Biological effects of and exposure to the peroxisome proliferating agent di(2-ethylhexyl)phthalate

Hubert Dirven

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Een wetenschappelijke proeve
op het gebied van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen
op woensdag 12 mei 1993,
des namiddags te 3.30 uur precies

door

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geboren op 11 januari 1962 te Dordrecht

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CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Dirven, Hubertus Adrianus Antonius Maria

Biological effects of and exposure to the peroxisome proliferating agent di(2-ethylhexyl)phthalate / Hubertus Adrianus Antonius Maria Dirven. - [S.I. : s.n.].-Ill.
Thesis Nijmegen. - With ref. - With summary in Dutch.
ISBN 90-9005900-8
Subject headings: plasticizers / biotransformation / toxicology.

The investigations described in this thesis were carried out at the department of Toxicology, Katholieke Universiteit Nijmegen, Nijmegen, The Netherlands.

Financial support by the TNO Institute for Toxicology and Nutrition, Chrompack and Biorad for publication of this thesis is gratefully acknowledged.

Contents

Abbreviations

ATP	adenosine triphosphate
BNF	ß-naphtoflavone
Br-mdmc	4-(bromo-methyl)-6,7-dimethoxycoumarin
BSA	bovine serum albumin
cDNA	complementary DNA
CoA	coenzyme A
Da	dalton
DCF	dichlorofluorescin
DEHA	di(2-ethylhexyl)adipate
DEHP	di(2-ethylhexyl)phthalate
DMF	dimethylformamide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EROD	ethoxyresorufin-O-deethylation
GC	gas chromatography
GS-MS	gas chromatography-mass spectrometry
h	hour
g	acceleration due to gravity
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HPLC	high-performance liquid chromatography
id	internal diameter
ip	intra peritoneally
MEHP	mono(2-ethylhexyl)phthalate
mRNA	messenger ribonucleic acid
MS	mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NMR	nuclear magnetic resonance
P450	cytochrome P450
RNA	ribonucleic acid
PPAR	peroxisome proliferator activated receptor
PBS	phosphate-buffered saline
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gelelectrophoresis
TRIS	tris(hydroxymethyl)aminomethane
UDS	unscheduled DNA synthesis
wt	weight

Chapter 1

General Introduction

In 1963 a massive increase in the number of dense particles within the cytoplasm of hepatocytes was described after treatment of rats with clofibrate (Paget *et al.*, 1963). Two years later the dense particles were identified as peroxisomes (Hess *et al.*, 1965; Svoboda and Azarnoff, 1966). In the following years it was shown that structurally not related xenobiotics could also produce this increase in the size and number of peroxisomes within liver cells. This effect is now known as peroxisome proliferation. Compounds classified as peroxisome proliferating agents have various commercial applications, including plasticizers such as di(2-ethylhexyl)phthalate (DEHP) and drugs as clofibrate and ciprofibrate.

Clofibrate was one of the first peroxisome proliferating compounds to be identified as a rat liver carcinogen (Reddy and Qureshi, 1979). In 1980 Reddy *et al.* suggested that peroxisome proliferators represent a novel class of hepatocarcinogens. As these chemicals are neither DNA-reactive nor mutagenic, mechanisms inducing carcinogenicity are not very well understood. In addition species differences in the sensitivity for peroxisome proliferation have been described. For these reasons it is hard to assess the hazard of these compounds to man.

During the last years a number of excellent reviews have been published about peroxisome proliferating compounds (Reddy and Lalwai, 1983; Thomas and Thomas, 1985; Turnbull en Rodricks, 1985; Albro, 1986; Hawkins *et al.*, 1987; Stott, 1988; Conway *et al.*, 1989; Moody *et al.*, 1991; Moody *et al.*, 1992). In this chapter a brief description of the current knowledge on the toxicity of peroxisome proliferating compounds is given. In the last paragraph an outline of our studies on the peroxisome proliferating compound DEHP is presented.

1.1 - Inducers of peroxisome proliferation

Peroxisome proliferating compounds can be identified by morphometric studies of liver tissue (figure 1) or by determining the activities of peroxisomal ß-oxidation enzymes in liver homogenates after treatment of rats or mice with these compounds. The activity of peroxisomal palmitoyl-CoA oxidase is the most sensitive marker for peroxisomal changes (Tomaszewski *et al.*, 1987).

