The potency estimate and no significant risk level associated with a lifetime cancer risk of 10^{-5} for a 70 kg adult are as follows:

Compound	Basis of Estimate			Human Cancer Potency		No Significant Risk Level
	Species	Route	Site			
DEHP	Mouse	Oral	Liver	All routes	0.0022 $(mg/kg\text{-}day)^{-1}$	310 μg/day

INTRODUCTION

Under the Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65, California Health and Safety Code Section 25249.5 *et seq.*), di(2-ethylhexyl)phthalate was listed as a chemical known to the State to cause cancer, effective January 1, 1988. In 1988, an NSRL for DEHP of 80 μg/day was adopted based upon the identification of male mice as the most sensitive sex and species for a hepatocarcinogenic effect and using a cancer potency value calculated by the U.S. Environmental Protection Agency (U.S. EPA, 1986). Since that time, a tremendous research effort by numerous laboratories and research institutions has gone toward clarifying the mechanism whereby DEHP elicits a carcinogenic effect and also the potential risk to humans from exposure to DEHP and other so-called peroxisome proliferators. The overall intent of this document is to update the previously adopted NSRL, providing a more confident estimate of carcinogenic risk to humans based upon the most current science. As it will become clear in the assessment which follows, there continue to be considerable uncertainties related to the risk assessment of DEHP (and other compounds termed "peroxisome proliferators") and it remains a highly active and controversial area of laboratory research in the scientific community. As future research developments clarify the human risk from exposure to this cancer-causing chemical, refinements to this assessment may be issued.

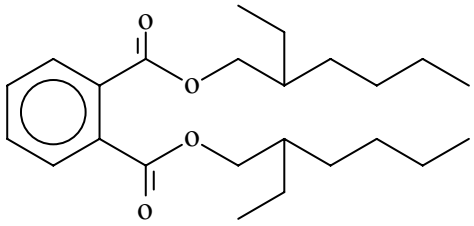
DEHP is a member of a group of structurally diverse compounds termed peroxisome proliferators for a characteristic set of pleiotropic (multiple) effects elicited by them. DEHP is thought to be metabolized to several proximate active compounds, the most well-studied of which is mono(2-ethylhexyl) phthalate (MEHP), formed by the deesterification of the parent compound by lipases of pancreatic origin (oral route) and by esterases found in the blood (oral and non-oral routes). These effects, predominantly on the liver, include increases in the number and size of cellular peroxisomes (a small membranous organelle containing numerous different oxidative enzymes present in all eukaryotic cells), enlargement of the liver, increases in enzymes associated with fatty acid metabolism, and liver tumors. A number of peroxisome proliferating chemicals also have serum hypolipidemic properties, thus providing a basis for their pharmaceutical use in the reduction of blood lipids.

Recent studies have strongly implicated a cellular protein receptor, called peroxisome proliferator activated receptor-α (PPAR-α), as a mediator of the carcinogenic effects for the peroxisome proliferator compounds. Since many of the chemicals in this category were classified prior to the identification of this receptor, for historical reasons they continue to be referred to as peroxisome proliferators, although the relationship between the phenomenon of peroxisome proliferation in the liver and the development of tumors remains unclear. At issue is whether intrinsic properties of peroxisomes are responsible for tumor development, or whether

peroxisome-independent effects or combinations of peroxisome dependent and independent factors may make contributions. For purposes of this assessment, this group of compounds will continue to be referred to as peroxisome proliferators. More precisely, this term will be applied to those compounds which exert physiological effects via the PPAR-α.

In this assessment, efforts have been made to identify the available evidence concerning the cancer risk from exposure to DEHP, particularly that which directly relates to DEHP and humans both *in vivo* and *in vitro*. In addition, we view the body of evidence regarding the effects which may be related to the carcinogenic process of other peroxisome proliferating chemicals as relevant, and in many areas, this evidence has been used to supplement that based upon studies of DEHP.

PHYSICOCHEMICAL PROPERTIES AND OCCURRENCE

CAS Registry Number	117-81-7
Synonyms	DEHP, bis(2-ethylhexyl)phthalate, BEHP, di-*sec*-octyl phthalate, dioctyl phthalate, DOP
Molecular Formula	$C_{24}H_{38}O_4$
Molecular Structure	
Molecular Weight	390.54 g/mol
Vapor Pressure	10^{-7} mm Hg at 25°C (Staples *et al.*, 1997)
Color/Form	light colored liquid
Octanol/Water Partition Coefficient	log K_{ow} = 4.89
Solubility	<0.01% in water at 25°C; 0.285 mg/l water at 24°C; miscible with mineral oil and hexane

CARCINOGENIC EFFECTS

Human Evidence of Carcinogenicity

No traditional epidemiological studies (case-control, prevalence, cohort) were found in the literature which examine the relationship between DEHP and cancer. U.S. EPA (1987) evaluated a single study which looked into toxic effects of workers exposed to DEHP:

> "Thiess et al. (1978) conducted a mortality study of 221 DEHP production workers exposed to unknown concentrations of DEHP for 3 months to 24 years. Workers were followed for a minimum of 5 to 10 years (mean follow-up time was 11.5 years). Eight deaths were reported in the exposed population. Deaths attributable to pancreatic carcinoma (1 case) and uremia (1 case in which the workers also had urethral and bladder papillomas) were significantly elevated in workers exposed for >15 years when compared

to the corresponding age groups in the general population. The study is limited by a short follow-up period and unquantified worker exposure. Results are considered inadequate for evidence of a causal association."

The design and conduct of epidemiological studies of the effects of DEHP is made difficult because DEHP is a ubiquitous environmental contaminant, and the clear identification of populations with substantially different exposures presents a challenge.

Candidate populations for study who are highly exposed to DEHP include patients undergoing various forms of dialysis (renal, peritoneal) or those in regular contact with polyvinyl chloride tubing containing DEHP. These populations tend to be poorly suited for studies of health outcomes as outlined by the U.S. EPA while examining the plausibility of conducting an epidemiologic study using dialysis patients. The agency specifically considered one using the End Stage Renal Disease Medical Information System (ESRD MIS) database (U.S. EPA, 1981). It was concluded that "the ESRD MIS is not suitable for the type of analyses required to determine the additional risk to ESRD patients." This is based on the lack of accuracy, completeness, and consistency in the database, susceptibility of the database to "fishing" for spurious associations, and limits to the amount of patient information which may be drawn from the database. It was also concluded that "…ESRD patients may not constitute a viable study group for an epidemiological investigation of the carcinogenic effect of exposure to DEHP for two reasons: 1) the inability to measure exposure to DEHP separately from exposure to carcinogens and possible etiologic factors; and 2) the extremely high combined competitive risk of mortality or morbidity from causes other than primary liver cancer and other malignancies." The authors of the document frame some of the limitations in the context of the commonly recognized criteria for causality. In this population, the possibility of finding a consistency and specificity of association of exposure to DEHP to effect is complicated by the concomitant exposure of this population to other potentially toxic agents. The establishment of the appropriate temporal relationship (generally long latency for chemically induced cancer) is complicated by the relatively short life expectancy of the ESRD population (7-10 years after chronic renal failure). Furthermore, animal models which may suggest biological plausibility are limited in their parallels to humans because of differences originating from the poor health of ESRD patients, thus compromising the coherence of association.

Thus, numerous issues have limited the ability to detect a possible carcinogenic effect in certain highly DEHP exposed patient populations.

Epidemiologic Studies of Other "Peroxisome Proliferators"

Pharmaceutical agents which have been identified as causing peroxisome proliferation in experimental animals have undergone some epidemiologic evaluation for potential carcinogenicity. Specific pharmaceutical agents which have been identified as having peroxisome proliferator properties include clofibrate and gemfibrozil.

Oliver *et al.* (1978) conducted a study on approximately 15,000 European males, one-third of who were treated with clofibrate for high cholesterol, one-third had high cholesterol but received a placebo, and a final untreated referent group drawn from the lower third of the cholesterol distribution. The patients were tracked over an average of 5.3 years, with test subjects receiving a daily dose of 1.6-g clofibrate. There was an increase in deaths due to cancer during the trial

among those receiving clofibrate relative to those receiving the placebo, however, no increase was observed within one year following the trial. Similarly, no increase in cancer diagnoses was observed in the year following the trial. The increase in mortality from cancer during the trial was not statistically significant. In regard to regional pathology, the authors noted that:

> "There were more deaths from diseases of the liver, gall bladder, and intestines, including malignant neoplasm of these sites, in the clofibrate-treated group than in the high cholesterol control group. Taken together with the significant excess in the treated group compared with both control groups of cholecystectomies for gall stones, there was therefore a possibility that clofibrate might be producing pathology in this area."

The studies by Oliver *et al.* (1978) were followed up with reports at two additional time points following the 5.3 year clinical trial, one 4.3 years following the trial (total follow-up: 9.6 years; Oliver *et al.*, 1980) and one 7.9 years following the trial (total follow-up: 13.2 years; Oliver *et al.*, 1984). An excess of cancer at all sites observed in the clofibrate treatment group was not significantly increased above that in the placebo-treated groups in either of these follow-up studies.

Frick *et al.* (1987) conducted an epidemiological study investigating the effects of the hypolipidemic agent (and peroxisome proliferator) gemfibrozil on a study population of approximately 4,000 males aged 40-55 years with primary dyslipidemia (high non-HDL cholesterol). Half the patients received gemfibrozil (1.2 g per day) and the other half a placebo for a period of five years. The five year treatment represented the maximum follow-up time for assessing cancer incidence. Five basal cell carcinomas of the skin were observed among the patients in the gemfibrozil group compared to none in the placebo group (marginally significant by Fisher's exact test, $p = 0.062$); however, using Finnish national cancer statistics, the expected number in a population this size is four to five. According to the authors: "There was no difference between the groups in the total death rate, nor did the treatment influence the cancer rates."

A follow-up to the Frick *et al.* (1987) study was conducted with a subset of the original study participants, including subjects who were originally treated with gemfibrozil ($n = 2002$) and untreated subjects ($n = 1992$) (Huttunen *et al.*, 1994). After 3.5 years (8.5 years after the study began), cancer mortality was higher in the gemfibrozil treatment group compared to the placebo group ($p = 0.08$). An 18-month follow-up beyond this point (at 10 years) showed a less significant increase in cancer mortality (35 cancer deaths in the gemfibrozil group *vs.* 31 cancer deaths in the untreated group). The authors suggested that the observed increase at 8.5 years was due to chance.

In a recent review of the available data concerning hypolipidemic agents and cancer, Newman and Hulley (1996) stated that: "Evidence of carcinogenicity of lipid-lowering drugs from clinical trials in humans is inconclusive because of inconsistent results and insufficient duration of follow-up." Further, they concluded that: "Longer-term clinical trials and careful postmarketing surveillance during the next several decades are needed to determine whether cholesterol-lowering drugs cause cancer in humans." These and other authors have also speculated that potential increases in cancer risk may be associated with the lowering of cholesterol levels itself,

rather than a treatment or chemical related effect (Dalen and Dalton, 1996). Overall, it appears that the safety of the hypolipidemic agents remains controversial and data are limited.

Implications of the Absence of Epidemiologic Data

Epidemiologic data can greatly assist in determination of causality. Unfortunately, in the case of DEHP and cancer, no useful epidemiologic data exist. If it were available, valid epidemiologic data could have provided answers to many questions about potential causality. To assess causality, however, relevant epidemiologic studies must exist. Then, if multiple relevant and valid epidemiologic studies are identified, meta-analysis techniques and causal criteria could be applied. Thus, epidemiologic evidence for risk assessment is the result of four steps: identification of relevant studies, assessment of the validity of the studies, application of meta-analysis techniques, and application of criteria for assessing causality (Krzyzanowski, 2000). While there are epidemiologic studies of interest to the topic of DEHP and cancer (see discussion of hypolipidemic agents above), none provide useful evidence for risk assessment because of limited relevance (*e.g*., not the same chemical) and/or scientific methods that fall short of acceptability (*e.g*., poor statistical power or limited follow-up period).

Thus, while epidemiologic data can be useful, such data are often not available, as is the case for DEHP. In this case, there is a substantial gap in the evidence for causality and the epidemiological evidence neither establishes nor rules out increased risk. In other words, the epidemiology is uninformative.

Human Case Studies of the Effects of Peroxisome Proliferators

Several investigators have attempted to assess the effects of peroxisome proliferating pharmaceuticals on human liver by way of biopsy of patients treated with these chemicals. These studies were initiated in an effort to examine in human liver the effects which had been observed in rodent studies. It is worth noting that the relevance of the measured endpoints to the carcinogenic process has not been established. Another important consideration regarding one of the hallmarks of treatment by these compounds, the peroxisomes themselves, is that there are structural differences between peroxisomes which form in humans compared to those observed in rodents. Human peroxisomes lack a "crystalloid core" that is commonly observed in rodent peroxisomes due to the absence of the enzyme urate oxidase, which humans do not have (Reddy and Lalwani, 1983). The significance of this difference for the assessment of cancer risk from exposure to peroxisome proliferating chemicals is not clear, although it presents difficulties for making direct morphological comparisons of peroxisomes between rodents and humans. The specific peroxisome proliferating agents in human case studies include gemfibrozil, clofibrate, and fenofibrate.

The few available studies are described below:

De La Iglesia *et al.* (1982) examined liver biopsy tissue from nine patients treated long-term with the hypolipidemic agent (and peroxisome proliferator) gemfibrozil. The authors noted that: "Detailed analysis of the peroxisome population showed matrix rarefaction, marginal plate formation, and spurious densities though no significant proliferation occurred. Distribution of peroxisomes in hepatocytes varied widely from cell to cell and in different lobular areas." … and … "Peroxisome proliferation, as seen in rodents when receiving gemfibrozil, did not occur and the structure of these subcellular organelles was not compromised." However, the authors also

noted that: “Since the population of this organelle varied within different regions of the cell and also from cell to cell, the numerical analysis by stereologic methods could confirm subjective findings and a preliminary report is available [citing De La Iglesia *et al.*, 1981].” This statement calls attention to a difficulty in the assessment of the degree of peroxisome proliferation in the liver. Because of regional variations, quantitative evaluation of the degree of effect within a given organ can be difficult.

Hanefeld *et al.* (1983) examined liver biopsy tissue from 16 patients (ten female, six male) treated for primary hyperlipoproteinemia with clofibrate (*p*-chlorophenoxyisobutyric acid; CPIB). Liver biopsies (Menghini technique) were obtained from all patients prior to initial treatment with the drug. Treatment consisted of daily doses of 2 g clofibrate. Twenty biopsies were subsequently obtained after treatment periods ranging from three to greater than 90 months. Samples were examined for serum lipid concentration, and number and volume of both mitochondria and peroxisomes. As expected, serum triglycerides and cholesterol were reduced by treatment using values obtained before and during treatment. Likewise, a significant increase in mitochondrial number (+38%) and volume (+34%) was observed, as was an increase in peroxisomal number (+50%). A 23% increase in peroxisomal volume was also observed but not statistically significant. With respect to the ultrastructural changes induced by clofibrate, the authors noted that “the extent of these changes is variable and peroxisomes especially show focal proliferation.” Evaluation of the time course of the effects suggested that the changes occurred during the first months of treatment.

Gariot *et al.* (1983) performed liver biopsies on 23 patients being treated for hyperlipoproteinemia either by diet alone (13 patients: 12 male, one female) or by treatment with the peroxisome proliferator fenofibrate (ten patients: seven male, three female). Patients received daily doses of 300 mg (n = 6), 400 mg (n = 2), or 600 mg (n = 2) with a mean duration of 9.01 ± 7.45 months (seven patients were treated for more than four months), which the authors calculated as an average daily dosage of 4 mg/kg. At the time of the biopsy, five of the ten patients treated with fenofibrate showed indications of pre-existing hepatomegaly, whereas only one of the diet alone group showed signs of hepatomegaly. No statistically significant differences in the number of peroxisomes were observed between the fenofibrate and diet treated groups. The authors observed that “[l]iver peroxisomes were not homogeneously distributed since in four patients the number of peroxisomes counted in the cells of the first block of liver was significantly different from that of the second block cells from the same biopsy sample.” This difficulty in assessing liver peroxisomes recalls that reported by the De La Iglesia *et al.* (1982) group at the cellular level, although this observation emphasizes variation at the tissue, rather than cellular level.

Ganning *et al.* (1984) reported briefly on biopsied liver tissue from two patients after one month and one year of dialysis. The authors noted that after one month “no deviation from controls and no sign of membrane induction is apparent,” however, after one year of dialysis, “peroxisomes, which are less characteristic in humans, since they lack a core, are present in a significantly higher number.” The authors noted difficulties in the quantitation in induction because of the “size of the required number of biopsies.” Clearly also, there is uncertainty regarding the health of the patients examined, the compounds to which they were exposed and the characterization and handling of the samples.

Overview of Case Studies

This is a limited data set involving humans exposed to different compounds for different periods. Convincing analysis of human tissues is subject to some of the limitations mentioned by the authors themselves (tissue variability, limited sample sizes, pre-existing medical conditions). Nonetheless, there is limited evidence for effects consistent with a peroxisome proliferation response, particularly that noted by Ganning *et al.* (1984) as a possible response to dialysis treatment and by Hanefeld *et al.* (1983) in response to treatment with clofibrate. An important caveat to many of these case studies is the temporal relationship of the tissue sampling to the administration/treatment of the subjects with the putative peroxisome proliferators. Peroxisome proliferation is a reversible phenomenon, with the peroxisomes themselves having a limited half-life, on the order of one to two days (Reddy and Lalwani, 1983; Sartori *et al.*, 1992). Rigorous evaluations of human subjects requires detailed information on the processing of the biopsy samples and the time of most recent exposure to the peroxisome proliferator, much of which is unavailable in the studies, as presented. Overall, no well-conducted, systematic evaluation of human responsiveness from *in vivo* exposure to DEHP has been conducted to date. Thus, this particular set of data does not provide strong evidence of either human responsiveness or a lack thereof.

Human Data Sets for Quantitative Estimates of Cancer Risk

No suitable data sets have been identified from *in vivo* human studies which are suitable for the assessment of risk from exposure to DEHP.

Animal Bioassays and Data Sets for Quantitative Estimates of Cancer Risk

The carcinogenicity of DEHP has been examined in several long-term bioassays in rodents and in one small set of studies in dogs: Carpenter *et al.* (1953), Harris *et al.* (1956), NTP (1982) [reported in Kluwe *et al.*, 1982], Schmezer *et al.* (1988), Rao *et al.* (1987; 1990), Ganning *et al.* (1991) and David *et al.* (1999, 2000a, 2000b). These studies are described briefly below, with an emphasis on those with useful data for the quantitative assessment of carcinogenic risk.

Carpenter *et al.* (1953)

The chronic oral toxicity of DEHP was examined in rats, guinea pigs, and dogs. Sherman rats (32/sex) were treated in their diet for up to two years with DEHP at concentrations of 0, 0.04, 0.13, and 0.4%. No significant increase in tumor incidence was noted.

Guinea pigs (22-24/sex) were administered DEHP in their diet for one year at concentrations of 0, 0.04, and 0.13% DEHP. No significant increase in tumor incidence was observed, although the exposure duration may not have been adequate to detect a carcinogenic effect under these study conditions.

Four cocker spaniels and four wire-haired terriers were randomly separated by breed and sex into two groups and administered DEHP in gelatin capsules for one year, receiving 0.03 ml/kg-day for the first four weeks (5 days/week) and 0.06 ml/kg-day for the remaining 48 weeks. No increase in tumor incidence was observed. However, the lifespan of dogs is generally considered to be on the order of 11 years, so this experiment only covered a fraction (~10%) of the expected lifespan and may have been inadequate to detect a carcinogenic effect.

This set of studies, although showing no carcinogenic effect of DEHP, is limited in several respects including potentially inadequate dosing (all species), limited reporting of data on tumors, small group sizes (especially with the dog studies), and inadequate study duration (guinea pigs, dogs).

Harris *et al.* (1956)
Wistar rats (43/sex/group) were maintained on diets containing 0, 0.1, or 0.5% DEHP (termed 2-ethylhexyl phthalate in the report), with serial sacrifices occurring at 3 months (4 rats/sex), 6 months (4 rats/sex), 12 months (10 rats/sex), and 24 months (24 rats/sex). No treatment related effect on mortality was observed, although overall mortality was high by the end of the experiment (85-96%). No significant increases in tumor incidence were reported.

