatant was then separated from precipitate by centrifugation at 850*g* for 20 min, radioactivity of acid-soluble ^{3}H-thymidine was measured in the 0.2*N* perchloric acid supernatant by liquid scintillation spectrometry, and DNA was extracted from the 0.2*N* perchloric acid precipitate for liquid scintillation measurement of DNA-incorporated ^{3}H-thymidine.

[b]Incorporation of ^{3}H-thymidine into DNA was measured for each lymphocyte culture as dpm/μg DNA. Values of ^{3}H-thymidine incorporation for control cultures of untreated lymphocytes were taken as 100%. Each reported value was obtained as the mean ± SD of three replicate cultures of treated lymphoid cells.

[c]Ratio of the mean values of acid-soluble to DNA-incorporated ^{3}H-thymidine obtained in three replicate lymphocyte cultures as dpm/μg DNA.

[d]Lymphoid cells were incubated for 1 hr without or with plasticizer.

[e]Cells were incubated for 2.5 hr without or with plasticizer plus 20% (v/v) S-9 microsomal mixture.

Table 3. Recovery of stimulated rates of ^{3}H-thymidine incorporation by splenic lymphoid cells treated with plasticizers.[a]

Compound	Concentration, *M*	% of control ^{3}H-thymidine incorporation[b]	
		0 hr	3 hr
Without S–9[c]			
MMS	10^{-3}	74 ± 8	32 ± 11
DEHP	10^{-5}	71 ± 10	96 ± 8
DEHP	10^{-3}	36 ± 4	58 ± 3
DEHA	10^{-2}	16 ± 8	55 ± 8
Ethyl-2-hexanol	10^{-4}	39 ± 1	105 ± 18
Hydroxyurea	10^{-4}	10 ± 1	110 ± 16
With S–9[d]			
DEHP	10^{-2}	22 ± 4	72 ± 17
DEHA	10^{-3}	24 ± 14	66 ± 9
Ethyl-2-hexanol	10^{-3}	13 ± 1	110 ± 22

[a]Concanavalin A-stimulated C57BL/6J splenic lymphoid cells were incubated in the absence or presence of S-9 microsomal mixture in medium with plasticizer, carcinogen, or hydroxyurea and were then incubated for the times indicated in fresh culture medium without plasticizer, carcinogen, or hydroxyurea. Lymphoid cell DNA was labeled with 10 μCi ^{3}H-thymidine per culture during the final 30 min of incubation.

[b]Incorporation of ^{3}H-thymidine into DNA was measured for each lymphocyte culture as dpm/μg DNA. Values of ^{3}H-thymidine incorporation for control cultures of untreated lymphocytes were taken as 100%. Each reported value was obtained as the mean ± SD of three replicate cultures of treated lymphoid cells.

[c]Lymphoid cells were incubated for 1 hr with carcinogen, plasticizer, or hydroxyurea prior to incubation in fresh culture medium without added carcinogen, plasticizer, or hydroxyurea.

[d]Cells were incubated for 2.5 hr with plasticizer plus 20% (v/v) S-9 microsomal mixture prior to incubation without plasticizer or S-9 microsomal mixture.

levels of both peroxisomal fatty acid β-oxidation and H_2O_2 (*24*). Increased H_2O_2 production by the proliferated peroxisomes could result in the production of OH· by the Haber-Weiss reaction catalyzed by cellular iron (*25*). It is suggested, therefore, that peroxisome proliferation in cells is accompanied by an increase in the rate of production and perhaps the steady-state levels of reactive oxygen species in the cells. Compatible with this proposal is the excessive accumulation of lipofuscin, indicative of increased lipid peroxidation, which occurs in liver parenchymal cells during Wy-14,643- (*24*) or methyl clofenapate- (*17*) induced liver tumorigenesis. Unstable oxygen species are highly reactive toward biological macromolecules, especially DNA. Oxidative degradation, strand breakage and crosslinking have been detected in isolated DNA upon interaction with H_2O_2 and OH· (*26, 27*). Also, H_2O_2 has been found to induce DNA scissions in intact prokaryotic and eukaryotic cells (*28, 29*) and chromatid breaks, chromatid exchanges and unscheduled DNA synthesis in intact mammalian cells (*30, 31*). The important possibility arises, therefore, that sustained peroxisome proliferation initiates cells for neoplastic transformation by increasing intracellular production of DNA-damaging oxygen species.

Further changes in peroxisomal enzyme profiles induced by the peroxisome proliferators can be cited which could serve to increase cellular reactive oxygen species. First, the increase in peroxisomal catalase activity is disproportionately small compared to the increase in peroxisome volume (*32*) and H_2O_2-generating fatty acid β-oxidation (*33*) induced by the peroxisome proliferators. It will be recalled that catalase catalyzes the degradation of H_2O_2. Second, a substantial level of uricase activity has been detected in the peroxisomes of rodent liver (*34*). Although serum and tissue levels of uric acid have yet to be systematically measured in animals treated with peroxisome proliferators, increased levels of hepatic uricase would result in decreased levels of uric acid. Uric acid is a powerful antioxidant and is a scavenger of oxygen radicals (*35*).

The occurrence of DNA damage by reactive oxygen species can be directly tested as a hypothesis for the initiation of cells in DEHP carcinogenesis. Such testing will include measurement of reactive oxygen formation, assessment of DNA damage (DNA strand breakage, DNA repair) by oxygen radicals, and examination of possible modulation by antioxidants (α-tocopherol, selenium) in phthalate ester hepatocarcinogenesis. In addition, work is required to examine possible covalent binding of isotopically labeled plasticizer to liver cell DNA, RNA and protein during chronic administration of plasticizer to rodents. Absence of consistent *in vitro* DNA-damaging activity does not necessarily exclude the formation of electrophilic metabolites from the plasticizers in liver of the intact animal. Finally, although no evidence has been reported to date for tumor promotion by the phthalate esters, the peroxisome proliferators Wy-14,643 and clofibrate have been observed to promote the appearance of hepatocellular carcinoma following initiation in F344 rats by diethylnitrosamine (*36*). Tumor promotion is a reversible process which, unlike initiation, appears not to depend upon covalent modification of biological macromolecules (*14*). However, mechanisms of tumor promotion are poorly understood, and proposed cellular/molecular mechanisms of promotion as of the date of this report (*14*) would add little to a basic understanding of phthalate ester carcinogenesis.

As a final note, we should stress that chemically induced peroxisome proliferation is not limited to rats and mice. Hepatic peroxisome proliferation has been induced by hypolipidemic drugs in hamsters, pigeons, chickens, cats, and rhesus monkeys (*37, 38*). Also, ultrastructural changes in the liver of human subjects on long-term (6 months to 7 years) clofibrate therapy included an elevated number of microbodies (peroxisomes) (*39*). Consequently, the potential importance of peroxisome proliferator carcinogenesis impacts on many species including the human.

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