To date more than 60 xenobiotics including hypolipidaemic drugs, herbicides, leukotriene antagonists and plasticizers have been classified as peroxisome proliferating compounds (for review: Moody *et al.*, 1991). The chemicals belonging to this group are structurally quite divers. Common to most of the compounds is that they either are, or can be converted to hydrophobic carboxylic acids by hydrolysis or by oxidation, and that these carboxylic acids can not be metabolized by mitochondrial ß-oxidation (Berge *et al.*, 1992).

In addition to xenobiotic chemicals, it has been observed that several nutritional and physiological states can induce peroxisome proliferation in the rat liver. These include high fat diets, vitamin E deficiency, alloxan diabetes, starvation and cold adaptation (for review: Hawkins *et al.*,1987). These factors may constitute a separate class of inducers since there appears to be a limit (i.e., circa twofold) to the increase in peroxisomal volume or peroxisomal enzyme activity caused by these factors, whereas induction by xenobiotics may be 30-fold or more (Moody *et al.*, 1991).

Not all the chemicals induce peroxisome proliferation to the same extend. Fenofibrate, ciprofibrate, Wy-14,643 and the perfluoro fatty acids are very potent inducers, whereas DEHP, DEHA and aspirin are much weaker (Barber *et al.*, 1987; Sharma *et al.*, 1988). Differences in absorption, metabolism, distribution and excretion kinetics may play a role in the varying potency of these chemicals to induce peroxisome proliferation in vivo. However, data obtained in vitro has also demonstrated significant differences in potency of these compounds (Gray *et al.*, 1983; Lake *et al.*, 1984). Conflicting results have been reported about sex differences in sensitivity for peroxisome proliferation (Watanabe *et al.*, 1989; Kawashima *et al.*, 1989; Sundseth and Waxman, 1992).

Peroxisome proliferation is tissue specific. The most pronounced effects are determined in hepatocytes, with a limited response also occurring in renal cortex and intestinal mucosa (Nemali *et al.*, 1988; Reubsaet *et al.*, 1990). Rat hepatocytes transplanted into the anterior chamber of the eye do still respond to peroxisome proliferating compounds (Rao *et al.*, 1986), suggesting that hepatocytes possess liver-specific control mechanisms required for the induction of peroxisome proliferation.

Induction of peroxisome proliferation may occur relatively rapid. Hepatic acyl-CoA oxidase mRNA was induced 13-fold and enzyme activities were 2-fold increased 24 h after dosing (Bell *et al.*, 1991a). The effects are rapidly reversible following the termination of dosing (review: Reddy and Lalwai, 1983; Stott *et al.*, 1988).

Many of the characteristics of peroxisome proliferation in the intact animal can readily be observed in rat hepatocytes, treated in vitro with peroxisome proliferating compounds (review: Bieri *et al.*, 1990).

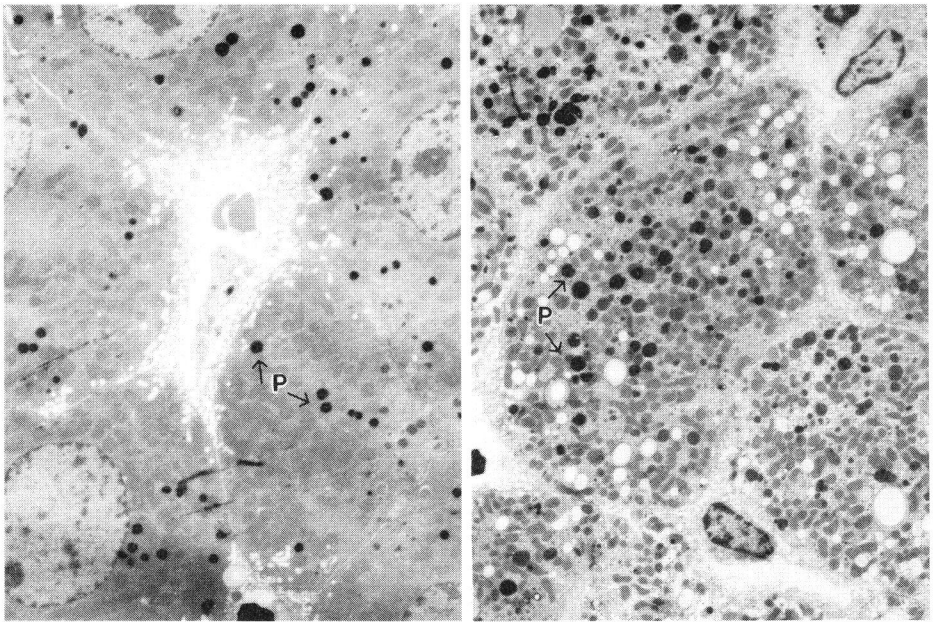

Figure 1 - Effect of feeding of DEHP on the number and volume of peroxisomes in the liver of rats. Peroxisomes are indicated by a P. Left: control liver, right: liver of DEHP treated rat (x ± 15,000). (photo by Jeanne Pertijs, Dept of Toxicology, University of Nijmegen).