National Toxicology Program (1982)
B6C3F1 mice and F344 rats (50/sex/group) were fed diets containing DEHP for 103 weeks. Levels in feed were 0, 3,000, or 6000 ppm (0, 0.3, or 0.6%) for mice and 0, 6,000, or 12,000 ppm (0, 0.6, or 1.2%) for rats. Significant increases in combined liver adenomas and carcinomas were observed in male and female mice at both doses. Increased incidences of carcinomas alone were observed in male mice in the high dose group and in female mice in both treated groups. Among male rats, a significant increase in combined liver carcinomas and neoplastic nodules was observed in the high dose group. Among female rats, the combined incidence of liver carcinomas and neoplastic nodules was increased in both DEHP treated groups. Significant increases in liver carcinomas and in neoplastic nodules were observed in the high dose group.

A slight, but not statistically significant, increase in the incidence of mononuclear cell leukemia (termed "myelomonocytic leukemia" in the NTP report) was observed in both groups of DEHP treated male rats. Two pancreatic acinar cell tumors (one carcinoma, one adenoma) were observed in the DEHP treated male rats.

NTP concluded: "Under the conditions of this bioassay, di(2-ethylhexyl)phthalate was carcinogenic for F344 rats and B6C3F1 mice, causing increased incidences of female rats and male and female mice with hepatocellular carcinomas, and inducing an increased incidence of male rats with either hepatocellular carcinomas or neoplastic nodules."

Table 1. Liver tumor incidence in B6C3F1 mice and F344 rats administered DEHP in feed for two years (NTP, 1982).

Tumor Site and Type		Control	Low Dose	High Dose
Male Mice				
Liver	Adenoma	6/50	11/48	10/50
	Carcinoma	9/50	14/48	**19/50**
	Adenoma or carcinoma	14/50		**29/50**
Female Mice				
Liver	Adenoma	1/50	5/50	1/50
	Carcinoma	0/50		**17/50**
	Adenoma or carcinoma	1/50		**18/50**

Tumor Site and Type		Control	Low Dose	High Dose
Male Rats				
Liver	Neoplastic nodule	2/50	5/49	7/49
	Carcinoma	1/50	1/49	5/49
	Nodule or carcinoma	3/50	6/49	**12/49**
Female Rats				
Liver	Neoplastic nodule	0/50	4/49	**5/50**
	Carcinoma	0/50	2/49	**8/50**
	Nodule or carcinoma	0/50		**13/50**

* Highlighted cells indicate a significant increase in tumor incidence relative to controls (Fisher's exact test, $p \leq 0.05$).

Table 2. Other lesions observed in F344 rats treated with DEHP in feed (NTP, 1982).

Tumor Site and Type		Control	Low Dose	High Dose
Male Rats				
Spleen	Mononuclear cell leukemia ("myelomonocytic leukemia")	13/50	20/50	17/50
Pancreas	Acinar cell adenoma or carcinoma	0/48	1/46	1/46

Rao *et al.* (1987)

Male F344 rats fed a diet containing 2% DEHP for 95 weeks showed an increase in liver nodules and/or hepatocellular carcinomas (see Table 3 below). Based upon the information in the publication, liver appears to have been the only organ examined for lesions.

Table 3. Tumors in F344 rats fed diet containing 2% DEHP for 95 weeks (Rao *et al.*, 1987).

Tumor Site and Type		Treatment*	
Male rats		**Control**	**2% DEHP**
Liver	Carcinoma or neoplastic nodule	0/8	**6/10**

* Highlighted cell indicates a significant increase in tumor incidence relative to controls (Fisher's exact test, $p \leq 0.05$).

Schmezer *et al*. (1988)

Syrian golden hamsters were treated by intraperitoneal injection or inhalation with DEHP. Briefly, groups of 50 male and female hamsters were treated with doses of 3 g DEHP/kg_{bw} once every week, once every two weeks, or once every four weeks. An untreated control group was included. Animals were observed for life or until moribund. Livers and organs showing abnormalities were examined histologically. The authors correlated the dose administered in their study with that used in NTP's positive rat and mouse bioassays, which they estimated to be 0.67 g/kg_{bw} for male rats, and 1.3 g/kg_{bw} for female mice. They concluded, "the total dose throughout the total course of the [NTP] experiment would be about 9-fold or 18-fold …higher than the total dose of our study." No significant increases in tumor incidence were observed for male or female hamsters.

Inhalation studies were conducted by continuous exposure of hamsters (60/sex) from the 12th week of life until their natural death to DEHP at a level of 15 ± 5 µg/m^3 (saturation at room temperature), which the authors calculated to be a dose of 0.007-0.01 g/kg_{bw}. Control groups (80/sex) were also included in the experiment. No significant treatment-related increases in tumor incidence were observed.

The likely inadequate dosing in both the intraperitoneal and inhalation studies for detection of a tumorigenic response suggests that, based on the evidence provided in this bioassay, the hamster should not necessarily be considered unresponsive to DEHP's carcinogenic effects, as these doses were below those which produced tumorigenic responses in rats and mice.

Rao *et al.* (1990)

Male F344 rats treated with diet containing 2% DEHP for 108 weeks showed a significant increase in liver tumors (combined carcinomas and neoplastic lesions) relative to untreated control animals (see Table 4 below). Rao *et al.* also observed that the "[p]ancreases from DEHP-treated rats showed either pseudoductular lesions or altered acinar-cell foci. In addition, in four rats, islet-cell adenomas were also present." While not stated, it is assumed that no pancreatic lesions were observed among the control animals.

Table 4. Tumors in F344 rats fed diet containing 2% DEHP for 108 weeks (Rao *et al.*, 1990).

Tumor Site and Type		Treatment	
Male rats		**Control**	**2% DEHP**
Liver	Carcinoma or neoplastic nodule	1/10	**11/14**
Pancreas	Islet cell adenoma	0/10	**4/14**

* Highlighted cell indicates a significant increase in tumor incidence relative to controls (Fisher's exact test, $p \leq 0.05$). The significance of the increase in pancreatic tumors is $p = 0.09$, by Fisher's exact test.

Ganning *et al.* (1991)

Adult male Sprague-Dawley rats were administered DEHP in feed at concentrations of 0, 0.02, 0.2, and 2% for two years. The number of animals per group was not explicitly stated in the report, although 520 animals were said to have been used in the investigation. The authors reported that "no hyperplastic nodules or primary liver carcinoma or other tumours could be observed in this series of experiments." Peroxisome proliferation was observed at one week in the high dose group and was "maintained thereafter." At 16 months, increases in peroxisome proliferation was also observed in the group treated with 0.2% DEHP. The low dose group showed no evidence of peroxisome proliferation after 16 months of treatment. The levels of liver palmitoyl-CoA oxidase were elevated in the high-dose group 8- to 12-fold over control animals over the course of the study, up to 8-fold over controls in the mid-dose group, and up to 2-fold over controls in the low-dose group. Catalase activity in the liver of rats in the high-dose group was initially lower than control rats. Near the middle portion of the study, catalase activity became higher than controls (~40% higher) before tapering off to near control levels by the end of the study. The lower level DEHP dose groups showed similar, but weaker, trends in catalase activity.

David *et al.* (1999, 2000a, 2000b)

Groups of Fischer rats or B6C3F1 mice were treated with DEHP in feed at various concentrations for 104 weeks (see Table 5 below for dose levels). Additional rats and mice (55/sex/group) were treated at the highest concentrations of DEHP (6000 ppm for mice and 12500 ppm for rats) for 78 weeks, then allowed to recover for 26 weeks. At 104 weeks, these animals were sacrificed and examined for tumors. Additional groups of ten animals per sex were treated at the two highest doses for 79 weeks, then sacrificed. Among rats in the control and high dose groups, additional animals (5/sex/group) and endpoints examined at one, two, and 13 weeks were liver labeling index, palmitoyl-CoA oxidation activity, and liver-to-body weight ratio. Among mice in the control, high-dose group, plus another higher dose (17500 ppm), additional animals (5/sex/group) were similarly examined at one, four, and 13 weeks.

Table 5. Liver tumor incidence in B6C3F1 mice and F344 rats administered DEHP in feed for two years (David *et al.*, 1999).

Tumor Site and Type		Dose (ppm in feed)					
		0	**100**	**500**	**1500**	**6000**	**Recovery**
Male mice							
Liver	Adenoma	4/70				**19/70**	3/55
	Carcinoma	4/70	5/60	9/65			**12/55**
	Adenoma or carcinoma	8/70	14/60			**37/70**	**14/55**
Female mice							
Liver	Adenoma	0/70	2/60	4/65		**34/70**	**13/55**
	Carcinoma	3/70	2/60	3/65		**16/70**	**23/55**
	Adenoma or carcinoma	3/70	4/60	7/65		**44/70**	**30/55**

Tumor Site and Type		**0**	**100**	**500**	**2500**	**12500**	**Recovery**
Male rats							
Liver	Adenoma	4/80	5/50	3/55	8/65	**21/80**	**12/55**
	Carcinoma	1/80	0/50	1/55	3/65	**24/80**	**7/55**
	Adenoma or carcinoma	5/80	5/50	4/55		**34/80**	**18/55**
Female rats							
Liver	Adenoma	0/80	3/50	1/55	2/65	**8/80**	**6/55**
	Carcinoma	0/80	1/50	0/55	1/65	**14/80**	**4/55**
	Adenoma or carcinoma	0/80	**4/50**	1/55	3/65	**22/80**	**10/55**

* Highlighted cells indicate a significant increase in tumor incidence relative to controls (Fisher's exact test, $p \leq 0.05$).

In addition to the liver tumors characterized above, other lesions of concern were also noted, particularly among male rats (see Table 6 below). These lesions include statistically significant increases in spongiosis hepatis in the two highest dose groups, an increase in pancreatic acinar cell adenoma in the highest dose group, and an increase in mononuclear cell leukemia in the two highest dose groups.

Table 6. Other lesions observed in F344 rats treated with DEHP in feed (David *et al.*, 2000a).*

Tumor Site and Type		Dose (ppm in feed)				
		0	**100**	**500**	**2500**	**12500**
Male rats						
Liver	Spongiosis hepatis	3/80	3/50	3/55		**11/80**
Pancreas	Acinar cell adenoma	0/60	0/17	0/14	0/18	**5/59**
Spleen	Mononuclear cell leukemia	15/65	13/50	16/55		**27/65**

* Highlighted cells indicate a significant increase in tumor incidence relative to controls (Fisher's exact test, $p \leq 0.05$).

Spongiosis hepatis is a pathological condition first described by Bannasch et al. (1981) thought to originate with the perisinusoidal liver cells (Stroebel *et al.*, 1995), and consisting of "multilocular formations filled with a finely granular or flocculent acidophilic material" and "often replace large areas of the liver parenchyma … or, sometimes, considerable portions of neoplastic hepatic nodules ... or hepatocellular carcinomas." More recently this lesion has been called spongiosis pericytoma and has been characterized as having "benign neoplastic behavior," due to the "persistence, the proliferative activity, and the slow expansive growth of these lesions." (Stroebel *et al.*, 1995). This lesion has been documented to occur in the liver of rats treated with hepatocarcinogenic compounds including *N*-nitrosomorpholine, dimethyl-nitrosamine, nitrosopyrrolidine, and diaminodiphenylmethane (Ito *et al.*, 1984; Zerban and Bannasch, 1983; Bannasch *et al.*, 1981).

In addition to the pancreatic adenomas observed by David *et al.* (2000), among DEHP treated rats in other studies, an increase in pancreatic islet cell adenomas was observed by Rao *et al.* (1990) and two acinar cell tumors (one adenoma and one carcinoma) were observed in the 1982 NTP study in male F344 rats. These observations suggest some level of reproducibility of this result in rats treated with DEHP, although the incidence tends to be low (the increases in the Rao *et al.* and NTP findings were not statistically significant). Pancreatic acinar cell adenoma is a proliferative lesion which has been observed in rats concomitant with hepatocellular tumors and Leydig cell tumors of the testes upon exposure to several compounds which interact with PPAR-α including Wy-14,643, ammonium perfluorooctanoate, methylclofenapate, and clofibrate (reviewed in Obourn *et al.*, 1997). The best long-term studies of the carcinogenic effects of DEHP have been conducted in F344 rats, a strain which is unsuitable for the study of Leydig cell tumors because of high spontaneous tumor incidence.

EVIDENCE FOR MODE OF ACTION

Relevant Metabolic and Pharmacokinetic Considerations

Pharmacokinetics and Metabolism Overview

Basic metabolic and pharmacokinetic issues have been recently reviewed in a document published by this office (OEHHA, 1997). A brief overview of key findings follows for the purpose of providing context for some of the discussions later in this document.

Limited studies in humans demonstrate that DEHP is absorbed following oral administration, metabolized, and excreted in the urine (Schmid and Schlatter, 1985). The compounds identified in the urine were de-esterified products of the parent compound, much of which was conjugated with glucuronide. An absorption fraction of 25% has been estimated based upon this study, although the extent of biliary excretion (with or without reabsorption) suggests that this level may be higher (see discussions below). Studies in rats have shown an oral absorption fraction of approximately 55% (Rhodes *et al.*, 1986). It has been speculated that DEHP is broken down to its major metabolites MEHP and 2-ethylhexanol prior to absorption at low doses, although absorption of intact DEHP may occur at higher doses (Albro *et al.*, 1982; Albro, 1986).

Distribution of DEHP or metabolites to a number of different tissues has been demonstrated in several species, including rat, pig, dog, and marmoset (Ikeda *et al.*, 1980; Elsisi *et al.*, 1989; Melnick *et al.*, 1987; Rhodes *et al.*, 1986). Distribution to various sites occurred within four days to tissues including liver, adipose tissue, and muscle. Mouse studies have demonstrated wide tissue distribution, with the exception of the central nervous system, bone, and thymus (Gaunt and Butterworth, 1982). There is little evidence for bioaccumulation of DEHP or its metabolites, although adipose tissue has been identified as containing certain DEHP metabolites (Tanaka *et al.*, 1975).

DEHP undergoes metabolism by several pathways, central to which is an initial de-esterification step leading to the formation of MEHP and 2-ethylhexanol. MEHP undergoes subsequent oxidation on the aliphatic ethylhexyl moiety by either ω- or (ω-1)-oxidation or by α or β-oxidation, resulting in a reduction in the chain length. Intact DEHP is not known to undergo oxidation, nor is the aromatic portion of DEHP known to be modified (Albro and Lavenhar, 1989). 2-Ethylhexanol may undergo β-oxidation, producing 2-ethylhexanoic acid or keto acid derivatives (Albro and Corbett, 1978). For the identity of the numerous specific metabolites found in both serum and urine (at least eight have been identified in humans), the reader is referred to any of numerous reviews, including Huber *et al.* (1996).

There is wide distribution of the esterases capable of converting DEHP to MEHP, including a presence in the liver, kidney, lungs, skin, plasma, pancreas, and the intestinal mucosa (Albro and Lavenhar, 1989; Albro, 1986; Gollamudi *et al.*, 1985). For DEHP administered by the oral route, the pancreatic and intestinal esterases are likely to be primarily responsible for its metabolic conversion prior to absorption. For DEHP exposures by parenteral routes, DEHP is likely to be primarily de-esterified by plasma or tissue esterases.

The conjugation of MEHP and various derivatives has been characterized by Albro and Lavenhar (1989). The primary conjugation activity identified in several species is the formation of glucuronides, with the relative level varying across species: rat (none) < mouse, hamster < primate (including humans). The relevance of conjugation to toxicological outcome is discussed in a later section of this document.

Elimination of DEHP or its metabolites is expected to occur by both fecal and urinary routes. Fecal elimination may occur following oral or parenteral routes of exposure due to the potential for biliary excretion. Estimates of the elimination half-life for DEHP in humans is on the order of 12 hours, based upon limited studies in two human volunteers (Schmid and Schlatter, 1985). Huber *et al.* (1996) have noted considerable variation in the estimations of the half-life of MEHP

in rat blood between different publications: 23.8 hours, 5 to 6 hours, and 2.8 to 3.2 hours (citing Oishi and Hiraga, 1982; Teirlynck and Belpaire, 1985; and Elsisi *et al.*, 1989, respectively).

There is evidence that DEHP and several of its metabolites, most notably MEHP, have peroxisome proliferation activity. *In vitro* studies, largely conducted in cell lines or primary cell cultures (mostly hepatocytes), have demonstrated that DEHP is active at a cellular level, indicating either that DEHP itself has some intrinsic activity in mediating the observed effects, or that cells have some capacity for conversion of DEHP to MEHP. Experimental data provide good evidence that MEHP is highly active in mediating many of the effects of DEHP. Few studies have been conducted *in vivo* with MEHP (reviewed in Thomas and Northup, 1982) and those which have been conducted have primarily focussed on the evaluation of teratogenic potential and reproductive endpoints (Yagi *et al.*, 1980; Curto and Thomas, 1982; Tomita *et al.*, 1982; Shiota and Mima, 1985), and thus are of limited value for assessing endpoints relevant to the carcinogenic process.

2-Ethylhexanol (2-EH) has demonstrated some properties characteristic of peroxisome proliferators, albeit weakly (Keith *et al.*, 1992; Astill *et al.*, 1996). Long-term bioassays in F344 rats and B6C3F1 mice of 2-EH administered by oral gavage showed some indication of hepatocarcinogenicity in mice only (Astill *et al.*, 1996). These authors concluded that "[w]hile 2EH may be a contributing factor in the hepatocellular carcinogenesis in female mice associated with the chronic administration of DEHA [di-(2-ethylhexyl) adipate] and DEHP, it is unlikely to be the entire proximate carcinogen."

Studies of other DEHP metabolites have been conducted in an effort to identify those compounds which may be the proximate peroxisome proliferators in rats (Sjöberg *et al.*, 1985a; citing the studies reported in Mitchell *et al.*, 1985):

> "… the (ω-1) oxidized metabolites, metabolites VI [mono-(2-ethyl-5-oxohexyl) phthalate] and IX [mono-(2-ethyl-5-hydroxyhexyl) phthalate], are as potent as MEHP in causing peroxisome proliferation in vitro, whereas no such effect was noted for metabolite V [mono-(5-carboxy-2-ethyl pentyl) phthalate] and mono-(3-carboxy-2-ethylpropyl) phthalate. This suggests that other metabolites than MEHP may be involved in DEHP-induced liver toxicity."

DEHP metabolites were identified in human urine from leukemia patients (data summarized from abstract; Peck *et al.*, 1978; Albro *et al.*, 1982). No intact DEHP was detected in the urine samples, suggesting that de-esterification, metabolism, and/or conjugation must occur prior to elimination by this route. The most frequent urinary metabolites identified included, in decreasing order of occurrence: metabolite IX (36.2%), MEHP (18.3%), metabolite VI (12.1%), metabolite VII (11.9%), metabolite VIII (8.1%), and V (5.3%). It is notable that the three most predominant metabolites were found to be active inducers of peroxisomal enzyme activity in *in vitro* assays in rodents, as reported by Mitchell et al. (1985).

Numerous studies which are specific to elucidation of the mode of action of peroxisome proliferators have been conducted with MEHP and/or DEHP. Such studies, where relevant, are described in detail in the sections below.

"Initiation" and "Promotion" Studies

Experiments have been conducted aimed at determining whether DEHP has properties which could be considered to fall into classic "initiation" and "promotion" roles in carcinogenicity.

Garvey *et al.* (1987) conducted two studies in Fischer 344 rats to assess DEHP's potential as an initiator. In one study, DEHP (10 g/kg_{bw}) was administered as a single oral dose by gavage, followed by two weeks of recovery, then treatment for two weeks with a diet containing 0.02% 2-acetylaminofluorene with a single oral dose of carbon tetrachloride (1.5 ml/kg_{bw}) at the midpoint of 2-acetylaminofluorene promotion. One week following the treatment, animals were examined for "altered hepatic foci" using six histological markers. No increase in foci was observed with the DEHP treatment regimen. Another study was conducted in which DEHP was administered in the diet at 1.2% for 12 weeks, followed by 39 weeks of treatment on a diet containing 0.05% phenobarbital. No increase in altered hepatic foci was observed compared to a control group treated with phenobarbital alone. In a second initiation study reported by Ward *et al.* (1986), $B6C3F_1$ mice were administered a single dose of DEHP (25 or 50 g/kg_{bw}) followed by six or 18 months of treatment with a diet containing 500 ppm phenobarbital. No increase in altered hepatic foci or liver carcinomas was observed.