1.2 - Peroxisomes

A number of enzymes are found in peroxisomes (for reviews on the biochemistry of peroxisomes: Tolbert, 1981; de Duve, 1983; Borst, 1989, van den Bosch *et al.,* 1992). Knowledge on the function of peroxisomes has expanded since a new group of genetic disorders have been described in which peroxisomal functions are impaired (for review: Schutgens *et al.*, 1986).

An important function of peroxisomes is their role in lipid metabolism. The peroxisomal fatty acid ß-oxidation system is very similar to the ß-oxidation system as present in mitochondria, however different enzymes are utilised. In mitochondria the first step in the ß-oxidation of fatty acids is catalysed by acyl-CoA dehydrogenases. The electrons are transferred to the respiratory chain and are used to produce adenosine triphosphate (ATP).

Lacking an electron transport system, the first enzyme of the peroxisomal ß-oxidation pathway, an acyl-CoA oxidase, transfers two electrons to molecular oxygen to form hydrogen peroxide. Hydrogen peroxide is degraded by catalase which is also located in peroxisomes (Tolbert, 1981; de Duve, 1983).

The function of the peroxisomal ß-oxidation system appears to be the shortening of fatty acids that are poorly oxidized by the mitochondria (e.g. long chain fatty acids) into fragments that can be oxidized by the mitochondria. In addition the peroxisomal ß-oxidation system has a high affinity for dicarboxylic acids and ω-hydroxy fatty acids, which are poorly metabolized by the mitochondrial ß-oxidation system (Reubsaet *et al.*, 1988; Vamecq and Draye, 1989; Poosch and Yamazaki, 1989; Suzuki *et al.*, 1989).

1.3 - Liver carcinogenesis

Anatomically, the liver lies on the main route by which substances absorbed from the intestine pass into the systemic circulation. Biochemically, it possesses an array of enzymes which are capable of metabolizing most xenobiotics and endogenous compounds. Because of this the liver is most frequently affected in carcinogenicity studies (IARC, 1987).

In two-stage models of liver carcinogenesis, chemicals may contribute to cancer risk by acting at the first stage (initiation) and/or the second stage (promotion) of tumour formation (review: Dragan and Pitot, 1992). Initiation is easily demonstrated for chemicals that are genotoxic and cause a modification of the DNA bases. Initiation involves the irreversible formation of individual cells possessing the potency to expand clonally under the influence of promoting agents. Promotion has been defined as an reversible enhancement of the proliferation and the genetic expression of initiated cells.

Mechanisms of non-genotoxic carcinogens are much less understood. Barrett *et al.* (1987) mentioned some possible mechanisms, i.e. 1) unusual metabolic activation, 2) indirect mutagenesis, for example by interfering with spindle assembly during mitose leading to chromosomal abberations, by reducing the efficiency of DNA repair mechanisms or by altering the methylating state of DNA and 3) alternatively, some non-genotoxic carcinogens might mimic the effect of natural signal transduction factors and therefore perturb normal cell growth and differentiation. Schulte-Hermann *et al.* (1983) suggested that non-genotoxic agents could enhance tumour growth in rodent bioassays by facilitating the development of tumours from cells that were spontaneously initiated. Cohen and Ellwein (1991) have suggested that non-genotoxic chemicals can initiate carcinogenesis if exposure leads to increased cell proliferation. This initiation may be attributed to a low risk of genetic alteration inherent with replicative DNA synthesis.

Numerous studies indicate that peroxisome proliferating compounds are non-genotoxic agents (for review: Turnbull and Rodricks, 1985; Stott, 1988; IARC, 1987). Occasionally positive responses have been reported but this is always marked by reservations with the experimental protocol and lack of reproducibility in other laboratories.