Several studies have also examined DEHP's potential to act as a tumor promoter. Generally, these studies follow a protocol of administering an initiating compound followed by longer term treatment with DEHP. Male Fischer 344 rats were administered a single injection of diethylnitrosamine followed after two weeks of recovery by six months of diet containing 1.2% DEHP (Popp *et al.*, 1985). No increase in altered hepatic foci was observed using six histological markers. In another study rats (n = 6) were administered 200 ppm 2-acetylaminofluorene in the diet for seven weeks, followed by four weeks of recovery, then treatment for another 24 weeks with a diet containing 1.2% DEHP (Williams *et al.*, 1987). Again, no increase in altered hepatic foci was observed using iron exclusion and hematoxylin-eosin staining criteria.

Groups of $B6C3F_1$ mice were treated with a single dose of diethylnitrosamine followed by up to six months of treatment with diet containing various concentrations of DEHP ranging from 0.3 to 1.2 % DEHP (Ward *et al.*, 1983). At the two highest doses of DEHP (0.6 and 1.2 %), liver tumor incidences and the size and number of altered hepatic foci were significantly increased above controls. Another study from the same group later reported significantly increased altered hepatic foci following a single dose of diethylnitrosamine followed by only 28 days of treatment with a diet containing 0.3% DEHP (Ward *et al.*, 1984).

The experimental results described above provide some evidence for tumor promotion properties of DEHP. In light of the experimental evidence described previously demonstrating that DEHP can act as a complete carcinogen, the limited evidence showing no "initiation" properties for DEHP has little relevance for the assessment of risk from exposure to DEHP.

Genotoxicity of DEHP/MEHP

Non-Mammalian Assays for Mutagenicity

DEHP and its metabolites have been studied in a number of bacterial mutagenesis assays. Most studies of DEHP and its metabolites were performed in the presence and absence of exogenous

metabolic activation provided by Aroclor 1254-induced rat liver S9. However, some studies used metabolic activation derived from other strains or systems. Aroclor 1254-induced Syrian hamster liver S9, ram seminal vesicle microsomes (a source of prostaglandin endoperoxide synthetase), phenobarbital/5,6-benzoflavone-induced or DEHP-induced rat liver S9 and cocultivation with either primary Sprague-Dawley rat hepatocytes or C0631 cells have all been used as metabolic activation sources (reviewed in Budroe and Williams, 1993). Several studies also investigated the mutagenicity of urine containing phthalate ester metabolites. DiVincenzo *et al.* (1985) tested urine samples from male Sprague-Dawley rats exposed by gavage to DEHP for 15 days in the presence and absence of both Aroclor 1254-induced rat liver S9 and β-glucuronidase/ arylsulphatase.

A few studies reported that several of the phthalate esters were weakly mutagenic. Tomita *et al.* (1982) concluded that DEHP and mono-2-ethylhexyl phthalate induced mutations in the *Salmonella* strain TA 100 in the presence of S9 for DEHP, and in the absence of S9 for MEHP in both plate incorporation and preincubation assays. However, although both agents caused a statistically significant increase in revertants compared to controls in the plate incorporation assay, neither caused a two-fold or greater increase in revertants, which is the commonly accepted measure of biological significance. In the preincubation assay (20 minutes preincubation, no S9), MEHP did induce a dose-dependent increase in mutants, with an approximate three-fold increase in revertants at the highest concentration tested (5 mM). In contrast to the above data, the majority of the published studies have found the phthalate esters and their metabolites to be non-mutagenic in the *Salmonella* mutation assay (reviewed in Budroe and Williams, 1993).

Data on DEHP mutagenicity from other bacterial systems is mixed. Yagi *et al.* (1976) stated in an abstract that MEHP induced DNA damage in *Bacillus subtilis* and mutagenicity in *Escherichia coli.* However, no experimental procedures or data were provided. Tomita *et al.* (1982) studied the effect of DEHP, MEHP, 2-ethylhexanol (2-EH) and phthalic acid in *B. subtilis* and *E. coli*. DEHP, 2-EH and phthalic acid had no effect in the *B. subtilis* Rec-assay at a concentration of 500 µg/disk. However, MEHP was positive at concentrations of 400 and 500 µg/disk (test range 50-500 µg/disk). It was reported that DEHP was positive in the Rec-assay when preincubated with mouse pancreas homogenate, but no supporting data accompanied this report. MEHP also induced dose-dependent mutations in a preincubation (20 min) assay without S9 using a tryptophan-requiring *E. coli* strain (WP2 B/r). Maximum revertants were induced at the highest concentration tested (5 mM DEHP).

DEHP did not display mutagenicity in the presence or absence of Aroclor 1254-induced rat liver S9 in a preincubation 8-azaguanine resistance assay using *S. typhimurium* performed by Seed (1982). Liber (1985) also found that DEHP did not induce mutants in an unspecified strain of *S. typhimurium* using 8-azaguanine resistance as a marker in the presence or absence of Aroclor 1254-induced rat liver S9.

Yoshikawa *et al.* (1983) found that DEHP and MEHP did not induce mutations in *E. coli* strain WP2 try$^-$ (uvrA$^+$ and uvrA$^-$) tested in the presence and absence of Aroclor 1254-induced rat liver.

Mammalian Cell Assays for Genotoxicity (*In Vitro *and* In Vivo*)

Significant increases in chromosomal aberrations were induced by the treatment of CHO cells with mono-2-ethylhexyl phthalate (MEHP) at concentrations of 1.4 mM without metabolic activation, and at concentrations of 1.2 and 1.4 mM with metabolic activation (Galloway *et al.*, 2000). Cell viability at these concentrations was ~20-40%.

The ability of MEHP to cause genetic damage was assessed in immortalized CHO (Chinese hamster ovary) and RL_4 (rat liver) cells (Phillips *et al.*, 1982). In CHO cells, MEHP at concentrations ranging from 0.5 to 10 mM caused dose-related increases in chromosomal aberrations characterized as gaps, breaks, and exchanges, although toxicity was noted at concentrations of 1.5 mM MEHP. A dose-related increase in chromosomal aberrations was observed in RL_4 cells, with growth inhibition only noted above 6.5 mM MEHP. Some variations in sensitivity were observed depending on the medium in which the cells were cultured.

Syrian hamster embryo cells were examined for chromosomal aberrations following treatment with 1 to 100 μM DEHP and 1 to 300 μM MEHP (Tsutsui *et al.*, 1993). Slight, but not statistically significant, increases in the frequency of aberrations were observed in DEHP and MEHP treated cells in the absence of metabolic activation by rat liver microsome fractions. Significant increases in chromosomal aberrations in cells undergoing metaphase were observed in DEHP and MEHP treated cells in the presence of metabolic activation.

Morphological Transformation of Mammalian Cells *In Vitro*

The morphological transformation of Syrian hamster embryo cells by DEHP or MEHP has been reported by a number of investigators (Inui *et al.*, 1976; Tomita *et al.*, 1982; Barrett and Lamb, 1985; Mikalsen *et al.*, 1990a; Mikalsen *et al.*, 1990b; Mikalsen and Sanner, 1993). Co-treatment of these cells with amitrole, an inhibitor of catalase activity, did not significantly increase the transformation potency of DEHP, but did with MEHP (Mikalsen *et al.*, 1990b). The authors also noted that the lack of effect of catalase inhibition suggests that peroxisome-derived H_2O_2 is unlikely to play a major role in DEHP/MEHP-induced morphological transformation in this model system.

Overview of Genotoxicity Data

Overall, DEHP does not induce mutations based upon the results of numerous *in vitro* and *in vivo* studies. There is evidence, however, that DEHP and a primary metabolite, MEHP, are capable of causing certain chromosomal changes and morphological transformation in rodent cells, which potentially may be involved in eliciting a carcinogenic response. No evidence of direct DNA damage or adduct formation is available.

Peroxisome Proliferator Activated Receptor-Alpha (PPAR-α) Mediated Carcinogenesis

Background

The coordinated response of rodents to the set of structurally unrelated compounds referred to as peroxisome proliferators suggested the involvement of a specific receptor. The primary effects from exposure to this set of chemicals included hypertrophy of liver cells, liver enlargement, carcinogenesis, and the induction of fatty acid metabolizing enzymes. The common finding of an increase in the number and size of subcellular organelles called peroxisomes led to the naming of this set of chemical compounds as "peroxisome proliferators." This class of

compounds includes phthalate esters, certain organic solvents, and hypolipidemic agents, among others (see Table 7 below).

Evidence for a Role for PPAR-α in Rodent Carcinogenesis

The peroxisome proliferator-activated receptor (PPAR) was identified by Issemann and Green in 1990 (Issemann and Green, 1990). Since that time, a considerable amount of research has gone toward identifying the normal function of this receptor and its potential role in the carcinogenesis caused by peroxisome proliferators. Discussion here will focus on the PPAR-α since this isoform has been most closely associated with the hepatocarcinogenic effects observed in rodents. The understanding of this receptor's role in the carcinogenic process is critical to moving forward in the cancer risk assessment of DEHP and other peroxisome proliferators.

The most compelling evidence for a role for PPAR-α in carcinogenesis induced by peroxisome proliferators has come from studies utilizing mice in which the PPAR-α gene has been effectively or functionally removed (so called 'knockout' mice). Lee *et al.* (1995) created a strain of mice (in a background of Sv/129 × C57BL/6N) which were constitutively disrupted by homologous recombination in the region of the PPAR-α gene coding for the ligand-binding domain. Treatment of these mice in their diet for two weeks with two peroxisome proliferators – Wy-14,643 (0.1%) and clofibrate (0.5%) – resulted in none of the effects characteristic of these chemicals in the wild-type mice, including hepatomegaly, peroxisome proliferation in the liver, and induction of acyl-CoA oxidase (ACO), bifunctional enzyme, cytochromes P-450 4A1 and 4A3. A "slight increase" in thiolase expression was observed in PPAR-α null (*i.e.*, knockout) mice treated with both chemicals. In the PPAR-α null mice there was also a notable "abundant accumulation of lipid droplets" compared to the wild-type treated animals, which the authors attributed to the disruption of lipid homeostasis caused by the loss of a functional PPAR-α. While the bioassay results presented in this study are limited due to the limited duration of the study, they do provide strong evidence for the involvement of this receptor in mediating effects characteristic of those caused by peroxisome proliferators, using two relatively potent peroxisome proliferating hepatocarcinogens.

Longer term studies were conducted using these PPAR-α null mice to investigate the potential involvement of this receptor in carcinogenesis (Peters *et al.*, 1997). Groups of PPAR-α null and wild-type male mice (4-10/group) were fed diet containing 0.1% Wy-14,643 for one or five weeks, or 11 months. Significant increases in liver weight in response to treatment were observed at all time points. Among wild-type mice observed for 11 months, none of the nine untreated mice developed liver tumors, whereas all of the six Wy-14,643 treated mice developed liver tumors (all six with adenomas, three with carcinomas). Among PPAR-α null mice, neither Wy-14,643 treated nor untreated control mice developed liver tumors (0/9 for each). Although the size of the experimental groups is small and the duration of exposure is less than lifetime, this study provides compelling evidence for a role of the PPAR-α in the hepatocarcinogenic response.

Studies using the PPAR-α knockout mouse to investigate the role of this receptor in mediating the toxic effects of DEHP were conducted by Ward *et al.* (1998). Briefly, both wild-type and knockout mice (five per group) were fed diet containing 0 or 12,000 ppm DEHP for four, eight or 24 weeks. Among wild-type mice, DEHP treatment resulted in increased liver weight at all

time points, and increased ACO, bifunctional enzyme, 3-ketoacyl-CoA thiolase, cytochrome P-450 4A1, and cytochrome P-450 4A3 messenger RNAs (mRNAs) (considered indicators of peroxisome proliferation) at eight weeks. Also, among wild-type mice treated with DEHP, the degree of hepatocytomegaly and cytoplasmic granular hepatocyte eosinophilia (deemed caused by peroxisome proliferation) and pigmented Kupffer cells were observed to increase in a time-dependent manner. None of these effects (liver weights, mRNAs indicative of peroxisome proliferation, and hepatic lesions) were observed among knockout mice treated with DEHP, suggesting a critical role for the PPAR-α in mediating these effects in mice. It was noted that at 24 weeks, the livers of knockout mice "appeared pitted," however, the authors did not comment on the toxicological significance of this finding. Tumorigenic endpoints were not examined in this study, although the limited duration of exposure to DEHP (24 weeks) indicates that this might not be informative. No longer term or lifetime bioassays of DEHP have been conducted in PPAR-α knockout mice. It is notable that in the PPAR-α null mice, treatment with DEHP did result in some toxicity to both the kidney and testes (albeit lower than that observed with wild type mice), suggesting that PPAR-α-independent toxicity remains an issue.

PPAR-α

The PPAR-α is a member of a nuclear receptor superfamily. Other closely related members of this family include PPAR-β/δ and PPAR-γ (isoforms γ1 and γ2), which show significantly different patterns of expression and ligand-binding. The studies described above with PPAR-α knockout mice, however, strongly implicated only the PPAR-α as a mediator of carcinogenesis by peroxisome proliferators. The normal physiological functions of PPAR-α have been inferred to include a role in lipid metabolism and the regulation of inflammation based upon both tissue expression and the spectrum of genes regulated by the receptor (see below).

The functional domains of the PPAR-α protein include a transcriptional activation domain (which is ligand independent and termed the A/B region), a DNA binding domain (zinc finger; C domain) which contains protein sequences which recognize with some degree of specificity DNA regions termed peroxisome proliferator response elements (PPREs) and hinge regions which couple the DNA binding domain (C) with the ligand binding domain (D).

Current evidence indicates that the constitutive or endogenous ligands for the PPAR-alpha include several saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, and eicosanoids (see Table 7). Synthetic or exogenous ligands vary widely in chemical structure; a selection of known or suspected ligands is presented in the table. While binding to the receptor has been demonstrated for a number of these compounds, binding has only been inferred by enzyme activity assays for many of the other chemicals.

Table 7. Partial List of Ligands for the PPAR-α (derived from Corton *et al.*, 2000a)

Constitutive PPAR-α Ligands	Synthetic/Exogenous PPAR-α Ligands
Palmitic acid	Wy-14,643
Stearic acid	Clofibrate
Palmitoleic acid	Ciprofibrate
Oleic acid	Gemfibrozil
Elaidic acid	Nafenopin
Linoleic acid	GW2331
Alpha-linoleic acid	Bezafibrate
Gamma-linoleic acid	Mono(2-ethylhexyl)phthalate
Dihomo-gamma-linoleic acid	Trichloroacetic acid
Arachidonic acid	ETYA (synthetic arachidonic acid)
Eicosapentaenoic acid	MK-571 (leukotriene B4 antagonist)
Docosahexaenoic acid	LY-17183 (leukotriene B4 antagonist)
PGA1, PGA2	KRP-297 (antidiabetic thiazolidinediones)
PGD1, PGD2	Indomethacin
PGJ1	Ibuprofen
	Fenoprofen

The PPAR-α functions by forming a transcriptionally active protein complex comprised of PPAR-α, the ligand, and a protein called retinoid X receptor (RXR) (reviewed in Schoonjans *et al.*, 1996b). This complex then binds to PPRE DNA sequences, generally found in the promoter regions of genes regulated by PPAR-α. Receptors other than PPAR-α may also bind to certain PPREs. The "consensus" PPRE DNA sequence has been shown to consist of a direct repeat of the sequence TGACCT with one base separation (reviewed in Corton *et al.*, 2000b). It should be noted that the consensus sequence represents the most likely sequence occurrence for a specific response element. In reality, within a given species, response elements which may control the expression of different genes may have different sequences. Interaction of the PPAR-α/RXR heterodimer with the transcription machinery is thought to require the participation of additional modulating factors (such as the cyclic AMP-responsive element binding (CREB)-binding protein/p300 or PPAR-γ coactivator-1), although there is still considerable uncertainty in this area (reviewed in Yeldandi *et al.*, 2000). A number of genes have been shown to be responsive to activation of the PPAR-α. Among them are three important peroxisomal enzymes involved in fatty acid β-oxidation, ACO, bifunctional enzyme, and ketoacyl-CoA thiolase. Some of the genes responsive to peroxisome proliferators via PPAR-α are indicated in Table 8 below.

Table 8. Partial List of Genes Responsive to PPAR-α Activation.

Gene Name	Reference(s)
Acyl-CoA oxidase	Tugwood *et al.*, 1992
Acyl-CoA synthetase	Schoonjans *et al.*, 1995
Apolipoprotein A-I	Staels and Auwerx, 1998
Apolipoprotein A-II	Vu-Dac *et al.*, 1995
Apolipoprotein C-III (down regulation)	Clavey *et al.*, 1999
Cytochrome P-450 4A1 (CYP4A1; early induction)	Aldridge *et al.*, 1995
Cytochrome P-450 4A6 (CYP4A6; ω-hydroxylase)	Muerhoff *et al.*, 1992; Palmer *et al.*, 1994; Hsu *et al.*, 1995
Cytochrome P-450 7A1 (CYP7A1; cholesterol 7α-hydroxylase)	Cheema and Agellon, 2000
Cytochrome P-450 4F14 (rat)	Cui *et al.*, 2001
Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bi-(tri-) functional enzyme	Bardot *et al.*, 1993; Bardot *et al.*, 1995
L-Fatty acid binding protein	Ockner *et al.*, 1993; Poirier *et al.*, 1997
3-Ketoacyl-CoA thiolase B	Kliewer *et al.*, 1992; Nicolas-Frances *et al.*, 2000
Lipoprotein lipase	Schoonjans *et al.*, 1996a
Malic enzyme	IJpenberg *et al.*, 1997
Medium-chain acyl-CoA dehydrogenase	Gulick *et al.*, 1994
Mitochondrial HMG-CoA synthetase	Rodríguez *et al.*, 1994
Muscle carnitine palmitoyltransferase I	Mascaro *et al.*, 1999
Sterol 12α-hydroxylase	Hunt *et al.*, 2000
Uncoupling protein-3 (UCP-3)	Acin *et al.*, 1999; Brun *et al.*, 1999

Tissue- and Species-Specific Expression of PPAR-α

There is considerable evidence that PPAR-α is expressed in some tissues of all mammalian species examined to date. Species examined include the rat, mouse, guinea pig, rabbit, and human.

PPAR-α Expression in Experimental Animals

One of the more comprehensive evaluations of the tissue-specific expression of PPAR-α in adult male and female rats has been reported by Braissant *et al.* (1996) who used *in situ* mRNA hybridization and immunohistochemical techniques to evaluate expression. Most broadly, PPAR-α is expressed in the liver (hepatocytes), digestive tract (stomach, intestinal enterocytes), retina, kidney (proximal tubules), spleen, and brain of rats, as indicated by the expression level of mRNA measured by *in situ* hybridization techniques. The authors noted that in the liver "expression varies between individuals" (ten animals were evaluated) and expression was higher in periportal regions compared to pericentric regions. A possible explanation for the variability was proposed by the authors based on studies showing that PPAR-α's expression may be modulated by stress, diurnal rhythm, and glucocorticoids (Lemberger *et al.*, 1994; Lemberger *et al.*, 1996a; Lemberger *et al.*, 1996b). Expression of PPAR-α in adult rat liver had previously been reported to vary 10-fold interindividually (unpublished data cited in Lemberger *et al.*, 1994). Other PPARs are expressed either more ubiquitously (PPAR-β/δ) or, in the case of PPAR-γ, with a different relative tissue distribution (primarily fat, spleen and intestine). The Lemberger *et al.* (1994, 1996a,b) studies showed that the glucocorticoid dexamethasone was able to induce expression of PPAR-α *in vitro* and *in vivo* and that stress (using an immobilization model) was similarly able to induce PPAR-α expression. A 4.5-fold increase in liver, but not

hippocampal, PPAR-α mRNA was induced by four-hour immobilization of Fischer 344 rats (Lemberger *et al.*, 1996b). Studies of the diurnal rhythm of PPAR-α by the same investigators showed that expression cycled daily with the lowest observed expression occurring in the morning (~9:30 a.m.) and the highest in the late afternoon (~6:30 p.m.), with a magnitude of change of approximately three-fold. The authors noted that this cycle corresponds well with the levels of circulating corticosterone in the plasma.

Mukherjee *et al.* (1994) used RNase protection assays of RNA samples from rat tissues (strain not stated) to show relatively high expression in muscle, heart, liver, and kidney, and relatively low expression in bone, brain, and lung. No transcripts were identified in rat spleen and testis.

Using RNase protection assays, the expression of PPARs was examined in male and female NMC mouse tissues (Jones *et al.*, 1995). PPAR-α mRNA expression was highest in liver, with somewhat less expression in the kidney and intrascapular brown adipose tissue. No sex differences in PPAR-α mRNA and protein expression in liver were found. Treatment of animals with methylclofenapate, a peroxisome proliferator, did not affect the expression of PPAR-α. These investigators also examined the expression of PPAR-α in 12 other mice strains identified as Bkl:TO, Bk:W, SWr/Bkl, FVB/NBkl, S129/Bkl, DBA/2/Bkl, C57BL/10Bkl, C57BL/6Bkl, C3H/HeBkl, CBA/CaBkl, BALB/cBkl and *Mus domestica* Zalende (Switz) Bkl. Absolute expression levels of PPAR-α mRNA were found to vary approximately three- to four-fold, which the authors termed "relatively constant."

The expression of PPAR-α mRNA by Northern blot and by *in situ* hybridization has been evaluated in rabbits, with particular attention to its expression in the urinary tract (Guan *et al.*, 1997). Heart, liver, and kidney tissue showed the highest expression levels, although all organs examined showed some level of expression. Within the urinary tract, PPAR-α expression was highest in the proximal tubules and medullary thick ascending limbs of the kidney, as well as ureter and bladder tissue.

More recent studies have demonstrated PPAR-α mRNA expression in non-human animal tissues including rat vascular smooth muscle cells (Diep *et al.*, 2000), rat and mouse brown adipocytes (Valmaseda *et al.*, 1999; Teruel *et al.*, 2000), and mouse mammary gland tissue (by Northern blot analysis) (Gimble *et al.*, 1998). A study examining chicken PPAR-α expression complemented that observed in other animals, that is, expression was primarily found in liver, heart, kidney (plus the lipogenic uropygial gland; Diot and Douaire, 1999).

PPAR-α Expression in Humans

Since the cDNA cloning of the PPAR-α from humans (Sher *et al.*, 1993; Mukherjee *et al.*, 1994), it has been clear that there is the potential for expression of this gene in human tissues and populations, and the potential for attendant responsiveness. The distribution of PPAR-α expression in human tissues, however, has not been examined as extensively as it has in experimental animals.

Subsequent to cloning the cDNA for PPAR-α from a human liver cDNA library, Mukherjee *et al.* (1994) examined its expression in mRNA from various adult human tissues using a commercially available Northern blot. The number of humans from which the panel of mRNAs

was produced was not stated. PPAR-α mRNA was "highly expressed in skeletal muscle, heart, liver, and kidney" and "at low levels in the brain and lung." Some PPAR-α expression in pancreas and placenta was also apparent. Expression of β-actin was also measured on the same blot, but varied considerably, making estimation of the relative expression levels difficult.

Auboeuf *et al*. (1997) examined the expression of PPAR-α mRNA using quantitative polymerase chain reaction (PCR) techniques in several human tissues including liver, small and large intestine, and kidney (from non-obese non-diabetic subjects; gender not identified), abdominal subcutaneous fat tissue (from morbid obese patients; three male, seven female), skeletal muscle and fat tissue (from lean subjects, five men, five women and diabetic subjects, three men, six women). The relative expression levels of PPAR-α for human tissues were as follows: liver > large intestine ≈ kidney ≈ skeletal muscle > small intestine > adipose tissue. Some level of PPAR-α mRNA expression was observed in all tissues examined and no condition-related (obesity, diabetes) differences in expression were found. The authors considered the mRNA expression levels for PPAR-α to be "low" in all tissues except liver, which they termed "high." Additional RNA samples derived from human cerebellum, adrenal, testis, monocytes, and fetal brain were also examined and found to express PPAR-α at "very low" levels.

Using *in situ* RNA hybridization and immunostaining techniques, Guan *et al.* (1997) examined the expression of PPAR-α in the kidney and ureter of a male accident victim and in bladder tissues removed from the benign margins of patients' bladder tumors (number not stated). Expression of mRNA in the kidney appeared mainly in the cortical tubules and outer medulla, (similar to the expression pattern observed in rabbits). No expression was observed in the medullary collecting duct. In the bladder, PPAR-α expression was detected in urothelial cells of the ureter, as well as the bladder itself.

Palmer *et al.* (1998) compared the expression of PPAR-α in human and mouse liver, as well as human cell lines, two derived from hepatomas (HepG2, Huh7) and another from a breast carcinoma (T47D). Twenty human liver samples from males and females, three to 66 years of age, which had been frozen within ten hours of death, were obtained from the University of Minnesota's Liver Tissue Procurement and Distribution System. The clinical histories of the patients varied, with five cases reported as having fatty liver, six on a variety of medications, and some liver function tests available for ten. CD-1 and BALB/c mouse liver samples were also prepared, although the authors did not report the time after death that the samples were frozen. Human PPAR-α mRNA expression levels, derived from a subset of ten subjects' liver samples, spanned an approximately three-fold interindividual range based upon RNase protection assays. An approximately ten-fold lower level of PPAR-α expression (relative to the housekeeping gene, β-actin) was observed between the ten human liver samples and the CD-1 and BALB/c mouse livers. The authors considered it unlikely that the lower level of expression resulted from "poor recovery" of RNA from the human liver samples because expression of the β-actin gene was similar to that in mice. PPAR-α expression in the Huh7 cell line approximated that found in the ten individual livers, expression in the HepG2 cell line was several fold below that, and no expression was observed in the T47D cell line. The issue of the stability of PPAR-α mRNA in liver following death was not addressed in the paper.

Palmer *et al.* also used electrophoretic mobility shift assays (EMSA) to evaluate the PPAR-α levels in the human liver samples. The authors reported that, in seven of 20 samples evaluated, PPAR-α protein levels (as measured in this particular assay) were approximately 10-fold below those observed in mouse liver samples (both CD-1 and BALB/c). The other 13 samples evaluated had even lower PPAR-α protein levels (with three samples undetectable by this assay).

The expression of PPAR-α was evaluated in human tissues by Su *et al.* (1998) using monoclonal antibodies for the N-terminal domain of the receptor. The expression of the protein paralleled that of RNA reported previous to this study; that is, expression in human tissue lysates was highest in skeletal muscle, liver, and kidney. Low levels were also detected in adrenals, placenta, and lung. These authors reported no detectable expression in lysates from heart and small intestine.

Tugwood *et al.* (1998) compared the expression of PPAR-α mRNA in a single "fresh" sample of total RNA from Alderley Park mouse, F344 rat, Syrian hamster, Hartley guinea pig, and human liver using a hybridization probe for mouse PPAR-α. No further characterization of the human mRNA sample (or others) was presented. Using the expression of serum albumin (as detected with a human gene probe) to standardize expression levels, the relative levels of PPAR-α expression (in arbitrary units) for the different species was 100 (mouse), 61 (rat), 69 (hamster), 13 (guinea pig), and 7 (human). Similar results were obtained using a probe for guinea pig PPAR-α.

Expression of PPAR-α mRNA has also been demonstrated in freshly isolated human monocytes as well as in their more differentiated form, macrophages, with a trend toward increased expression as the cells reached a more differentiated phenotype (Chinetti *et al.*, 1998). The increase in transcription was confirmed by protein analysis (Western blot). Immunofluorescence studies indicated that PPAR-α protein was localized in the macrophage cytoplasm. Transfection of macrophages with a PPRE driven reporter plasmid accompanied by treatment with activators of PPAR-α demonstrated that a functional PPAR-α protein was produced by the cells.

Low passage human carotid artery endothelial cells derived from six patients were shown by immunohistochemical techniques to express PPAR-α protein (Marx *et al.*, 1999). *In situ* hybridization, as well as Western blot analysis of nuclear and cytoplasmic cellular fractions, demonstrated that PPAR-α expression was localized in the nucleus.

The expression of PPAR-α was evaluated in skeletal muscle and fat tissues obtained from lean, obese, and Type II diabetic subjects (Loviscach *et al.*, 2000). Muscle tissue generally expressed five- to nine-fold more PPAR-α protein (by Western blot) than fat in these biopsy specimens. No difference in the baseline expression of PPAR-α protein in muscle was observed between Type II diabetic (n = 14) and non-diabetic (n = 22) subjects.

Expression of PPAR-α was examined in prostatic tissues from 49 patients with prostate cancer (Collett *et al.*, 2000). Using *in situ* hybridization and immunohistochemical techniques, PPAR-α mRNA and protein were weakly or not expressed in the prostatic epithelial cells (*vs.* stromal cells) of the normal tissue surrounding the tumors. Expression of PPAR-α in the tumor correlated with histological grade, with considerably higher expression associated with more

advanced prostatic tumors. These investigators also found that PPAR-α levels in an epithelial cell line derived from a human prostate cancer (LNCaP) in which expression was constitutively high were inhibited by the addition of the synthetic androgen mibolerone.

Expression of PPAR-α has been demonstrated in human endothelial cells of umbilical vein and aortal origin (Inoue *et al.*, 1998). The expression of PPAR-α in these cells was inducible by benzafibrate, eicosapentaenoic acid, or dexamethasone, and could be inhibited by insulin. PPAR-α expression (measured by reverse transcriptase-polymerase chain reaction) was considered low compared to its expression in the liver.

Studies using cell lines, especially those derived from tumors, should be viewed cautiously for the interpretation of the potential correlation with human expression or responsiveness *in vivo*, as there is the potential for heritable mutations or aberrations which may produce artifactual effects not relevant to the *in vivo* situation. It remains clear, however, from studies of human tissues, that PPAR-α is widely expressed at both the mRNA and protein level in a number of human tissues, including the liver. To date, the vulnerability of human PPAR-α expression to the same modulatory influences observed in experimental animal cells (steroids, stress, and diurnal rhythms) has not been examined in any study, with the exception of that observed *in vitro* by Inoue *et al.* (1998).

Tissue- and Species-Specific Responsiveness

A review of the scientific literature regarding the responsiveness of cells derived from various species and tissues to DEHP (and other peroxisome proliferators) has produced some evidence for differences among species, with investigators examining the responses of liver cells from species including rodents, guinea pigs, dogs, marmosets, maccaca, and humans (Cornu-Chagnon *et al.*, 1995, citing Bieri *et al.*, 1988; Blaauboer *et al.*, 1990; Elcombe and Mitchell, 1986; Cornu *et al.*, 1992).

Evidence in the literature supports some degree of human responsiveness to peroxisome proliferators *in vitro.* The clinical use of several peroxisome proliferators as hypolipidemic agents also points to a responsiveness of humans. Based on the data available at this time, it would be inappropriate to conclude that humans are unresponsive or refractory to peroxisome proliferators. Further investigation and characterization of the extent to which human tissues are responsive to peroxisome proliferators is needed. The relevance of those responses which have been observed to the carcinogenic process also needs to be established. The available studies have sought to examine the effects of DEHP and other peroxisome proliferating compounds on human cells *in vitro* and have included studies of the effects of DEHP on primary cultures of human hepatocytes, as well as established cell lines derived from human tumors.

Experimental Animal Studies with DEHP/MEHP and Other Peroxisome Proliferators

Numerous studies have examined the effects of DEHP, MEHP, and other peroxisome proliferators on many endpoints in experimental animals, including some which have attempted to do side-by-side interspecies comparisons of responsiveness. Studies in which human and experimental animal cells are compared are described in the "Human" sections below. Described briefly in this section are some of the important findings (from studies other than cancer bioassays) of the pleiotropic effects on experimental animals which have come to characterize the peroxisome proliferator response.

Exposure to DEHP and other peroxisome proliferators causes peroxisome proliferation in rodents, a response consisting of increases in liver size and in number and size of peroxisomes in the liver cells (visible microscopically), frequently accompanied by increases in mitochondria and lysosomes. Biochemical changes include increases in enzymes associated with oxidative metabolism of fatty acids in both peroxisomes (oxidase enzymes) and mitochondria (β-oxidation). In assays for such activities following exposure to peroxisome proliferators, rodents have shown a greater responsiveness than other mammalian species, including primates. Effects from two week administration of DEHP at 2% in feed was examined in male and female Sprague-Dawley rats, male Wistar rats, male mice (strain not stated), and guinea pigs (strain not stated) (Osumi and Hashimoto, 1978). Significant increases in palmitoyl-CoA oxidase were observed among male Wistar rats (22-fold), male Sprague-Dawley rats (16-fold), female Sprague-Dawley rats (5.5-fold), and mice (7-fold). No significant increase in palmitoyl-CoA oxidase activity was observed in guinea pigs as a result of this treatment.

Male Sprague-Dawley rats and Syrian hamsters were compared in their response to oral treatment for two weeks with MEHP or clofibrate at doses of 500 mg/kg each (Lake *et al.*, 1984). Rats treated with MEHP showed an induction of palmitoyl-CoA oxidase activity (14-fold) and CAT activity (21-fold). Treatment of hamsters with MEHP resulted in only a 1.7-fold increase in palmitoyl-CoA oxidase activity and 1.6-fold increase in CAT activity. Both rats and hamsters also showed a significant increase in relative liver weight and succinate dehydrogenase activity. Carnitine palmitoyltransferase activity was increased 1.6-fold in hamsters, but not rats.

The response of a number of species including cats, chickens, pigeons, and rhesus and cynomolgus monkeys to treatment with the peroxisome proliferator ciprofibrate for three to seven weeks was examined (Reddy *et al.*, 1984). In all species, an induction of enzyme indicators of peroxisome proliferation including peroxisomal catalase, carnitine acetyl transferase (CAT), heat-labile enoyl-CoA hydratase, and enzymes of the fatty acid β-oxidation system was observed.

Adult rhesus monkeys were examined for responsiveness to a hypolipidemic agent called DL-040 [4-(((1,3-benzodioxol)-5-yl)methyl)amino-benzoic acid] (Lalwani *et al.*, 1985). Briefly, monkeys were treated at 300 mg/kg for one week or at 400 mg/kg for 11 weeks. Significant increases in peroxisome volume density, and peroxisomal enzyme activities including catalase (1.9-fold), CAT (20-fold+), enoyl-CoA hydratase (6.4-fold), palmitoyl-CoA oxidase (7.2-fold), and urate oxidase (1.9-fold).

Rats and marmosets were also compared in responsiveness to DEHP administered either orally or intraperitoneally (Rhodes *et al.*, 1986). DEHP administered at 5 mmol/kg_{bw} for two weeks did not result in discernable morphological changes to the liver or increases in peroxisomal β-oxidation enzymes, although catalase activity was significantly increased in the monkeys.

Male Fischer 344 rats and cynomolgus monkeys were compared in responsiveness to DEHP treatment for three weeks (Short *et al.*, 1987). Rats treated with 1000 ppm DEHP showed increases in peroxisome proliferation, whereas monkeys treated by oral gavage at doses up to 500 mg/kg_{bw} showed no indications of peroxisome proliferation or induction of palmitoyl-CoA oxidase or CAT activities.

Primary cultures of marmoset hepatocytes were compared with those of rat in responsiveness to treatment with nafenopin (Bieri *et al.*, 1988). Treatment of marmoset hepatocytes with concentrations of nafenopin ranging from 1 to 30 μg/ml did not result in a significant increase in peroxisomal β-oxidation activity compared to controls, although a significant increase in unscheduled DNA synthesis was observed. Rat hepatocytes showed both the induction of peroxisomal β-oxidation activity as well as increases in unscheduled DNA synthesis.

The effects of nafenopin administered for three weeks by gastric intubation on the liver was examined in male Sprague-Dawley rats (n = 5; 0.5 – 50 mg/kg-day), Syrian hamsters (n = 6; 5 – 250 mg/kg-day), Dunkin-Hartley guinea pigs (n = 6; 50 and 250 mg/kg-day), and marmosets (n = 3; 50 and 250 mg/kg-day) (Lake *et al.*, 1989). Significant increases in relative liver weight were observed in rats and hamsters. An increase in relative liver weight was also observed in marmosets, although the increase was not statistically significant. Rats showed significant increases in palmitoyl-CoA oxidase, enoyl hydratase, and CAT activity in multiple doses. Hamsters showed increases in palmitoyl-CoA oxidase and CAT, although to a less extent than that observed in rats. Guinea pigs and marmosets showed significant increases in palmitoyl CoA-oxidase, carnitine palmitoyl transferase and CAT (guinea pigs only) at 250 mg/kg-day. Thus, the relative responsiveness was as follows: rat >> hamster > marmoset ≈ guinea pig. No effects on catalase activity were observed. In each species, nafenopin treatment also resulted in significant increases in microsomal lauric acid 11-hydroxylase activity and 12-hydroxylase activity (except marmoset). Marmosets showed significant induction of ethylmorphine N-demethylase and cytochrome P450 activity as a result of nafenopin treatment. Guinea pigs showed significant induction of cytochrome P450, ethylmorphine N-demethylase, and 7-ethoxycoumarin O-deethylase activities as a result of nafenopin treatment. Rats and hamsters did not show induction of these enzyme activities except rats which showed slight induction of cytochrome P450 activity.

Marmosets (14/sex/group) were treated for three years by oral gavage with ciprofibrate at doses of 0, 2, 10, or 20 mg/kg_{bw} (Graham *et al.*, 1994). Significant increases in peroxisomal β-oxidation, CAT, and carnitine palmitoyl transferase activity were observed in both male and female marmosets in the two highest dose groups, with increases noted in the range of 1.7- to 2.5-fold, maximally. No histological changes to the liver were observed.

Marmosets were examined for toxicological effects caused from oral exposure to DEHP (Kurata *et al.*, 1998). The marmosets (four/group) were treated with 0, 100, 500, or 2500 mg/kg DEHP for 13 weeks. An additional group was treated with clofibrate at a single dose (250 mg/kg) for reference. An examination of the livers of treated animals generally showed no increases in organ weight, hypertrophy, or peroxisome volume, number, morphology, or peroxisomal enzyme activity (D-amino acid oxidase, cyanide-insensitive ACO, carnitine-dependent acetyl transferase, and carnitine-dependent palmitoyltransferase). Among males in the mid- and high-dose groups, however, 33 and 36% increases ($p < 0.05$) in mean peroxisome volume were observed, respectively. In the same study, among female marmosets treated with 250 mg/kg clofibrate (the only dose tested), ACO and CAT activities were elevated 91% and 67%, respectively ($p < 0.01$), and among similarly treated male marmosets, carnitine-dependent palmitoyltransferase activity was increased 80% ($p < 0.05$).

Choudhury *et al*. (2000) examined the effects of short-term administration of methylclofenapate and Wy-14,643 on hamsters and guinea pigs. Treatment for three day with 50 mg/kg of each agent resulted in significant increases in relative liver weight in hamsters, but not guinea pigs. Doses of 25 mg/kg of each agent resulted in increases in blood triacylglycerols in both guinea pigs and hamsters, however. Mice treated with methylclofenapate showed increased expression of CYP4 mRNA, however, guinea pigs similarly treated showed no increased expression of this gene.

Isenberg *et al.* (2000) examined the effect of treatment of male rats, mice, and hamsters with DEHP in the diet at levels ranging from 500 to 20,000 ppm DEHP for varying lengths of time up to six weeks on endpoints including peroxisomal β-oxidation activity, gap-junctional intercellular communication (GJIC), and replicative DNA synthesis. In all three species, treatment for several weeks resulted in statistically significant increases in relative liver weight and peroxisomal β-oxidation activity. In rats, peroxisomal β-oxidation activity increased up to 20-fold relative to controls following treatment for six weeks at 6,000 ppm DEHP. In mice, peroxisomal β-oxidation activity increased up to 14-fold following treatment for two weeks with 500 ppm DEHP. In hamsters, peroxisomal β-oxidation activity increased up to 2.4-fold over controls following treatment for two weeks with 1000 ppm DEHP.

Measurements of GJIC by *in situ* dye transfer in liver slices from the different rodents showed that in rats and mice, but not hamsters, DEHP in the diet inhibited GJIC, although slight inhibition was observed in hamsters. Replicative DNA synthesis was assessed in periportal and centrilobular hepatocytes independently in each of the rodents. Dose related increases in both periportal and centrilobular hepatocyte replicative DNA synthesis were observed in both rats and mice. A small, but statistically significant, increase in replicative DNA synthesis in centrilobular hepatocytes was observed in hamsters treated with 6000 ppm DEHP for two, but not four weeks.

Human Studies *In Vitro* with DEHP/MEHP

The set of studies examining the potential responsiveness of human cells to DEHP/MEHP for obvious reasons been limited to studies of isolated hepatocytes. Described below are several studies which have compared the responsiveness of human and non-human hepatocytes with respect to several endpoints which may be relevant to the hepatocarcinogenic process.