The evidence of carcinogenicity of DEHP comes from a National Toxicology Program bioassay (NTP, 1982). In this study rats and mice were fed a diet containing DEHP for two years. The incidence of hepatocellular carcinomas was statistically significant in female rats and male and female mice. The International Agency for Research on Cancer (IARC, 1987) has evaluated the carcinogenicity studies performed with peroxisome proliferators and has classified DEHP and nafenopin as a group 2b carcinogen (= "agent is possibly carcinogenic to humans") and di(2-ethylhexyl)adipate (DEHA) and clofibrate as a group 3 carcinogen (= "agent is not classifiable as to its carcinogenicity to humans"). Also for other peroxisome proliferating compounds (positive) carcinogenicity studies have been reported, although these compounds have been tested with small numbers of animals (for review: Stott, 1988; Conway *et al.*, 1989). The carcinogenic potency of various peroxisome proliferating compounds differs considerable. For example rats fed a diet containing 0.1% Wy-14,643 produce a 100% liver tumour incidence compared with none in rats fed a diet with 1.2% DEHP (Marsman *et al.*, 1988).

Tumorigenesis induced by genotoxic carcinogens follows a progression from altered foci, neoplastic nodules to hepatocellular carcinomas (Pitot, 1990). In tumours induced by peroxisome proliferating compounds a similar progression is observed. Peroxisome proliferator-induced altered cells have like cells induced by other hepatocarcinogens, decreased ATPase and glucose-6-phosphatase activities and are resistant to iron accumulation during dietary overload. However, these cells do not have enhanced activities of γ-glutamyl transpeptidase, glutathione S-transferase π, or α-fetoprotein (for review: Reddy and Rao, 1987).

1.4 - Possible mechanisms in hepatocarcinogenesis by peroxisome proliferating compounds

Two theories have been advanced to explain by which mechanisms peroxisome proliferating compounds induce carcinogenesis. One theory explains the carcinogenicity as a direct result of peroxisome proliferation and the other theory proposes that peroxisome proliferators have a promotor activity (for review: Conway *et al.*, 1989).

1.4.1 - Peroxisome proliferating compounds and oxidative stress

A correlation exists between the ability of compounds to induce proliferation of peroxisomes and their hepatocarcinogenicity upon chronic administration to rodents (Reddy and Lalwai, 1983; Barber *et al.*, 1987). The oxidative stress hypothesis tries to link peroxisome proliferation with

DNA damage (review: Rao and Reddy, 1987). In this model, the enhanced production of hydrogen peroxide by the peroxisomal ß-oxidation system is larger than the ability of hydrogen peroxide degrading enzymes, and results in a slow accumulation of oxidative damage to the genome. Activities of enzymes like glutathione peroxidase, glutathione transferase and superoxide dismutase are decreased by peroxisome proliferating compounds, which might result in higher concentrations of hydrogen peroxide in the cytosol (Furukawa *et al.*, 1985; Lake *et al.*, 1987). However, hepatic capacities of hydrogen peroxide degrading enzymes may still exceed the hydrogen peroxide-generating levels (Tamura *et al.*, 1990).

A number of studies provides evidence for this theory. Treatment of rats with a peroxisome proliferating compound simultaneously with the antioxidants ethoxyquin and 2(3)-tert-butyl-4-hydroxyanisole prevents the formation of tumours (Rao *et al.*, 1984). The number of 8-hydroxydeoxyguanosine and 5-hydroxymethyl-2-deoxyuridine DNA adducts were increased after treatment of rats with peroxisome proliferating compounds (Kasai *et al.*, 1989; Takagi *et al.*, 1990 and 1991; Srinivisan and Glauert, 1990). Unidentified DNA alterations were detectable by the ^{32}P post-labelling assay after chronic administration of ciprofibrate and Wy-14,643 to rats (Randerath *et al.*, 1991).

DNA damage induced by activated oxygen might result in DNA breaks, which can be detected by alkaline elution of cellular DNA. Inconsistent results have been reported about these experiments after treatment of rats with peroxisome proliferating compounds (Bentley *et al.*, 1987; Kornbrust *et al.*, 1984; Tamura *et al.*, 1991; Nilsson *et al.*, 1991). Attempts to induce DNA repair or unscheduled DNA synthesis by peroxisome proliferating agents, which might be expected if DNA-modifying active oxygen radicals were formed, have, so far, been unsuccessful (Glauert *et al.*, 1984; Smith-Oliver and Butterworth, 1984; Cattley *et al.*, 1988; Nilsson *et al.*, 1991). Numerous tumours were observed in rats fed Wy-14,643, but none in rats fed DEHP despite the fact that DEHP produced only 25% less peroxisome proliferation than Wy-14,643 (Marsman *et al.*, 1988). In old rats treated with nafenopin more tumours were observed than in young rats, although no differences in parameters associated with oxidative stress were found (Huber *et al.*, 1991).

Lipid peroxidation products like diene conjugate formation have been reported after long-term treatment of rats with peroxisome proliferating compounds and a correlation was found between conjugated diene formation and carcinogenicity (Lake *et al.*, 1987; Conway *et al.*, 1989a; Goel *et al.*, 1986; Cattley *et al.*, 1987). The formation of the pigment lipofuscin might be a predictor for tumour formation by peroxisome proliferating compounds (Lake *et al.*, 1987; Conway *et al.*, 1989a; Marsman *et al.*, 1992). It has been proposed that lipofuscin is generated by the polymerization of oxidized lipid within the lysosome, and might be used as a marker for oxidative stress (Reddy et al., 1982). There appears to be a correlation between the potency of a peroxisome proliferating carcinogen and the level of accumulated hepatic lipofuscin (Cattley *et al.*, 1987;

Conway *et al.*, 1989a; Marsman *et al.*, 1992).

Summarizing: although there is some evidence that peroxisome proliferator-induced hepatocarcinogenicity is associated with oxidative stress, definitive evidence that this is the primary mechanism responsible for tumour formation is lacking.

1.4.2 - Peroxisome proliferating compounds act as promotors

The rate of cell proliferation is considered to be important in non-genotoxic carcinogenesis since cell proliferation might lead to increased chances of fixation of DNA damage as well as an enhanced conversion rate of initiated cells to tumour cells. Quantification of mitotic figures, autoradiographic quantification of labelled nuclei after pulse labelling with tritiated thymidine and scintillation counting of thymidine incorporation into liver DNA are methods to study cell proliferation (Ward *et al.*, 1984; Smith-Oliver and Butterworth, 1987; Butterworth *et al.*, 1984; Butterworth *et al.*, 1987 and Marsman *et al.*, 1988). These studies indicate that DEHP and other peroxisome proliferating compounds cause a rapid burst in cell proliferation during the first 7 days of treatment and then cell proliferation reverts to normal levels despite continued administration of the compound. Differences in potency to induce tumours are not reflected in differences in DNA synthesis during the first 7 days of treatment with DEHP and Wy-14,643 (Marsman *et al.*, 1988), so it has been argued that it is unlikely that this rapid burst in cell proliferation is related to the carcinogenicity of these compounds (Conway *et al.*, 1989).

Chronic elevation of the hepatocyte replication rate have been related to the carcinogenicity of peroxisome proliferating compounds (Marsman *et al.*, 1988). However, persistent replicative DNA synthesis has only been reported for Wy-14,643 but not for other potent carcinogenic, peroxisome proliferating compounds like clofibrate, nafenopin and LY-171883 (Marsman *et al.*, 1988; Eacho *et al.*, 1991; Marsman *et al.*, 1992).

It has been suggested that sustained replication of subpopulations of hepatocytes are involved in the carcinogenic process. Two cell types, large cells, uniformly basophilic (Cattley *et al.*, 1991; Marsman *et al.*, 1992) and small cells, heterogeneously basophilic (Kraupp- Grasl *et al.*, 1990 and 1991) might be involved in this process.

Recently, it has been reported that after treatment with Wy-14,643 and with nafenopin more tumours were found in the livers of old rats when compared to young rats (Kraupp-Grasl *et al.*, 1991, Cattley *et al.*, 1991). Since old rats might have higher number of spontaneously initiated cells these experiments support the theory that peroxisome proliferating compounds are promotors of carcinogenesis.

The use of initiation-promotion protocols can provide more insight into

the carcinogenic action of peroxisome proliferating compounds (for review: Popp *et al.*, 1987; Conway *et al.*, 1989). Quantification of altered hepatic foci (AHF) is commonly used as an indicator of carcinogenicity (Pitot, 1990). However, this method is less useful in studying peroxisome proliferating compounds since the ratio of AHF and tumours is lower with peroxisome proliferating compounds than with genotoxic hepatocarcinogens (review: Conway *et al.*, 1989). This may explain the variable results in initiating/promoting studies which only measured altered cell transformation. In studies in which tumour formation was used as an endpoint peroxisome proliferating compounds were identified as promoters (review: Conway *et al.*, 1989). Initiating activity has not yet been identified. Studies in which initial treatment with DEHP in rats (Garvey *et al.*, 1987; Williams *et al.*, 1987) or mice (Ward *et al.*, 1986) and ciprofibrate and Wy-14,643 in rats (Cattley *et al.*, 1989; Glauert and Clark, 1989) were followed by various promotion regimes failed to reveal any initiating activities of these compounds.