Butterworth *et al.* (1984) used primary cultures of human hepatocytes prepared by perfusion of liver samples obtained from discarded surgical material to examine the effect of several carcinogens, including DEHP and other peroxisome proliferators, on the DNA repair response and the induction of enzymes associated with peroxisome proliferation. Hepatocytes from three individuals failed to show an increase in DNA repair response to concentrations of DEHP ranging from 0.1 to 10 mM. Similarly, hepatocytes from four individuals failed to show a response in this assay to MEHP and to the known peroxisome proliferator Wy-14,643. The ability of these cells to exhibit a measurable DNA repair response was demonstrated following treatment with other carcinogens such as aflatoxin B1 and 1,6-dinitropyrene. Rat hepatocytes treated with DEHP also failed to show an increase in DNA repair response in these studies. Hepatocytes from two individuals showed no increase in palmitoyl-CoA oxidase or CAT following treatment with clofibric acid, MEHP, or Wy-14,643. An approximately 10-fold induction of these enzymes was observed in rat hepatocytes treated with the same concentration.

Elcombe and Mitchell (1986) reported on the responsiveness of primary cultures of rat, guinea pig, marmoset, and human hepatocytes to treatment with MEHP, using induction of palmitoyl-CoA oxidase as the marker of response. Human liver slices were obtained from renal transplant donors and hepatocytes were isolated by enzyme digestion of the slices. Only rat hepatocytes responded to treatment with MEHP at concentrations ranging from 0.05 to 0.5 mM MEHP with induction of palmitoyl-CoA oxidation (15-fold induction). Marmosets showed a slight increase in palmitoyl-CoA oxidation activity at 0.25 mM MEHP, although a higher dose showed no induction. The human hepatocytes were considered to be viable based upon parallel cultures which showed phenobarbitone-induced cytochrome P-450 mediated ethoxycoumarin-*O*-deethylase activity.

Bichet *et al.* (1990) investigated the effects of MEHP on human and rat hepatocytes as part of a study focussed primarily on two other peroxisome proliferators, benzbromarone and clofibric acid. Human hepatocytes obtained from surgically resected tissue in liver cancer patients (n = 3) treated *in vitro* with MEHP (0.5-1.0 mM) showed neither induction of palmitoyl-CoA oxidase nor any indication of peroxisome proliferation as assessed by electron microscopy.

Goll *et al.* (1999) compared the effects of several peroxisome proliferators including DEHP on the induction of DNA synthesis, ACO activity, and CAT activity in primary cultures of Sprague-Dawley rat hepatocytes isolated by collagenase perfusion and human hepatocytes isolated from three liver cancer patients undergoing partial hepatectomy (collagenase digestion without perfusion). In rat hepatocytes, DEHP at 100 µM for 72 hours was found to increase ACO activity approximately 2-fold over controls and CAT activity approximately 1.2-fold over controls. These increases in rat ACO and CAT activity were both statistically significant. A concentration of 250 µM DEHP showed an approximately 1.6-fold increase in rat ACO activity and 1.3-fold increase in CAT activity, although only the increase in CAT activity was statistically significant. DEHP at 500 µM did not produce significant increases in either enzyme activity. Other peroxisome proliferators tested all showed higher levels of induction of these enzymes (ciprofibrate = nafenopin > bezafibrate > clofibric acid > DEHP). DEHP induced a 1.2- to 1.5-fold increase in DNA synthesis (as measured by 5-bromo-2'-deoxyuridine (BrdU) incorporation) in rat hepatocytes relative to controls following treatment at concentrations of up to 250 µM DEHP for 48 hours. Spontaneous apoptosis (as measured by DNA fragmentation) was significantly inhibited 60-80% by each of the peroxisome proliferators tested, although a concentration dependence was not observed (except with nafenopin). Rat hepatocytes treated with peroxisome proliferators showed a significant apoptotic response following treatment with transforming growth factor-β (TGF-β), but not with tumor necrosis factor-α (TNF-α)/α-amanitine (an inhibitor of RNA polymerase), a treatment which has also been demonstrated to induce apoptosis in rat and human hepatocytes. The authors suggested that these findings indicate that the RNA synthesis or the TNF-α response pathway may play a role in inhibition of the apoptotic response observed with peroxisome proliferators.

In human hepatocytes, incubation with 250 µM DEHP for 72 hours increased ACO activity 1.5-fold over controls, although the increase was not statistically significant. A slight, but not statistically significant, decrease (15%) in human CAT activity was observed with the same DEHP treatment. The other peroxisome proliferators were less active than DEHP in inducing ACO activity in human hepatocytes. No increases in human CAT activity or DNA synthesis and

no decrease in spontaneous apoptosis were observed in response to any of the other peroxisome proliferators. Human hepatocytes treated with the peroxisome proliferators also did not show an apoptotic response following stimulation with either TGF-β or TNF-α/α-amanitine.

Hasmall *et al.* (1999) compared the responsiveness of primary cultures of hepatocytes derived from surgically resected human liver (n = 3) and male Fischer 344 rats to MEHP and diisononylphthalate (DINP), measuring replicative DNA synthesis and apoptosis as endpoints. Results regarding the induction of peroxisomal β-oxidation in both species are presented, but the methods and endpoints used for measurement were not included in the paper. No clear dose-response relationship for replication of DNA was observed in human hepatocytes prepared from three separate donors in response to 250 to 750 μM MEHP or DINP, although statistically significant increases were observed in a single donor at the low dose of MEHP (250 μM) and the mid-dose of DINP (500 μM). Treatment with a positive control substance for DNA synthesis, epidermal growth factor (EGF) at 25 ng/ml, resulted in an approximately 2-fold induction of DNA synthesis in each of the three human hepatocyte preparations. Rat hepatocytes showed a dose-dependent increase in DNA synthesis following treatment with 250 to 750 μM MEHP or DINP. No positive control results were reported for the rat hepatocyte studies.

Human hepatocytes obtained from liver surplus from surgical resection and cultured *in vitro* were treated with MEHP and compared to similarly treated Fischer rat hepatocytes (Hasmall *et al.*, 2000). Rat hepatocytes responded to MEHP with induction of peroxisomal β-oxidation at concentrations of 0.25, 0.5, and 0.75 μM MEHP, although no significant increases were observed at 1 or 2 μM MEHP due, according to the authors, to "non-specific toxicity." Human hepatocytes showed no significant increases in peroxisomal β-oxidation activity. Rat hepatocytes also showed induction of DNA replication at 0.5, 0.75, and 1 μM MEHP, whereas human hepatocytes showed no induction. Significant induction of DNA replication was observed in both rat and human hepatocytes following treatment with 25 ng/ml EGF, a positive control for DNA synthesis. Apoptosis induced by TGF-β1 was significantly inhibited by MEHP at doses of 0.25-1 μM MEHP in rat hepatocytes, whereas no effect on apoptosis was observed in human hepatocytes at any dose. TGF-β1-induced apoptosis was effectively suppressed in the human hepatocytes by treatment with EGF.

Table 9. Summary of studies comparing responsiveness of primary cultures of rat and human hepatocytes to DEHP or MEHP.[*]

Study	Compound, Dose	Endpoint	Animal Response	Human Response	Notes
Butterworth *et al*., 1984	DEHP 0.1-10 mM	DNA repair	–	–	
		Palmitoyl-CoA oxidase	++	–	3 humans
		CAT	++	–	
		Positive control	+	+	Aflatoxin, 1,6-DNP for DNA repair
Elcombe & Mitchell, 1986	MEHP < 0.5 mM	Palmitoyl-CoA oxidase	++	–	3 humans
		Positive control	+	+	P-450 activity
Bichet *et al*., 1990	MEHP 0.5-1.0 mM	Palmitoyl-CoA oxidase	+	–	3 humans
		Peroxisome proliferation	+	–	
		Positive control	–	–	
Goll *et al*., 1999	DEHP 100 and 250 µM	Palmitoyl-CoA oxidase	++	Slight ↑[*]	[*]Not statistically significant
		CAT	+	Slight ↓[*]	
		DNA synthesis	+	–	3 humans
		Apoptosis suppression	+	–	
		Positive control	–	–	
Hasmall *et al*., 1999	MEHP 0.25-1 µM	β-oxidation	++	–	3 humans
		DNA synthesis	+	±[*]	[*]No dose-response
		Apoptosis suppression	+	–	
		Positive control	–	+	EGF (25 ng/ml)
Hasmall *et al.*, 2000	MEHP 0.25-0.75 µM	β-oxidation	+	–	
		DNA replication	+	–	
		Apoptosis suppression	+	–	
		Positive control	+	+	EGF (25 ng/ml)

[*] The endpoints examined in these studies are among those frequently associated with peroxisome proliferator action in rodents. The relevance of these endpoints to carcinogenesis has not been clearly established, although theoretical links have been made.

Human Studies with Other Peroxisome Proliferators

A number of investigators have examined the potential responsiveness of humans to peroxisome proliferators other than DEHP/MEHP, sometimes through the use of primary cultures of parenchymal hepatocytes isolated from intact liver tissues and other times through the use of cell lines usually established from human tumor tissue.

Slices of human liver obtained from renal transplant donors (n = 2) were digested with collagenase *in vitro* and plated in culture dishes (Elcombe, 1985). No induction of cyanide

insensitive palmitoyl CoA oxidase was observed following treatment with up to 6 mM trichloroacetic acid. The authors indicated that these cells responded to treatment with phenobarbitone and β-naphthoflavone with induction of mixed function oxidase activity (data not presented). Metabolic conversion of trichloroethylene to trichloroacetic acid by the human hepatocytes was also observed indicating metabolic competence of the cells.

The pesticide and peroxisome proliferator fomesafin [5-(2-chloro-α,α,α-trifluoro-4-tolyloxy)-*N*-methylsulphonyl-2-nitrobenzamide] was tested for its ability to induce peroxisomal β-oxidation in mouse, rat, hamster, guinea pig, and human hepatocytes (Smith and Elcombe, 1989). Of these species tested, only the rat and mouse showed a significant response.

Elcombe and Styles (1989; abstract only) reported briefly on a comparison of rat, guinea pig, and human hepatocytes' responsiveness to methylclofenapate. Rat hepatocytes showed a significant increase in S-phase DNA synthesis, lauric acid hydroxylation (an indicator of cytochrome P-452 activity), and cyanide insensitive palmitoyl-CoA oxidation following treatment with 5-150 μM methylclofenapate, whereas human and guinea pig hepatocytes showed no effect.

Blaauboer *et al.* (1990) compared the responsiveness of human, Wistar rat, and *Macaca cynomolgus* monkey hepatocytes to the peroxisome proliferators beclobric acid and clofibric acid. The human hepatocytes were isolated post-mortem from three organ donors (two males, 40 and 42 years old, and one female, 43 years old). The hepatocytes from rat and monkey were isolated by a two-step *in situ* collagenase perfusion method. A dose-related increase in peroxisomal β-oxidation (as measured by NAD^+ generation) and number of peroxisomes was observed in rat hepatocytes treated with doses of up to 300 μM clofibric acid. Human and monkey hepatocytes showed no increase in either of these markers following treatment with clofibric acid. Treatment with beclobric acid also increased peroxisomal β-oxidation and number of peroxisomes in rat hepatocytes, although the response was highly variable at high doses (300 μM). Human and monkey hepatocytes showed no responses to beclobric acid. Providing an indication of cell viability and responsive potential, monkey cells responded to treatment with EGF and inducers of cytochrome P450 isozymes. Data from the human hepatocytes in similar tests were "not available" at the time of publication.

Parzefall *et al.* (1991) examined the DNA synthesis response of isolated human hepatocytes to nafenopin (among other agents). Hepatocytes were isolated from liver tissue of seven subjects (four female, three male; aged 35 to 77 years) which was obtained during the course of partial hepatectomy due to liver tumors or from transplants. Cell viability ranged from 32 to 70%. Nafenopin did not increase DNA synthesis in any of the hepatocyte preparations over controls. Previously published reports indicated increased DNA synthesis in rat hepatocytes exposed to nafenopin (Bieri *et al.*, 1984). The authors also noted an order of magnitude lower responsiveness of human hepatocytes compared to rat hepatocytes to EGF, which served as a positive control. There was little evidence of a dose-response for DNA synthesis to EGF (concentrations between 10 and 100 ng/ml) in the human hepatocytes.

Scotto *et al.* (1995) examined the responses of two liver tumor cell lines, one from a human (Hep EBNA2) and one from a rat (FaO), to the hypolipidemic agent and peroxisome proliferator clofibrate. Two endpoints were examined, catalase activity and fatty ACO activity. Relatively slight induction of catalase activity was observed in both cell lines (1.2-fold for the human and

1.7-fold for the rat) and the response showed little time- or dose-dependence. Fatty ACO, however, was induced 2.4- to 3-fold in the human cell line and 6- to 11-fold in the rat cell line, and the increase was both time- and dose-dependent. The increase in the biochemical activity was supported by increases in mRNA for ACO.

Cornu-Chagnon *et al*. (1995) showed no induction of peroxisomal palmitoyl-CoA oxidase in the cultured human hepatoma cell line, HepG2, following treatment with the peroxisome proliferator fenofibric acid at concentrations up to 1.0 mM. Primary cultures of rat hepatocytes showed an approximately 7-fold induction in this enzyme at a concentration of 0.5 mM.

Richert *et al.* (1996) examined the effects of oxadiazon and clofibric acid (both peroxisome proliferators) on the activity of palmitoyl-CoA oxidase and CAT in human and rat hepatocytes *in vitro*. Rat hepatocytes showed induction of palmitoyl-CoA oxidase of 1.5- to 1.9- fold and CAT of 1.6- to 7.3-fold. Human hepatocytes showed no significant induction of either of these enzymes. According to the authors, slight decreases in palmitoyl-CoA oxidase activity observed at the high dose of oxadiazon tested (10^{-4} M) in both rat and human cells may have been caused by cytotoxicity.

Elcombe *et al.* (1996) examined the effects of several peroxisome proliferators, including methylclofenapate, fomesafen, clofibric acid, 2-ethylhexanoic acid, and mono(2-ethyl-5-oxohexyl)phthalate, on palmitoyl-CoA oxidase activity and S-phase DNA synthesis in primary cultures of both rat (ApfSD strain), guinea pig (Alpk: Dunkin Hartley strain) and human hepatocytes. The human hepatocytes were obtained from surgical resection material and then isolated and viability was estimated at approximately 80%. The human and guinea pig cells did not show induction of palmitoyl-CoA oxidase activity or S-phase DNA synthesis following three days of exposure *in vitro*, whereas the rat hepatocytes showed up to nearly 20-fold induction of palmitoyl-CoA oxidase activity with some agents.

Perrone *et al*. (1998) used cultured hepatocytes obtained from human male transplant donors to examine the effects of several peroxisome proliferating agents on peroxisomal fatty ACO, DNA synthesis, and apoptosis. Comparisons were made with Fischer 344 rat hepatocytes cultured similarly. ACO activity tended to be quite variable using homogenates prepared from both rat and human hepatocytes, although the activity (in units of 10^{-9} moles/μg protein × min) was consistently higher in rats than humans (~10-fold). Ciprofibrate induced a significant increase in ACO activity in five hepatocyte preparations isolated from different individuals; clofibric acid induced significant increases in four preparations. Both compounds displayed a clear dose-response relationship in the 0.1 to 1.0 mM range of concentrations, with induction in the 2- to 3-fold range. Rat hepatocytes showed a similar dose-response with 2- to 3-fold induction. Hepatocytes from both rats and humans showed responsiveness to EGF as evidenced by an increase in DNA synthesis after 48 hours exposure. Addition of clofibric acid or ciprofibrate had no effect on rat hepatocyte DNA synthesis whereas human hepatocytes responded with an inhibition of DNA synthesis (2- to 3-fold). Cultures were examined for effects on apoptosis induced by TGF-β by both peroxisome proliferators. Whereas rat hepatocytes showed responsiveness with an attenuation of the apoptotic response, human hepatocytes did not show this effect.

Primary cultures of human hepatocytes isolated from surplus tissue in transplantation procedures were isolated by collagenase perfusion methods (Hasmall *et al.*, 1998). The basal rate of apoptosis in these cells was not affected by 50 μM nafenopin. TGF-β induced apoptosis in these cells was also not suppressed by treatment of the cells with 50 μM nafenopin. Replicative DNA synthesis was induced in these cells by treatment with EGF, although no data were presented and the extent of induction was not presented.

Cultured human endothelial cells were treated with peroxisome proliferators including fenofibrate and Wy-14,643 (Marx *et al.*, 1999). Pretreatment of cultured cells with both of these agents was able to reduce TNF-α induced expression of vascular cell adhesion molecule-1. The effect of fenofibrate was shown to be dependent on both the concentration and time of pretreatment. While no link to carcinogenic modes of action has been proposed for this endpoint, it remains notable that human cells have been shown to be responsive for each of these peroxisome proliferators.

Rodriguez *et al.* (2000) compared the responsiveness of a rat hepatoma cell line (Morris) to that of a human hepatoma cell line (HepG2). They reported no induction of ACO mRNA in the human cell line compared to that in the rat cell line upon treatment of the cells with ciprofibrate, clofibrate, or bezafibrate.

Cimini *et al*. (2000) examined the effects of the peroxisome proliferator perfluorodecanoic acid (PFDA) (alone and in combination with all-*trans*-retinoic acid) in a human glioblastoma cell line called Lipari (LI). In treated cells, PFDA led to significant increases in numerical density of peroxisomes (~50%) and significant decreases in profile mean diameter and "circular shape factor." No change in overall volume density was reported. Treatment with 0.1 μM PFDA showed a 3-fold increase in ACO activity three days following treatment, but not at one or two days.

Cultures of human endothelial cells derived from either the umbilical vein or from aorta were shown to induce Cu^{2+}/Zn^{2+}-superoxide dismutase mRNA and protein (a scavenger of superoxide) in response to treatment with bezafibrate (Inoue *et al.*, 2001).

Factors Which May Influence Responsiveness or PPAR-α Expression

As described above, the expression of PPAR-α has been shown in experimental animals to be modulated by a number of agents including synthetic chemicals (Wy-14,643), steroids (dexamethasone) and protein hormones (insulin), as well as other factors such as stress and diurnal rhythm. Factors modulating the expression of PPAR-α in humans have not been investigated.

Sher *et al.* (1993) originally described the cloning of the human PPAR-α gene from a human liver cDNA library. Using co-transfection assays of mouse cells, these investigators determined that the human PPAR-α gene product was capable of activating both the mouse ACO promoter and a rabbit cytochrome P-450 4A6 promoter following stimulation of the transfected cells with several peroxisome proliferators (Wy-14,643, nafenopin, and clofibrate), indicating that the human PPAR-α is functional and capable of activating genes via (at least) two different (non-human) genetic response elements.

Experiments such as those conducted by Owens *et al.* (1997) have demonstrated that the human PPAR-α is capable of activating the PPRE and driving the expression of PPAR-α responsive genes in a human cellular environment. These investigators used cotransfection studies into the human hepatoma cell line HepG2 with a plasmid bearing the human PPAR-α gene and a plasmid bearing the rat ACO gene and promoter followed by treatment with Wy-14,643 to demonstrate that active protein was produced under these conditions.

The investigators who characterized the relative expression of PPAR-α in human liver samples and compared it to that of mice (Palmer *et al.*, 1998) also observed that humans express second minor mRNA transcript of PPAR-α. They found that this smaller transcript formed as a result of an alternate splicing of the RNA which would, if translated into protein, result in the production of a non-functional PPAR-α. Whether the production of this alternatively spliced PPAR-α mRNA has any biological significance has not been established, although it has been proposed by these authors to be a factor in diminished PPAR-α expression in humans.

Allelic variants of the PPAR-α gene have been identified in human DNA (Sapone *et al.*, 2000). These allelic variants include mutations in the DNA binding region of the protein and were found in high frequencies in a Northern Indian population. *In vitro* assays suggested that these variant proteins were not as responsive to Wy-14,643 as wild-type PPAR-α. The authors noted that the biological significance of these allelic variants remains to be established. The frequency of variants in the general population has not been established.

Differences in PPREs

Varanasi *et al.* have extensively characterized one of the genes regulated by PPAR-α, the human ACO gene (Varanasi *et al.*, 1994). These investigators further characterized this gene using *in vitro* reporter assays to determine its potential activity in humans as a response to stimulation by peroxisome proliferators (Varanasi *et al.*, 1996[2]). The human promoter was found to be active when cloned into reporter gene constructs and introduced into a cellular environment containing rat PPAR-α protein. Recombinant PPAR-α and RXR-α were found to bind to the human promoter. These authors concluded that "[t]he presence of a PPRE in the promoter of this human peroxisomal ACOX gene and its responsiveness to peroxisome proliferators suggests that factors other than the PPRE in the 5'-flanking sequence of the human ACOX gene may account for differences, if any, in the pleiotropic responses of humans to peroxisome proliferators."