Summarizing: It is likely that peroxisome proliferating compounds have a promotor activity in carcinogenesis, however it has not been demonstrated that chemically induced cell proliferation is the primary mechanism by which these compounds induce cancer (Melnick, 1992). Peroxisome proliferators have liver tumour incidence rates approaching 100%, which have not been reported for other promoters (Moody *et al.*, 1991). For this reason it is likely that other factors play a role in the carcinogenic process as well.

1.5 - Cytochrome P450

Cytochrome P450 is a family of haem based enzymes which perform a wide range of oxidative reactions (for review: Porter and Coon, 1991). The enzymes are membrane bound in the smooth endoplasmic reticulum or in a few cases, the mitochondrial inner membrane. A classification was devised by comparing amino acid sequences of individual cytochrome P450 enzymes with each other. Of the 27 gene families so far described, 10 exist in all mammals (Nebert *et al.*, 1990). Six of these are small families of one or two members involved in pathways of steroidogenesis in highly specialized tissues or in bile acid synthesis. Three families encode drug metabolizing enzymes, and one family (Cytochrome P450 4) consist of P450s that hydroxylate fatty acids (for review: Gibson, 1989).

1.5.1 - Cytochrome P450 4A1

A large increase in the terminal hydroxylation of fatty acids was reported in 1980 after administration of the peroxisome proliferators clobuzarit and methylclofenapate to rats (Parker and Orton, 1980). Subse-

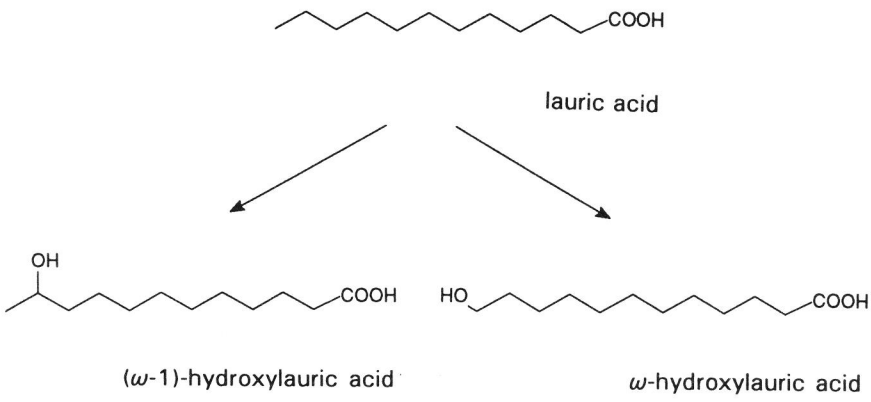

Figure 2 - Lauric acid is the marker substrate for the determination of cytochrome P450 4A1 activity. After incubation of microsomal liver fractions and lauric acid, in the presence of NADPH, ω-hydroxylauric acid and (ω-1)-hydroxylauric acid are formed. Lauric acid ω-hydroxylation activity is most characteristic for cytochrome P450 4A1 enzymes.

quently, an cytochrome P450 enzyme was purified to homogeneity from clofibrate induced rat liver. This enzyme was termed cytochrome P-452, based on the wavelength absorption maximum of the ferrous-carbonmonoxide adduct of the haemoprotein. The pure protein has a molecular weight of 51.500 Da and possesses an extremely high substrate specificity, the preferential substrate being lauric acid. Lauric acid is metabolized to ω-hydroxylauric acid, with trace amounts of the (ω-1)-hydroxylauric acid being formed (Tamburini *et al.*, 1984) (figure 2). According to the classification of Nebert *et al.* (1990) cytochrome P452 is now named P450 4A1 (before 1990: P450 IVA1). Cytochrome P450 4A1 has a highly structured active site that steric suppresses (ω-1)-hydroxylation in order to deliver the oxygen to the thermodynamically disfavoured terminal carbon (CaJacob *et al.*, 1988). The nucleotide sequences of the cDNAs coding for cytochromes P452 and P450LAw, an enzyme purified and characterized by Hardwick et al., 1987, have been shown to be virtually identical (Hardwick *et al.*, 1987; Earnshaw *et al.*, 1988).

The precise number of genes in the P450 4 family are not known at present. At least 3 clofibrate-inducible cytochrome P450 4 gene products are found in liver and kidney of rats (Kimura *et al.*, 1989a en b). Cytochrome P450 4 enzymes are constitutively expressed in both liver and kidney. The enzymes are inducible in both tissues (Kimura *et al.*, 1989a and b; Sharma *et al.*, 1989).

1.6. Mechanisms in peroxisome proliferation

Many studies were aimed at discovering the initial trigger for peroxisome proliferation. A substrate overload-perturbation of lipid metabolism is mentioned in most theories (for review: Lock *et al.*, 1989: Berge *et al.*, 1992). This substrate overload is either a result of lipolysis occurring outside the liver with a subsequent influx of fatty acids into the liver, or is a consequence of the peroxisome proliferating compounds or their metabolites perturbing lipid metabolism within the liver, for example inhibition of mitochondrial fatty acid oxidation (Veitch *et al.*, 1989; Foxworthy and Eacho, 1988). In this latter model the inducer directly inhibits mitochondrial ß-oxidation enzymes, and/or the carnitine acyltransferase responsible for transport of fatty acids across the mitochondrial membrane.

A variant on the substrate overload theory postulates that peroxisome proliferation by xenobiotics and by different metabolic states is mediated by increased levels of long-chain acyl-CoA (Berge and Aarsland, 1985). Since many peroxisome proliferating compounds possess a carboxylic acid group, or a group that can be oxidized to a carboxylic group, several authors have dealt with the idea that formation of the CoA esters of peroxisome proliferators is a prerequisite for peroxisome proliferating activity (Hertz *et al.*, 1985; Bronfman *et al.*, 1986; Aarsland and Berge, 1991; Berge *et al.*, 1992).

1.6.1 - Peroxisome proliferation and cytochrome P450 4A1

Studies with peroxisome proliferators in rats have demonstrated an excellent correlation between induction of microsomal P450 4A1 and lauric acid ω-hydroxylation on the one hand and peroxisomal volume and peroxisomal palmitoyl-CoA oxidation on the other (Lake *et al.*, 1984 and Sharma *et al.*, 1988). Sharma *et al.* (1988) have hypothesized that cytochrome P450 4A1 induction is mechanistically interrelated to peroxisome proliferation (figure 3). According to this model induction of cytochrome P450 4A1 gives an increase in cellular ω-hydroxy fatty acids that are subsequently oxidized to dicarboxylic acids. These dicarboxylic acids are good substrates for the peroxisomal ß-oxidation system, but not for the mitochondrial ß-oxidation system (see paragraph: 1.2). According to this model peroxisome proliferation is a response of peroxisomes to a substrate overload of one of its preferred substrates, in which P450 4A1 is a necessary requisite. After treatment with peroxisome proliferating compounds P450 4A1 RNA is detected earlier than acyl-CoA oxidase RNA (Bieri *et al.*, 1990; Milton *et al.*, 1990; Bell *et al.*, 1991a and b). Inactivation of cytochrome P450 4A1 by 1-aminobenzotriazole inhibits peroxisome proliferation (Ortiz de Montellano *et al.*, 1992). The induction of cytochrome P450 4A1 therefore appears to be an early and key event in peroxisome proliferation.

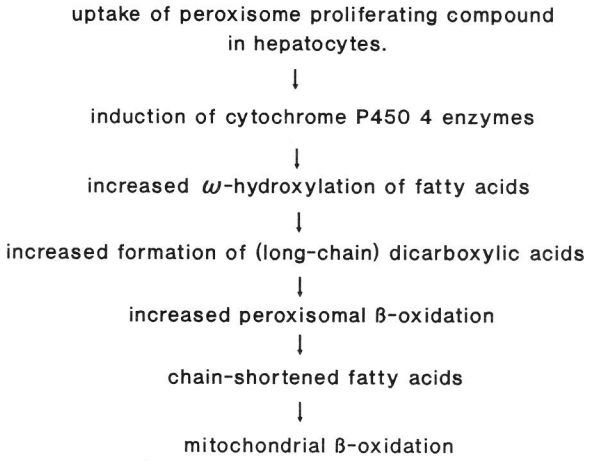

Figure 3 - Scheme to show the proposed inter-relationship of microsomal and peroxisomal changes in fatty acid metabolism after treatment of rats with peroxisome proliferating compounds, according to the model of Sharma *et al., 1988.*