Woodyatt *et al.* (1999; Lambe *et al.*, 1999) also cloned the promoter region of the human ACO gene from a liver biopsy sample. When a fragment of the human DNA was incorporated into a plasmid for assessing its transcription promoting potential, the human ACO promoter was not found to be active relative to a similarly incorporated rat ACO promoter. These investigators

[2] A PPRE DNA sequence was reported in this paper. Over concerns that this sequence may have been obtained from a mutant sample, Varanasi *et al.* resequenced their plasmids and source material. In a published erratum (Varanasi *et al.*, 1998), the reporting of the response element DNA sequence in the original Varanasi *et al.* (1996) paper was found to contain an error (rather than a mutation) caused by a "typographical transposition of three nucleotides." The correct sequence was found to be identical to that reported in later studies (Woodyatt *et al.*, 1999). Thus, the results of Varanasi *et al.* (1996) appear valid in spite of the erroneous reporting of the promoter sequence.

identified a DNA sequence different from that published previously (Varanasi *et al.*, 1994, 1996). Analysis of 22 different human DNA samples showed they all contained the same ACO promoter sequence.

Clearly, the findings of these two groups of investigators present conflicting indications of the potential for humans to respond to peroxisome proliferators with the induction of ACO transcription and activity. Possible reasons for the discrepancy include the use of slightly different reporter assay plasmid constructs which contain different extents of the promoter regions, thus providing different potential regions for repression of expression in the *in vitro* assays.

Cheema *et al.* (2000) compared the promoter regions of the mouse and human cholesterol 7α-hydroxylase genes. Their studies showed that the promoters for each of these genes respond differently to stimulation by fatty acids, with the mouse sequence conferring significantly more activity than the human sequence. It was found that the mouse promoter region contained an additional binding site for PPAR-α, which the authors speculated might confer additional responsiveness of this gene to peroxisome proliferating chemicals.

Collett *et al.* demonstrated that the human prostatic cancer cell line LNCaP contains a functional PPAR-α (Collett *et al.*, 2000). Using plasmids containing the PPRE-bearing promoter region of the human genes ApoA-II and muscle carnitine palmitoyltransferase I coupled to the reporter gene luciferase, transfection studies indicated that stimulation with Wy-14,643 resulted in expression of the reporter gene *in vitro*.

Overview of Human Responsiveness

The body of data examining responses of human cells to peroxisome proliferators other than DEHP/MEHP is mixed. There are numerous cases where human cells appear refractory to the effects of peroxisome proliferators. Yet there are other cases where responsiveness, as measured by the traditional indicators of peroxisome proliferation, appears to occur. Specifically, the studies by Perrone *et al.* (1998) examining the effects of the hypolipidemic agents clofibrate and ciprofibrate on primary cultures of hepatocytes from five human donors induced significant increases in ACO activity, albeit to a lesser level than that observed in primary cultures of rat hepatocytes. Scotto *et al.* (1995) demonstrated increased ACO enzyme activity and induction of mRNA by clofibrate in a human hepatoma cell line. Cimini *et al.* (2000), using a cell line derived from a human glioblastoma, found significant induction of peroxisomal density and an increase in ACO activity following treatment with perfluorodecanoic acid. As noted earlier, results observed in cell lines, particularly those derived from tumors, must be viewed cautiously. However, these studies do indicate that the cellular "machinery" required to bring about some of the typical peroxisome proliferator effects can be induced in human cells.

There are frequent references in the scientific literature to the lack of responsiveness of humans and statements that humans are refractory to the effects of DEHP and other peroxisome proliferators, largely based on the body of data described above. While many investigators appear willing to assert a lack of human responsiveness (and thus carcinogenic risk) from exposure to peroxisome proliferators, other investigators are not convinced that a lack of human responsiveness has been established with sufficient certainty:

> "It is not yet clear whether humans should be considered unresponsive, and there is concern about the long-term safety of fibrates." (Rodríguez *et al.*, 2000)

> "The issue of human risk associated with exposure to chemicals that belong to the peroxisome proliferator group remains controversial. Despite several major advances in understanding the mechanisms of how these chemicals cause liver tumors in rodents, the issue of toxicity to human population is far from being resolved." (Rusyn *et al.*, 2000)

> "There is no conclusive evidence that humans are not responsive to peroxisome proliferation. Although the indirect experimental data reported until now suggests that humans should be included among the nonresponsive species, the issue remains controversial and concern has been expressed about the long-term safety of drugs such as fibrates [citing Cattley *et al.*, 1998]." (Rodríguez *et al.*, 2000)

> "While the pathological consequences of moderate levels of DEHP exposure in human populations are uncertain, DEHP is an established … hepatocarcinogen … in rodents." (Maloney and Waxman, 1999)

> "Peroxisome proliferators are a diverse group of non-genotoxic chemicals, many of which are hepatocarcinogenic upon chronic administration to rats and mice [citing Reddy and Lalwani, 1983]. Despite the widespread use and potential for human exposure to PP [peroxisome proliferators], their mechanism of action remains poorly understood and minimal information is available on the susceptibility of humans exposed to these chemicals. The overall objective of this work is to elucidate the mechanism(s) of PP

action in an attempt to assess human risks to these chemicals." (Karam and Ghanayem, 1997)

Theories of Mode of Action and Supporting Data

The identification of the PPAR-α as a central mediator of the action of peroxisome proliferators has been a highly significant development toward gaining an understanding of the risks associated with exposure to DEHP and other peroxisome proliferators. Prior to the clarification of the role of PPAR-α in rodent carcinogenesis, a long history of hypothesizing occurred regarding the possible modes of action, none of which has been invalidated by the PPAR-α studies, and, in fact, multiple modes of action may be in play at the same time. The most prominent among the hypotheses are modes of action involving DNA damage induced by oxidative stress and those involving changes in cell proliferation (both growth stimulation and inhibition of cell death).

Direct DNA Binding/Damage

There is little evidence that DEHP interacts directly with DNA or is likely to induce direct damage to DNA. No DNA binding occurred in nucleic acids (DNA, RNA) and purified proteins isolated from rat liver (Albro *et al.*, 1984). To assess the covalent binding potential of DEHP, rats were treated orally with DEHP (either with or without pre-treatment with 1% dietary DEHP for four weeks), then their livers were evaluated at 16 hours (von Däniken *et al.*, 1984; Lutz, 1986). An appraisal of a "covalent binding index" suggested that the level of DNA binding observed with DEHP was well below that associated with compounds with genotoxic potential.

"Initiation" and "Promotion"

DEHP has been tested in experimental protocols which suggest that it has some potential for tumor promoting activity (Ward *et al.*, 1983; Ward *et al.*, 1984). However, DEHP and other peroxisome proliferators are clearly "complete" carcinogens, that is, they have the potential to bring about neoplastic transformation without required exposure to another initiating agent. An early observation by Reddy and Lalwani (1983) follows:

> "Although the peroxisome proliferators do promote hepatocarcinogenesis in animals preexposed to a carcinogen, we do not favor the suggestion that peroxisome proliferators are tumor promoters for the following reasons. First, all carcinogens have initiating and promoting activity if they influence cancer by themselves. Therefore, the fact that clofibrate, Wy-14,643, nafenopin, or possibly other peroxisome proliferators enhance the incidence of liver tumors in rats already initiated with a carcinogen such as diethylnitrosamine does not necessarily mean that peroxisome proliferator-induced carcinogenesis is because of their ability to promote the expression of cells initiated by ambient environmental factors. Second, studies by Lalwani et al. [1981] demonstrated a 100% incidence of liver tumor induction in rats fed Wy-14,643 in a semipurified diet to rule out the possibility of interference with an extraneous initiating chemical. These results also do not support the argument that peroxisome proliferators are tumor promoters. Finally, all six peroxisome proliferators tested in our laboratory induced nearly 100% incidence of liver tumors in both rats and mice, whereas most known tumor promoters such as TCDD, phorbol esters, and phenobarbital are not carcinogenic by themselves or induce a very low incidence of neoplasms." [Reddy and Lalwani, 1983]

> "Therefore, we conclude that peroxisome proliferators, like most carcinogens used under conditions leading to cancer, induce initiation and promotion. Because the initiation of carcinogenesis by peroxisome proliferators does not appear to conform to the concepts currently in vogue for the majority of chemical carcinogens, the peroxisome proliferators may prove to be useful tools for the elucidation of alternate mechanisms of carcinogenesis." [Reddy and Lalwani, 1983]

There is little in the wealth of evidence relating to the carcinogenicity of peroxisome proliferators which has followed which would permit a clearer classification of DEHP and other peroxisome proliferators into "initiator" or "promoter" categories.

Oxidative Stress Induced DNA Damage

Historically, the most prominent hypothesis for peroxisome proliferator action has been that espoused initially by Reddy *et al.* (1979; 1980) (also recently reviewed in Yeldandi *et al.*, 2000). The foundation of this hypothesis is that compounds which cause the proliferation of peroxisomes in rodents (initially a group of hypolipidemic pharmaceuticals) lead to increases in peroxisomal and mitochondrial oxidases which produce hydrogen peroxide, a compound capable of causing the generation of lipid and hydroxyl radicals and singlet oxygen leading to oxidative damage to DNA. This induction of peroxide generating enzyme activity is not offset by increases in the enzyme responsible for the breakdown of hydrogen peroxide (catalase). This imbalance in the capacity to generate versus the ability to break down hydrogen peroxide results in a sustained potential for production of peroxide, and oxidative DNA damage. The oxidative DNA damage causes heritable changes to DNA, which according to this hypothesis is ultimately responsible for the induction of tumors by peroxisome proliferators.

The oxidative stress hypothesis is supported by:

- Strong induction of expression and activity of enzymes associated with hydrogen peroxide production (ACO and enoyl-CoA hydratase) (Reddy *et al.*, 1986).

- Good correlations between the carcinogenic potential of peroxisome proliferating chemicals with the induction of steady-state hydrogen peroxide (Tomaszewski *et al.*, 1986).

- Evidence of oxidative damage in cells treated with peroxisome proliferators. This damage manifests itself as increases in 8-hydroxy-2'-deoxyguanosine adducts characteristic of oxidative DNA damage (Kasai *et al.*, 1989; Takagi *et al.*, 1990), and cellular lipid peroxidation (Conway *et al.*, 1989; Marsman *et al.*, 1992).

- Reductions in ciprofibrate-induced liver tumors in rats by treatment with the antioxidant ethoxyquin (Rao *et al.*, 1984).

- Potential for over-expression of ACO in NIH-3T3 cells *in vitro* to induce a transformed phenotype (anchorage-independent growth and tumorigenicity in nude mice) (Chu *et al.*, 1995).

However,

- Treatment of B6C3F1 mice for seven days with 100 ppm Wy-14,643 in the diet did not result in increased liver F_2-isoprostane production in spite of 16-fold increases in peroxisomal β-oxidation accompanied by only two-fold increases in catalase activity. The generation of F_2-isoprostanes, produced from the peroxidation of arachidonic acid and catalyzed by free-radicals, has been proposed as an indicator of oxidative stress (Soliman *et al.*, 1997). Certain indicators of oxidative injury are only modestly increased in response to peroxisome proliferators, for example, the low induction of F_2-isoprostanes and the low amount of ethane (a product of lipid peroxidation) in exhaled air of mice treated with Wy-14,643 (Conway and Popp, 1995).

- There is a relatively poor correlation between formation of 8-hydroxy-2'-deoxyguanosine, an indicator of oxidative DNA injury, and multiplicity of tumors (Cattley and Glover, 1993), including some studies showing no significant induction of 8-hydroxy-deoxyguanosine following treatment of male rats with nafenopin (Hegi *et al.*, 1990).

- Sprague-Dawley rats treated intraperitoneally with the peroxisome proliferator perfluorooctanoate five days prior to evaluation at a dose associated with the induction of peroxisomes did not result in increased production of hydrogen peroxide, as measured by *in situ* perfusion of the animals' livers, whereas ACO activity was increased several fold (Handler *et al.*, 1992). F344 rats treated in feed with bezafibrate (0.1%), clofibrate (0.25%), and DEHP (2%) for up to 78 weeks showed a 1.2- to 1.3-fold increase in hydrogen peroxide content at the time of maximal peroxisomal fatty ACO stimulation (12- to 20-fold at 2-4 weeks), although the increase was not time-dependent and could not be correlated with fatty ACO activity (Tamura *et al.*, 1990).

Mitogenesis/Cell Proliferation

The hypothesis that the stimulation of DNA synthesis and cell division by peroxisome proliferators plays an important role in the carcinogenic process is supported by the following lines of evidence:

- Studies of Fischer 344 rats treated for one year with either Wy-14,643 or DEHP in their diet showed a good correlation between replicative DNA synthesis and multiplicity of tumors in the liver, whereas peroxisome proliferation and enzyme activity associated with peroxisomal β-oxidation was only poorly correlated with tumor multiplicity (Marsman *et al.*, 1988).

- More pre-neoplastic foci are present in older rats treated with Wy-14,643 compared to younger rats, suggesting the possibility that there are more spontaneously initiated cells in the older animals (Cattley *et al.*, 1991; Kraupp-Grasl *et al.*, 1991). The induction of basophilic foci, which are associated with increases in proliferation rates, correlates with tumorigenicity of Wy-14,643 (Marsman and Popp, 1994) [reviewed by Rose *et al.*, 2000].

- Treatment of male Sprague-Dawley rats with Wy-14,643 in the diet resulted in increased expression of certain genes associated with cell proliferation, including $p34^{cdc2}$ kinase (CDK1) and DNA polymerase δ-PCNA (Ma *et al.*, 1997). However, the induction of other genes associated with inhibitory signals of cell growth (including the tumor suppressor gene, p53 and the CDK inhibitory protein $p21^{Waf1}$) suggest that this peroxisome proliferator leads to discordant growth regulation rather than simple stimulation.

- Apoptosis was suppressed in cells treated with peroxisome proliferating chemicals (Marsman *et al.*, 1992; Gerbracht *et al.*, 1990, and Bayly *et al.*, 1994, *in vitro*; James *et al.*, 1998).

However, these findings are complicated by findings such as those below:

- Some suggestion of a discrete cell population of rat hepatocytes vulnerable to stimulation by certain non-genotoxic carcinogens, including DEHP (Hasmall and Roberts, 2000).

- Different localization in the liver of the induction of peroxisomal enzymes and CYP4A (centrilobular; Bell *et al.*, 1991) compared to the site of mitogenesis (periportal; Barrass *et al.*, 1993).

Recent Mechanistic Developments

Further Evidence for a Role of Lipid Homeostatic Pathways in Hepatocarcinogenesis

Supporting evidence for the involvement of lipid regulatory pathways has also come from studies utilizing mice which have the ACO gene knocked out (Fan *et al.*, 1996; Fan *et al.*, 1998). In the reported studies, all the ACO knockout mice developed liver tumors by 15 months of age. A striking implication of this study is that the ACO gene is not necessarily permissive in the peroxisome proliferation and hepatocarcinogenic pathway mediated by PPAR-α, but that ACO may play a critical role in the regulation of other genes (particularly those regulated by PPREs) which may be participatory in the cascade of events leading to hepatocarcinogenesis.

It has been speculated that the loss of down-regulation of certain activators of PPAR-α associated with the loss of the ACO gene results in the overexpression of endogenous PPAR-α ligands (such as ω_6-unsaturated fatty acids and prostaglandins), some of which may have growth stimulatory properties (Corton *et al.*, 2000b, citing Masters, 1996).

Kupffer Cell Involvement in Hepatocarcinogenesis

A role for the liver's Kupffer cells (phagocytic cells of the liver sinusoids; part of the reticuloendothelial system) in the hepatocarcinogenic process induced by peroxisome proliferators has been proposed based on the following lines of evidence:

- TNF-α is a known trigger of hepatocyte proliferation; the primary site of TNF-α production in the liver is the Kupffer cell. Increases in TNF-α mRNA in liver cells stimulated by Wy-14,643 is blocked by methyl palmitate (an inactivator of Kupffer cells) and glycine (a calcium signaling blocker which blunts the activity of Kupffer cells) (Rose *et al.*, 1997a; Rose *et al.*, 1997b).

- Increases in hepatocyte DNA synthesis, but not peroxisome proliferation (as measured by ACO activity), caused by Wy-14,643 were blocked by methyl palmitate in Sprague-Dawley rats, suggesting a dependence for the DNA synthesis response on Kupffer cells and suggesting that different mechanisms or pathways mediate the DNA synthesis and peroxisome proliferation responses (Rose *et al.*, 1997b).

- Pretreatment of Fischer 344 rats with anti-TNF-α antibodies blocked cell proliferation in the liver (as indicated by BrdU uptake) induced by intragastrically administered Wy-14,643 (Bojes *et al.*, 1997).

- Wy-14,643 activates nuclear factor-κB (NF-κB), a key stimulator of TNF-α in Kupffer cells (Rusyn *et al.*, 1998).

- Isolated Kupffer cells were found to be activated to produce superoxide (from the stimulation of NADPH oxidase) by Wy-14,643 and MEHP at approximately equal levels, but not by DEHP or ethylhexanol (Rose *et al.*, 1999). The induction of superoxide by Wy-14,643 was found to be dependent on protein kinase C as indicated by inhibition studies with staurosporine. Twenty-one day treatment *in vivo* of male Sprague-Dawley rats in their diet with 0.1% Wy-14,643, but not 1.2% DEHP, resulted in a two-fold induction in superoxide.

- A recent study has identified an increase in hydroxyl radicals by the spin-trapping technique in the bile following acute treatment of rats *in vivo* with DEHP (Rusyn *et al.*, 2001). The observed increase occurred before the induction of peroxisomal oxidases (within four hours). Pretreatment of the animals with glycine (to inactivate Kupffer cells) prevented radical formation. The observation that hydroxyl radicals were not detected in NADPH oxidase-knockout mice treated with DEHP lead the study's authors to suggest that this provides evidence for NADPH oxidase in Kupffer cells as a source of free radicals from treatment with peroxisome proliferators. PPAR-α null mice showed the same response as their wild-type counterpart, demonstrating that PPAR-α is not required for the induction of hydroxyl radicals by DEHP.

The mechanism by which the peroxisome proliferator enhances superoxide formation is unclear. Recent studies have shown that neither PPAR-α mRNA nor protein are detectable in Kupffer cells from female Sprague-Dawley rats either untreated or treated orally with diet containing 0.1% Wy-14,643 for one week (Peters *et al.*, 2000). The lack of expression of PPAR-α in activated macrophages has also been reported by Ricote *et al.* (1998). Peters *et al.* reported that PPAR-α expression was induced approximately two-fold in the parenchymal hepatocytes of these Wy-14,643 treated rats. Kupffer cells isolated from PPAR-α knockout mice and wild-type mice also showed stimulation of superoxide production *in vitro* by either phorbol 12-myristate-13-acetate or by Wy-14,643 to an equal extent, further suggesting that PPAR-α does not have a role in the Kupffer cell response. These authors speculated that the effect of peroxisome proliferators on the liver parenchymal cells may be dependent on a factor of Kupffer cell origin (TNF-α) which is stimulated via factors released from the parenchymal cells via the PPAR-α. They also asserted the need for *in vivo* models which replicate the cell types which may be operating in the peroxisome proliferation response, saying that: "*In vitro* culture systems that lack functional Kupffer cells are likely to produce equivocal results since interaction between parenchymal and Kupffer cells *in vivo* is required."

Recent studies have also directly implicated NADPH oxidase as central to the superoxide production associated with the stimulation of Kupffer cells by peroxisome proliferators (Rusyn *et al.*, 2000). An inhibitor of NADPH oxidase, diphenyleneiodonium, was able to effectively inhibit the activation of NF-κB and cell proliferation resulting from *in vivo* treatment of Sprague-

Dawley rats with Wy-14,643. These investigators also demonstrated that the mechanism of induction of cell proliferation was independent of that from peroxisome proliferation (as manifested by increased ACO activity). In rat liver, diphenyleneiodonium inhibited Wy-14,643 induction of cell proliferation (measured as percent of BrdU positive hepatocytes), whereas no inhibition of the induction of ACO activity was observed.

Further evidence for a role for non-parenchymal cells of the liver in the proliferative response to peroxisome proliferators has come from studies in which isolated parenchymal cells from Wistar rats have been separated from non-parenchymal cells by Percoll gradient centrifugation (Parzefall *et al.*, 2001). Using this separation technique, populations of 99.9% pure hepatocytes were obtained. In populations of purified parenchymal hepatocytes, Wy-14,643 and nafenopin were able stimulate ACO activity (two-fold for Wy-14,643), but not DNA synthesis. Using cell culture medium conditioned from isolated Kupffer cells treated with Wy-14,643, parenchymal hepatocytes showed stimulation of DNA synthesis *in vitro*, suggesting that cytokines released from Kupffer cells may be responsible for this stimulation.

Overall, there are data which support a role for both the generation of reactive oxygen species and for the induction of cell proliferation by peroxisome proliferators. There are also indications that these two mechanisms are independently regulated at the cellular level. No data suggest that either of these modes operates exclusively of the other. Moreover, some studies have suggested that the mechanism by which peroxisome proliferators induce their hepatocarcinogenic effect may involve the interaction of two (if not more) cell types (see Figure 1).

PPAR-α has already been closely associated with the induction of a relatively large number of genes as demonstrated by increases in both RNA expression and active proteins. It remains to be seen whether some, if any, of the genes involved in the carcinogenic process have been identified already. In a recent review of the carcinogenicity of peroxisome proliferators, Cattley *et al.* captured the uncertainty regarding the pathways which may be involved:

> "Although not yet proven, it is likely that peroxisome proliferators elicit liver growth and hepatocarcinogenesis by the regulation of as yet undefined gene networks. The PPARα/RXR complex would be a key component of such a mechanism." (Cattley *et al.*, 1998)

Efforts to identify some of these as yet unidentified "gene networks" are underway using toxicogenomic analysis (Hamadeh *et al.*, 2001). Using the gene expression profiles generated from cells which have been treated with peroxisome proliferating chemicals compared to those untreated (or treated with other classes of chemical), this analytical technique has the potential to assist in the identification of novel genes and ultimately of biochemical pathways involved in peroxisome proliferator-mediated carcinogenesis.

A schematic representation of some of the processes which may contribute to the overall carcinogenic process induced by peroxisome proliferators is presented in the figure below.

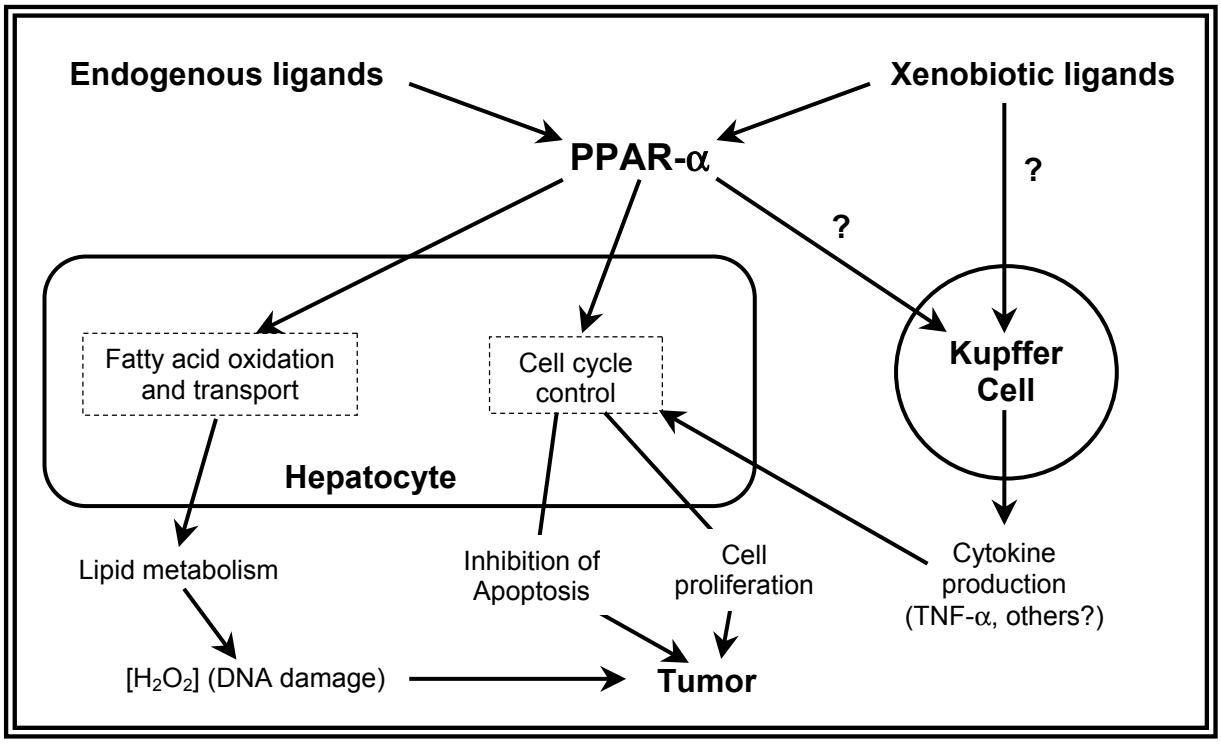


Figure 1. Schematic representation of events in the liver related to peroxisome proliferator induced carcinogenesis (adapted from Gonzalez *et al.*, 1998).

Route-Specific Modifications of Risk

It has been postulated that since a majority of ingested DEHP is metabolized to MEHP in the intestinal tract of rats by pancreatic and intestinal lipases and, since this represents a considerably greater conversion to MEHP than that observed in certain studies in rats (Pollack *et al.*, 1985b) following parenteral DEHP administration, that such non-oral exposures are less significant with respect to human exposure and risk. Specifically, it has been posited that intravenous exposures to DEHP will circumvent metabolism to MEHP and any resulting toxic effects. There is substantial evidence, discussed below, for significant formation of MEHP (and other active metabolites) in humans following parenteral exposure including those routes related to exposures from medical devices (intravenous, intraperitoneal).

Biliary Excretion and Enterohepatic Circulation

DEHP, as well as many other phthalates, undergo significant biliary excretion which can confound estimates of total absorption (reviewed in OEHHA, 1997). Intestinally absorbed DEHP is, upon reaching the liver, partially excreted back into the intestine via the bile completing an enterohepatic circulation. Such enterohepatic circulation has the effect of prolonging the time for elimination of DEHP from the body and providing additional opportunity for its metabolism in the intestine and in the liver. Similarly, DEHP reaching the systemic blood via non-oral routes of exposure such as inhalation, percutaneous absorption, or direct intravenous exposure via medical devices is also subject to biliary excretion into the small intestine and additional metabolism, absorption, etc. While such considerations are important in assessing risk to humans from rodent experimental data, they are also vitally important in simply understanding

rodent metabolism. This point is well illustrated by the recent attempts to model the metabolism of DEHP to MEHP in the rat.

Keys *et al.* (1999) developed a physiologically-based pharmacokinetic (PBPK) model of DEHP uptake, metabolism to MEHP, and excretion in the rat. They compared the PBPK model predictions with experimental data on oral or intravenous doses of DEHP and MEHP. The usual flow-limited model structure failed to adequately simulate the experimental observations. Variation of the model structure to accommodate diffusion-limited membrane transport, enterohepatic circulation, or MEHP ionization all significantly improved predictions of DEHP and MEHP blood concentrations versus the flow-limited model. The authors concluded, "the shape of the liver concentration-time curve was not well predicted. This may be because diffusion-limitation or enterohepatic circulation or both is occurring in the liver." In their model the metabolic activity in the small intestine was predicted to be much more quantitatively significant than metabolic conversion of DEHP in the blood or liver. However, no quantitative values were provided.

The authors caution that more data on DEHP and MEHP blood and tissue concentrations under more environmentally relevant exposure conditions are necessary for a more complete model validation. This paper indicates the potential importance of biliary excretion and enterohepatic circulation in understanding DEHP dosimetry. It also indicates that parenteral administration of DEHP may lead to its metabolism to MEHP in the blood or liver or in the intestine. Therefore, while parenteral exposures of rats to DEHP may result in less MEHP tissue exposure, there will clearly be some exposure. The rat PBPK model mentioned above is new and has not yet been extended to make direct predictions of human DEHP/MEHP exposure and dosimetry (*i.e.,* from a human PBPK model). Using the parameters identified in the Keys *et al.* rat PBPK model employing enterohepatic circulation of DEHP and MEHP, OEHHA found that exposure to one ppm DEHP in air for 8 hr can lead to a relative MEHP liver dose of about 17 percent (2.6 *vs.* 15.5 μM×hr, area under the concentration-time curve). While this is not a fully validated model, it indicates that parenteral exposures of DEHP may result in substantial MEHP internal doses. Furthermore, the rat model is expected to under-predict MEHP levels in humans resulting from parenteral exposures, based upon human experimental observations (Sjöberg *et al.*, 1985b).

Hepatic and Extrahepatic Metabolism of DEHP

Lake *et al.* (1977) demonstrated the hydrolysis of a variety of phthalate diesters including DEHP to their monoester analogs by both hepatic and intestinal preparations from several species. In the hepatic studies, a quantitative species difference was observed in that phthalate diester hydrolase activity generally decreased in the order baboon > rat > ferret. The authors note that mammalian liver contains both acid and alkaline nonspecific esterases but that only the latter were measured in the study. Albro and Thomas (1973) studied DEHP esterase activity from a wide variety of rat tissues. Pancreas, liver, and intestinal mucosa contained the large majority of DEHP hydrolase activity in male CD rats. Significant activity was also detected in lung, kidney, and adipose tissues. Since the rat liver phthalate ester hydrolase can also hydrolyze MEHP at about two percent of its DEHP hydrolysis rate, the urinary metabolites of orally administered DEHP are all derivatives of MEHP and 2-EH, except for a small amount of free phthalic acid.

Ex Vivo Metabolism of DEHP

DEHP may be metabolized to MEHP in plastic blood bags during storage. Sjöberg *et al.* (1985b) reported the exposure of newborn infants to DEHP and MEHP during exchange transfusions. The amounts of DEHP and MEHP inadvertently infused varied from 1.7 to 4.2 and 0.2 to 0.7 mg/kg body weight, respectively.

In Vivo Metabolism of DEHP to MEHP

Pollack *et al.* (1985a) studied the exposure of 11 patients undergoing hemodialysis for treatment of renal failure. The amount of DEHP leached from the dialyzer during a four-hour dialysis session was estimated by measuring the DEHP blood concentration gradient across the dialyzer. Circulation concentrations of the products of DEHP de-esterification, MEHP and phthalic acid, were also determined. On average 105 mg DEHP was extracted from the dialyzer during a single session. The average blood concentration of DEHP during dialysis was 1.91 ± 2.11 μg/ml and for MEHP was 1.33 ± 0.58 μg/ml. The authors concluded that in humans significant mono-de-esterification may occur after systemic intake (*i.e.*, parenteral) of DEHP as indicated by the comparable concentrations of DEHP and MEHP observed during dialysis. The notable contradiction with findings from the same laboratory in rat studies (Pollack and Shen, 1984; Pollack *et al.*, 1985b) has been noted by the authors:

> "… we have further data in hemodialysis patients which suggest that neither oral nor intraperitoneal administration of I [DEHP] in the rat exactly mimic the situation during clinical exposure. Studies in a group of renal failure patients on maintenance hemodialysis showed that the circulating concentrations of derived II [MEHP] were comparable to the parent diester during a 4-h dialysis session (AUC ratio of 2.42 ± 0.66). This suggests that mono-deesterification of I [DEHP] in the systemic circulation occurs much more readily in humans than in rats. Alternatively, II [MEHP] may be eliminated much more slowly (relative to the parent compound) in humans than in rats." (Shen and Pollack, 1984)

In the Sjöberg *et al.* (1985b) study discussed above, immediately following transfusions which took 1-2 hours, the DEHP plasma concentrations in individual infants ranged from 3.4 to 11.1 μg/ml, and MEHP from 2.4 to 15.1 μg/ml. Thus, the amount of DEHP infused was about 10 times the infused amount of MEHP, but in the plasma samples of the newborns the DEHP levels were only twice those of MEHP. The relatively long elimination half-life of DEHP indicates that exposure to MEHP continues for a relatively long time following transfusion (Sjöberg *et al.*, 1985b). Plonait *et al.* (1993) also detected DEHP in the plasma of newborn infants subjected to exchange transfusion or extracorporeal membrane oxygenation. The levels ranged from undetectable (<1 μg/ml) before transfusion to 6.1 to 21.6 μg/ml (mean 12.5, n = 16) after a single transfusion.

Mettang *et al.* (1996) evaluated 21 patients on hemodialysis for at least six months for DEHP exposure and uremic pruritus. Pre-hemodialysis serum concentrations of DEHP and MEHP ranged from 0.156 to 0.17 μg/ml and 0.025 to 0.035 μg/ml, respectively. Post-hemodialysis values were 0.54 to 0.83 μg/ml and 0.12 to 0.17 μg/ml, respectively. While the latter values for MEHP were somewhat lower than those reported by Pollack *et al.* (1985a) above, they provide additional evidence of substantial conversion of DEHP to MEHP. Whether this represents metabolism and/or another degradation mechanism is unclear.

In a follow up investigation, Mettang *et al.* (1999) studied the metabolism of DEHP in 10 patients receiving continuous ambulatory peritoneal dialysis for at least six months. Effluent dialysate, urine and blood samples were taken for analysis of DEHP, MEHP, and 2-ethylhexanol (EH). In addition, the degree of glucuronidation of the phthalic acid (PA) esters in urine was determined. In blood serum, dialysate, and urine, PA was the predominant metabolite of DEHP: 0.205 ± 0.067 mg/L; 0.284 ± 0.18 mg/L; and 1.34 ± 1.00 mg/L, respectively. Concentrations of MEHP were lower: 0.01 ± 0.0056, 0.022 ± 0.008, and 0.011 ± 0.0064 mg/L, respectively. Urinary MEHP was glucuronidated 15 percent. PA was 35 percent eliminated as a glucuronide. In this respect the dialysis patients differed from normal healthy subjects who excrete mainly MEHP and MEHP metabolites.

Peck and Albro, in a 1982 review of the known data on the metabolism of DEHP, commented on the potential for significant conversion of DEHP to MEHP:

> "The studies of DEHP disposition reviewed in this paper demonstrate that DEHP administered parenterally to primates and humans is rapidly converted to conjugated hydrolyzed oxidation products of MEHP which are rapidly excreted in the urine. These metabolic processes account for elimination of more than 50% of the infused DEHP by 8 hr after infusion, while elimination of the entire dose is virtually complete in 4-5 days." (Peck and Albro, 1982)

It has been postulated that species differences in the level of metabolic conjugation of DEHP may have a bearing on its toxicity. An early report on the metabolism of DEHP by different animal species identified conjugated metabolites in the urine of all species examined but rats (Albro *et al.*, 1982). The species examined included rats, mice (CD), guinea pig, green monkey, hamster and man. The only DEHP metabolite conjugates identified were glucuronides (glycine, taurine, and sulfate conjugates were not found). Humans and green monkeys demonstrated the highest level of DEHP metabolite conjugation (~80%); mice and guinea pigs also demonstrated high levels (~65%). Hamsters demonstrated relatively low levels of conjugated metabolites of DEHP (~15%). While there are striking differences in the levels of these urinary conjugates, most notably between rats and humans, this does not appear to be a basis for differences in sensitivity to carcinogenic effects, since mice, which here are reported to have glucuronide conjugate levels in urine nearly as high as humans, are the most sensitive species tested to the development of liver tumors from oral exposure to DEHP (see below). The influence of route of exposure on the conjugation of DEHP metabolites is not known; however, the level of conjugation would be expected to depend on the circulating levels of MEHP in the blood.

Evidence from Occupational Exposures

Dirven *et al.* (1993) studied the uptake and metabolism of workers exposed to DEHP in the polyvinylchloride processing industry. They measured the concentrations of four DEHP metabolites in the urine samples of the workers: MEHP; mono(5-carboxy-2-ethylpentyl) phthalate; mono(2-ethyl-5-oxohexyl) phthalate; and mono(2-ethyl-5-hydroxyhexyl) phthalate. DEHP concentrations in air were determined by personal air sampling. Nine workers exposed to a maximum of 1.2 mg DEHP/m^3 air showed an increase in urinary concentrations of all four metabolites over the course of the workshift, all increases except MEHP were statistically significant. The production of each of the detected metabolites would be expected to require a de-esterification step, likely involving the transient generation of MEHP. Six other workers

exposed to the same maximum DEHP air concentration also showed one to four-fold increases in the four metabolites, but these increases were not statistically significant. It seems clear from this study that chronic inhalation exposure to DEHP can result in metabolism of DEHP to a variety of products, probably including MEHP.

Conclusions Regarding Metabolic Issues

- Phthalates, including DEHP and MEHP, are subject to biliary excretion and enterohepatic circulation.

- While the intestinal tract is a dominant site of DEHP hydrolase activity, many other tissues (including blood) also contain esterases that can convert DEHP to MEHP and in some cases further convert MEHP to additional metabolites.

- Parenteral exposures to DEHP can result in metabolism to MEHP via the action of internal esterases of blood and other tissues and may also be subject to intestinal lipase action following biliary excretion into the small intestine.

- MEHP generated in the intestine following biliary excretion of DEHP is available for gastrointestinal absorption, just as oral DEHP and MEHP are.

- MEHP may be generated *in vitro* in human blood stored in poly(vinylchloride) (PVC) blood bags and in PVC tubing, presumably via the action of blood esterases, resulting in inadvertent exposures via transfusion and extracorporeal dialysis. This is of particular concern for newborn infants who have underdeveloped liver metabolism and biliary excretion.

- Human occupational inhalation exposures to DEHP in PVC processing industries indicate internal metabolism to a number of DEHP metabolites, including MEHP, which are excreted in urine.

Based upon the available data and the reasons above, there is no basis for concluding that intravenous or other parenteral exposures to DEHP are free of subsequent internal exposures to MEHP, or more specifically, that internal doses of MEHP are solely a result of oral route exposures. While anticipated MEHP doses from parenteral DEHP may be less than from oral route DEHP exposures, there is too little information to assert that these internal doses of MEHP are toxicologically insignificant.

Furthermore, preliminary estimates of internal exposure levels suggest that a parenteral route of exposure does not necessarily produce a lower area-under-the-curve (AUC) compared to the oral route of administration. It is also unclear how the relative proportion of DEHP to its primary metabolite under different exposure route scenarios may influence the risk of toxicity, in light of evidence that DEHP itself has cellular effects, whether they be from intracellular metabolism to active moieties such as MEHP or by direct interaction with a cellular receptor, and evidence that metabolites other than MEHP have peroxisome proliferator properties.

Overall data from human subjects suggest that somewhat less rapid metabolism of DEHP to its more active metabolite MEHP occurs following administration via parenteral routes (here

representing intravenous and intraarterial administration) compared to oral routes of exposure. This is supported by PBPK models developed for experimental animals. The available human data, however, indicate that following parenteral exposures of infants, DEHP and MEHP levels eventually come to approximately the same level, with area under the curve levels for DEHP and MEHP falling within a factor of two. Furthermore, analysis of the urine of human subjects has failed to specifically identify intact DEHP, suggesting that metabolism (likely via MEHP) occurs prior to elimination (with the possible exception of some fecal elimination of DEHP pursuant to biliary excretion, although human data are not available). It is expected that because of the pancreatic esterases in humans and rodents, oral route administration would produce predominantly MEHP in the blood (and thus also at the liver). The applicability of the PBPK analyses to humans clearly suffers from lack of data regarding similarities between humans and rodents with respect to certain pharmacokinetic parameters. Development of data on human parameters would enable a more confident assessment of differences in relevant metabolites relating to different routes of exposure.

Methodological Approach to Assessing Cancer Risk in Animals

Implications of Low-Dose Extrapolation of Receptor-Mediated Effects

Briefly setting aside the debate regarding the sensitivity of humans to DEHP's carcinogenic effects; a useful step in the risk assessment process is to establish the level of cancer risk that experimental animals face from exposure to DEHP. General principles for the estimation of risk from exposure to carcinogens dictate that no level of exposure is without risk, in the absence of information suggesting otherwise. This is reflected in the Proposition 65 regulations (Title 22, CCR §12703(a)(5)) concerning quantitative risk assessment for chemicals known to cause cancer:

> "The absence of a carcinogenic threshold dose shall be assumed and no-threshold models shall be utilized. A linearized multistage model for extrapolation from high to low doses, with the upper 95 percent confidence limit of the linear term expressing the upper bound of potency shall be utilized. Time-to-tumor models may be appropriate where data are available on the time of appearance of individual tumors, and particularly when survival is poor due to competing toxicity."

The current data regarding DEHP's carcinogenic effects suggest that the process is receptor-mediated (via PPAR-α), although the precise mechanism by which activation of this receptor causes tumors to develop remains unclear (Cattley *et al.*, 1998). The appropriate method for the extrapolation of cancer risk at low doses from exposure to carcinogens which act via cellular receptors is controversial. Several investigators have recently summarized the key issues under consideration in generic terms, rather than specifically for the case of DEHP or peroxisome proliferators. Gaylor and Zheng (1996) have noted that:

> "Nongenotoxic cytotoxic carcinogens that increase cell proliferation rates to replace necrotic cells are likely to have a threshold dose for cytotoxicity below which necrosis and hence, carcinogenesis do not occur. Thus, low dose cancer risk estimates based upon nonthreshold, linear extrapolation are inappropriate for this situation. However, a threshold dose is questionable if a nongenotoxic carcinogen acts via a cell receptor. Also, a nongenotoxic carcinogen that increases the cell proliferation rate, via the cell division

rate and/or cell removal rate by apoptosis, by augmenting an existing endogenous mechanism is not likely to have a threshold dose."

And Cohen has observed (Cohen, 1998):

> "There are numerous chemicals which are nongenotoxic, however, which appear to produce a carcinogenic response without producing direct cellular toxicity. These frequently are related to direct mitogenic processes and involve hormones and/or growth factors and their interactions with cellular receptors. In cell receptor biology, it is well accepted that a significant proportion of the receptors on the cell membrane must be occupied before a cellular response is generated. The percentage of receptors required to be occupied has been estimated in the range of 1% to as high as 30%. Regardless of the actual percentage, the fundamental principle is that a single receptor on the cell surface being occupied is insufficient to generate the cellular response. Thus, these processes can also be visualized as threshold phenomena.
>
> However, for chemicals that produce cancer through such cell receptor-mediated processes, the endogenous, physiologic ligands binding to receptors must be taken into account. For a substance such as estrogen, which clearly is mitogenic and carcinogenic for certain cell types, one additional molecule of administered estrogen could conceivably lead to a cellular response (cell division) since there already is in the blood stream a potentially high concentration of estrogen, either from exogenous consumption, or, more likely, from endogenous production. In these circumstances, the cell receptor-mediated process is actually a non-threshold phenomenon related to administered dose or exogenous exposure, since the threshold has already been exceeded by the level of the substance in the untreated animals."

Using the "classification" scheme for carcinogens described above, peroxisome proliferator-induced carcinogenesis most appropriately falls into the mode-of-action category in which the tumorigenic response is mediated by a cellular receptor for which there are endogenous physiological ligands present. Thus, according to these assessment schemes, there would likely be no threshold for carcinogenic effect for peroxisome proliferators. There is considerable uncertainty resulting from lack of scientific data regarding the most significant endogenous ligands for PPAR-α, their levels, and the resulting level of normal physiological "background" activity of this receptor.

Traditional Mathematical Models for Carcinogenesis

A number of lines of evidence support the linear extrapolation of risk to rodents from low dose exposure to DEHP:

- No evidence of a threshold in the dose-response relationship has been observed in the cancer results of bioassays in rodents (*i.e.*, no upward curving, non-linear dose-response data).

- DEHP-induced carcinogenesis in rodents appears to be a PPAR-α (receptor)-mediated phenomenon. PPAR-α activation by exogenous ligands (such as DEHP) is likely to be additive with activation by endogenous ligands (fatty acids, eicosanoids, or other as yet unidentified ligands).

- Evidence from *in vitro* studies which suggests a potential for DEHP to cause genetic damage (clastogenicity). Data from *in vivo* studies are lacking.

For these reasons, a model which assumes low-dose linearity (the linearized multistage model) was applied to the data sets showing a carcinogenic effect of DEHP in rodents. Two studies which showed a carcinogenic effect of DEHP in rats, Rao *et al.* (1987) and Rao *et al.* (1990), were not presented here for quantitative risk assessment, as they only examined a single dose level, thus providing more limited data for the estimation of the carcinogenic potency.

Table 10. Animal potency estimates from primary studies of DEHP in rodents.

Study	Sex	Dosing (mg/kg feed)	Calculated Dose (mg/kg-day)*	Combined liver tumors	q1* (mg/kg-day)$^{-1}$
Rat Studies					
NTP (1982)	Male	0, 6000, 12000	0, 240, 480	3/50, 6/49, 12/49	0.00068
	Female	0, 6000, 12000	0, 300, 600	0/50, 6/49, 13/50	0.00068
					0.0013
					0.00059
Mouse Studies					
					0.0013
					0.00079
David *et al.* (1999)	Male	0, 100, 500, 1500, 6000	0, 12, 60, 180, 720	8/70, 14/60, 21/65, 27/65, 37/70	0.0012
	Female	0, 100, 500, 1500, 6000	0, 13, 65, 195, 780	3/70, 4/60, 7/65, 19/65, 44/70	0.0016

* Dose rates were calculated based upon feed consumption as a fraction of body weight: male rats - 4%, female rats - 5%, male mice - 12%, female mice - 13%.

The potency estimates derived from the male and female rat and mouse studies are strikingly similar, with estimates in a comparison of the same sex and strain differing by less than two-fold between the NTP and David *et al.* studies.

The most sensitive sex and strain when using the linearized multistage model is the female mouse, with a potency estimates of 0.0016 (mg/kg-day)$^{-1}$ in the David *et al.* studies. The highest potency estimate derived from rat studies is 0.0013 (mg/kg-day)$^{-1}$ (also from the David *et al.* studies). While these two potencies are fairly close in magnitude, traditional allometric scaling between these two rodent species (based on body weight) would suggest a higher potency from the studies in mice than in the rats. Thus, of the rodent data sets available, the female mice should be considered the most sensitive sex and species by this methodological approach.

Alternative Approaches to Modeling Carcinogenic Risk

A recent review produced as a follow-up to a workshop on the assessment of human risk from exposure to peroxisome proliferators suggested several endpoints other than tumor development itself that may be appropriate for modeling the dose-response (Cattley *et al.*, 1998). These parameters included measures of hepatomegaly (*e.g.*, changes in relative liver weight),

peroxisome proliferation (*e.g.*, morphometric measure of peroxisome volume), and cell proliferation (*e.g.*, replicative DNA synthesis as measured by BrdU nuclear labeling).

Enzymatic Surrogates/Markers

As one of the primary downstream target genes of the PPAR-α, ACO activity would appear to be a good candidate for assessment or correlation of risk. Other candidates would include other PPAR-α activated enzymes (see Table 8). However, inconsistent correlations with carcinogenic potency have been noted (Conway *et al.*, 1989).

The studies in Sprague-Dawley rats reported by Ganning *et al.* (1991) point to a potential problem with using ACO as a surrogate for carcinogenic risk. In this long-term study, the rats were treated orally with DEHP at four doses for up to two years. While significant induction of ACO activity was observed in the rats (as high as 12-fold), no liver tumors or nodules were observed in this strain. By comparison, F344 rats in the David *et al.* (1999) studies only showed increases in ACO activity on the order of 4- to 6-fold after two years of exposure, yet significant increases in tumors were observed.

A study was conducted in which male Fischer 344 rats were treated in the diet with 1.2% DEHP or 0.1% Wy-14,643 for one year (Marsman *et al.*, 1988). Groups of 5-10 animals were sacrificed after 1, 4, 8, 18, 39, 77, 151, and 365 days of exposure. A clear hepatocarcinogenic effect was observed in the group treated with Wy-14,643 whereas the DEHP-treated group showed little indication of liver nodules or carcinogenesis. Although this study is of limited duration, it points to a difference in carcinogenic potency between these two compounds. In spite of this, peroxisomal volume density and peroxisomal β-oxidation enzyme activities were significantly increased in rats treated with both chemicals, suggesting that each of these parameters is a poor indicator of hepatocarcinogenic potential.

Cell Proliferation

A number of uncertainties and problematic areas related to the use of cell proliferation data for the estimation of carcinogenic risk have been identified by Cohen and Elwein (1991). One concern is whether measurement of proliferation the appropriate cell type has been done and whether the test model incorporates them. "…[T]he relevant proliferation is only that which occurs in the stem cell (pluripotential) population of the tissue, not in the differentiated cells." Given the uncertainties regarding the growth stimuli (PPAR-α-dependent and –independent) and cell types involved in peroxisome proliferator effects (parenchymal hepatocytes, Kupffer cells, or possibly other cell types; see sections above), it appears premature to use cell proliferation in estimating the potency of DEHP. Furthermore, as these authors note, the assessment of the degree of proliferation must not only take into account the mitotic rate, but also any changes in organ or cell size, both of which occur in the liver in response to treatment with peroxisome proliferators.

Extrapolation of Risk to Humans

According to Proposition 65 regulations (Title 22, CCR §12703(a)(6) and (7)) concerning the quantitative risk assessment:

> "(6) Human cancer potency shall be derived from data on human or animal cancer potency. Potency shall be expressed in reciprocal milligrams of chemical per kilogram of

bodyweight per day. Interspecies conversion of animal cancer potency to human cancer potency shall be determined by multiplying by a surface area scaling factor equivalent to the ratio of human to animal bodyweight, taken to the one-third power. This is equivalent to a scaling factor of 14 when extrapolating from mouse data, and a scaling factor of 6.5 when extrapolating from rat data."

"(7) When available data are of such quality that physiologic, pharmacokinetic and metabolic considerations can be taken into account with confidence, they may be used in the risk assessment for inter-species, inter-dose, and inter-route extrapolations."

For the purpose of this assessment, an initial interspecies scaling factor using a mouse to human body weight ratio to the third power will be applied to the potency derived from the most sensitive site, sex, and strain for tumor development in experimental animals (liver, female mice). This adjustment is applied to account for presumed similarities between rodents and humans in physiological, anatomical, and biochemical parameters.

It is proposed that the interspecies adjusted cancer potency be further adjusted by a factor of ten to account for the apparent reduced sensitivity at the cellular level of human cells compared to those of mice. This factor is based upon the following observations:

- Multiple studies of human tissues showing lower levels of PPAR-α expression (mRNA and protein), generally on the order of ten-fold, or greater (Palmer *et al.*, 1998; Tugwood *et al.*, 1998).

- *In vitro* evidence from human cells compared to rodent cells showing lower induction of enzyme markers of peroxisome proliferator responsiveness (ACO activity), on the order of ten-fold (Scotto *et al.*, 1995; Perrone *et al.*, 1998).

Some lines of evidence suggest that within a given species, expression of PPAR-α may vary three- to four-fold (Jones *et al.*, 1995) or even up to ten-fold (Lemberger *et al.*, 1994). Limited data available for human expression of PPAR-α indicate that expression also varies in this range (Palmer *et al.*, 1998), although clearly, due to limitations in the availability and quality of human tissues, these studies cannot be controlled and validated as well as those in experimental animals. This clearly remains an area of uncertainty with respect to the estimation of human carcinogenic risk.

This proposal that the human extrapolated cancer potency for DEHP be modified by a scaling factor of ten to account for the apparent interspecies differences in sensitivity to its effects on enzyme induction and DNA synthesis relies on the assumption that the carcinogenic effects of this compound are dependent on and limited by the expression of PPAR-α. A ten-fold factor would appear, based upon the data available from the studies of PPAR-α expression in humans by Palmer *et al*. (1998), to capture the difference between humans with relatively high expression and that of mice.

Thus the estimated human cancer potency becomes a product of the following expression, where bw_{human} and bw_{mouse} are the body weights of humans (70 kg) and mice (0.03 kg), respectively,:

$$\text{human cancer potency} = q_1^* \times \text{allometric scaling} \times \text{sensitivity scaling}$$

$$\text{human cancer potency} = q_1^* \times \left(\frac{bw_{human}}{bw_{mouse}} \right)^{1/3} \times \frac{1}{10}$$

$$\text{human cancer potency} = 0.0016 \text{ (mg/kg-day)}^{-1} \times 13.3 \times 0.1 = 0.0022 \text{ (mg/kg-day)}^{-1}$$

Further, based upon the discussion above concerning route-specific differences in metabolism of DEHP, no additional reduction to the carcinogenic potency will be applied for exposure by parenteral routes (*i.e.*, those that bypass the gut on initial exposure). This is based on evidence that humans have a greater capacity than rats to de-esterify DEHP in the blood compartment and on evidence that intact DEHP is not eliminated by humans, but eventually converted to de-esterified and oxidized metabolites, several of which appear to be active.

Thus, the estimated human cancer potency for exposures to DEHP by parenteral routes is also 0.0022 (mg/kg-day)$^{-1}$.

Areas of Uncertainty

There remain numerous areas of uncertainty related to the estimation of carcinogenic risk to humans from exposure to DEHP and other peroxisome proliferating chemicals. In this assessment, we have assumed that the carcinogenic process is dependent upon the activation of the cellular receptor PPAR-α and that the expression levels of this receptor govern the responsiveness of different species and also the carcinogenic risk. Some researchers have raised concern that using PPAR-α expression as a surrogate for sensitivity to peroxisome proliferators is an oversimplification:

> "The extensive information that has accumulated on the mechanism of PP [peroxisome proliferator] action in rodents, and the responses of humans to these compounds, has yet to provide a definitive explanation for species differences. However, since its discovery, PPARα has been shown to be an essential mediator of PP-induced responses in rodents and humans." … "The possibility that PPARα holds the key to species differences is still a plausible premise irrespective of whether the quantity or the quality of the receptor is more relevant. In fact, the most likely explanation is that both qualitative and quantitative factors are important to human and rodent responses to PPs. While it may be tempting to conclude that a lack of induction of certain classes of genes in humans is due solely to reduced PPARα levels, there is a considerable weight of evidence to suggest that this is an over-simplification." (Holden and Tugwood, 1999)

Some key uncertainties include the possibility that differences in response may occur as a result of other factors such as differences in DNA response elements in genes activated by PPAR-α, the contribution of alternatively spliced mRNA leading to the production of some fraction of inactive PPAR-α protein, or that there may be interspecies differences in the spectrum of genes regulated by PPAR-α. At present, there are enough conflicting data such that quantitative adjustments to the carcinogenic potency based on these factors cannot be made with confidence.

An important uncertainty relates to the recent finding that PPAR-α-dependent as well as PPAR-α-independent phenomena (induction of oxygen radicals in Kupffer cells; production of TNF-α) appear to occur in bringing about increases in DNA synthesis upon treatment of experimental animals. Whether these receptor-independent events are "rate limiting" for carcinogenic risk is unclear, as is the precise contribution of cell proliferation (as caused by stimulation of DNA synthesis) to the carcinogenic process.

Another area of uncertainty is the true range and distribution of PPAR-α expression in human populations and its susceptibility to modulating influences such as stress and hormones. Evidence of human responsiveness has also relied largely on evidence from *in vitro* studies of isolated parenchymal hepatocytes. In light of recent observations that the stimulation of DNA synthesis by peroxisome proliferators *in vivo* may be dependent on an interaction between two cell types in the liver (Kupffer cells and parenchymal hepatocytes), historical *in vitro* data need to be evaluated carefully since the degree of Kupffer cell "contamination" in primary cell cultures is generally not known or reported. If preparations of human hepatocytes were, for methodological reasons, to lack a Kupffer cell presence, it is unclear whether fair assessments of their response potential for this endpoint have been made. The range of sensitivity of humans to activators of PPAR-α may be influenced by certain factors which appear to potentially influence the sensitivity of rodents (via changes in PPAR-α expression):

> "It is not known whether PPARα expression can be induced by as-yet unidentified factors in human liver as it is in rat liver by glucocorticoids (Lemberger *et al.*, 1994). If so, elevated expression of [human] PPARα may increase the sensitivity of human liver to peroxisome proliferators." (Palmer *et al.*, 1998)

Human data on PPAR-α receptor expression are limited to studies of a few dozen individuals. The possibility that there may be subpopulations at risk because of enhance PPAR-α expression has not been adequately addressed in the available literature.

As part of their assessment following the identification of certain mutant alleles of PPAR-α in certain human populations (as described above), Sapone *et al.* (2000) observed that:

> "It is reasonable to suspect that variant and mutant PPARα alleles exist in the human population since PPARα-null mice are viable and capable of reproduction (Lee *et al.*, 1995). Since the PPARα probably functions in controlling many of the same metabolic pathways in these two species, humans deficient in PPARα or having altered expression due to variant alleles, might be expected to have an altered capacity to metabolize fatty acids." "While PPARα serves a critical function in modulating lipid homeostasis in both species, the role of this receptor in peroxisome proliferator-induced carcinogenesis in humans is not clear."

Gonzalez *et al.* (1998) has recently offered an appraisal of the current state of the science regarding peroxisome proliferators and carcinogenicity from the regulatory perspective:

> "Whether long-term exposure to peroxisome proliferators that are potent rodent hepatocarcinogens represents a hazard to humans is unknown [citing Cattley *et al.*, 1998].

> On the basis of this uncertainty, the regulatory agencies are especially cautious in licensing chemicals that exhibit peroxisome proliferation and carcinogenesis. Since peroxisome proliferation and enzyme induction can be demonstrated after short-term administration of test compounds to rodents, avoiding development of peroxisome proliferators has become standard practice in the pharmaceutical and chemical industries, especially if long-term human exposures are anticipated. To determine with certainty whether peroxisome proliferators can be carcinogenic in humans, however, the mechanisms of carcinogenicity and species differences in response need to be determined. Studies of peroxisome proliferator activated receptor α (PPARα) have brought us closer to this goal." (Gonzalez *et al.*, 1998)

As more experimental data become available in this rapidly developing area, further refinements to the estimation of carcinogenic risk to humans from exposure to DEHP may be made.

CALCULATION OF RISK SPECIFIC INTAKE

No Significant Risk Level Calculation Method

The intake level (I, in mg/day) associated with a cancer risk R, from exposure to a carcinogen is

$$I = \frac{R \times bw_h}{q_{human}}$$

where bw_h is the body weight, and q_{human} the theoretical cancer potency estimate for humans.

Daily intake levels associated with lifetime cancer risks at or below 10^{-5} are considered to pose no significant risk of cancer under Proposition 65 (Title 22 California Code of Regulations, Section 12703). Thus for a 70 kg adult, the intake level posing no significant cancer risk under Proposition 65 is given by

$$NSRL = \frac{10^{-5} \times 70\,kg}{q_{human}}$$

No Significant Risk Levels for DEHP

Potency estimates for rats and mice, in units of $(mg/kg\text{-}day)^{-1}$, derived from data on tumor incidence after oral exposure to DEHP are shown in Table 10. Evidence of a ten-fold lower level of PPAR-α expression in humans compared to mice suggests a corresponding reduction in human cellular sensitivity to the effects of DEHP. Using this value as a scaling factor applied to the human-extrapolated animal potency, $0.0022\ (mg/kg\text{-}day)^{-1}$ was estimated as the cancer potency for a 70 kg human. Based on this potency, the oral intake level associated with lifetime cancer risk of 10^{-5} for DEHP for a 70 kg adult is 310 µg/day.

ABBREVIATIONS

ACO	Acyl Co-A oxidase
ApoA	Apolipoprotein A
AUC	Area under the curve
BrdU	5-Bromo-2'-deoxyuridine
bw	Bodyweight
Cal/EPA	California Environmental Protection Agency
CAS	Chemical Abstracts Service
CAT	Carnitine acetyl transferase
CCR	California Code of Regulations
CDK	Cyclin dependent kinase
CHO	Chinese hamster ovary (cells)
DEHA	Di(2-ethylhexyl) adipate
DEHP	Di(2-ethylhexyl) phthalate
DINP	Diisononyl phthalate
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
2-EH	2-Ethylhexanol
EMSA	Electrophoretic mobility shift assay
ESRD MIS	End Stage Renal Disease Medical Information System
GJIC	Gap-junctional intercellular communication
HDL	High density lipoprotein
Hg	Mercury
kg	kilogram
MEHP	Mono(2-ethylhexyl)phthalate
mg	milligram
mM	millimolar
mol	mole
mRNA	Messenger ribonucleic acid
NAD^+	Nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB
NSRL	No significant risk level
NTP	National Toxicology Program
OEHHA	Office on Environmental Health Hazard Assessment
PCNA	Proliferating cell nuclear antigen
PFDA	Perfluorodecanoic acid
PGA1, PGA2	Prostaglandin A1, Prostaglandin A2
PGD1, PGD2	Prostaglandin D1, Prostaglandin D2
PGJ1	Prostaglandin J1
PPAR	Peroxisome proliferator activated receptor
PPRE	Peroxisome proliferator response element
PVC	Poly(vinylchloride)
RNA	Ribonucleic acid
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TGF-β1	Transforming growth factor-β1

TNF-α	Tumor necrosis factor-α
UCP-3	Uncoupling protein-3
U.S. EPA	U.S. Environmental Protection Agency

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