1.6.2 - Peroxisome proliferation is receptor mediated

Which control mechanisms are influenced to result in a proliferation of hepatic peroxisomes is unknown at present. The idea of a receptor mediated activation of specific genes is supported by the tissue-specific nature of proliferation, the uniform enzymatic responses, the rate of the increase in transcriptional activation, and induction of peroxisome proliferation in cultured or transplanted hepatocytes (Moody *et al.*, 1991).

A member of the nuclear hormone receptor family termed PPAR (Peroxisome Proliferator Activated Receptor) was activated by the addition of several peroxisome proliferators (Isseman and Green, 1990). Nuclear hormone receptors are intracellular proteins that bind their ligand with high affinity and specificity and in addition recognize short DNA motifs. Binding of the ligand-receptor complex to DNA can either activate or repress specific gene expression (for review: Green, 1992). A good correlation was observed between the ability of peroxisome proliferating compounds to activate PPAR and their potency as either peroxisome proliferators or rat liver carcinogens. The tissue specific expression of PPAR compares well with the tissue specific induction of acyl-CoA oxidase by peroxisome proliferators (Isseman and Green, 1990). Recently, it was demonstrated that PPAR is a peroxisome proliferator activated transcription factor for the acyl-CoA oxidase gene (Tugwood *et al.*, 1992).

Recently, the cloning from the rat of a gene homologous to that encoding mouse PPAR have been described (Göttlicher *et al.*, 1992). This receptor was activated by arachidonic acid, linoleic acid and saturated fatty acids.

1.7 - Species differences in the effects of peroxisome proliferating compounds

Peroxisome proliferation has been demonstrated in most mammalian species that have been tested, however the dose required to produce recognizable proliferation varies widely among species (review: Rodricks and Turnbull, 1987; Stott *et al.*, 1988). The mouse and rat are most responsive; the hamster has an intermediate response; and the guinea pig, marmoset, and other non-human primates are weakly responsive (Orton *et al.*, 1984; Lake *et al.*, 1984; Watanabe *et al.*, 1989; Lake *et al.*, 1989).

A morphological analysis of liver biopsy tissue from hyperlipidaemic patients receiving therapeutic dose level of clofibrate (20-40 mg/kg.day), revealed a 1.5 fold increase in the number, and a 23% increase in the volume density of hepatocellular peroxisomes (Hanefeld *et al.*, 1983). In patients treated with gemfibrozol and fenofibrate no increase in hepatocellular peroxisomes was found (de la Iglesia *et al.*, 1981; Gariot *et al.*, 1987; Blane and Pinaroli, 1980; Blümcke *et al.*, 1983). Measurements of peroxisomal enzymes have not been reported. An epidemiological study did not show an increase in cancer rate after the use of certain hypolipidemic drugs (Muldoon *et al.*, 1990). Several in vitro studies suggest that human and non-human primate hepatocytes are relatively unresponsive to hypolipidemic drugs and phthalate ester plasticizers (Butterworth *et al.*, 1989; Blaauboer *et al.*, 1990; Foxworthy *et al.*, 1990). Mechanisms behind these species differences in sensitivity for peroxisome proliferation are not very well understood (see chapter 6).

Studies on species differences in the stimulation of liver cell replication have been very limited (Bieri *et al.*, 1988; Styles *et al.*, 1988). Stimulation of DNA synthesis has been observed in non-human primate hepatocytes at concentrations that do not cause peroxisome proliferation (Bieri *et al.*, 1988).

1.8 - Outline of the studies described in this thesis

In a panel discussion held in 1988 between scientists and regulatory policy experts no consensus could be achieved regarding several critical issues on the effects of DEHP on human health (Schulz, 1989).

In figure 4 a scheme is presented which might be used in assessing the health effects of peroxisome proliferating compounds on humans. If peroxisome proliferation is indeed an important step in the development of cancer it is necessary to determine if man/primate are also sensitive for this effect. Since peroxisome proliferating agents are non-genotoxic carcinogens, extrapolation of the effects as found in rat/mouse to man might be based on the determination of a no-effect level. When this no-effect level is known, it is possible to calculate a dose with no increased risk on the development of malignancy. When in addition information is

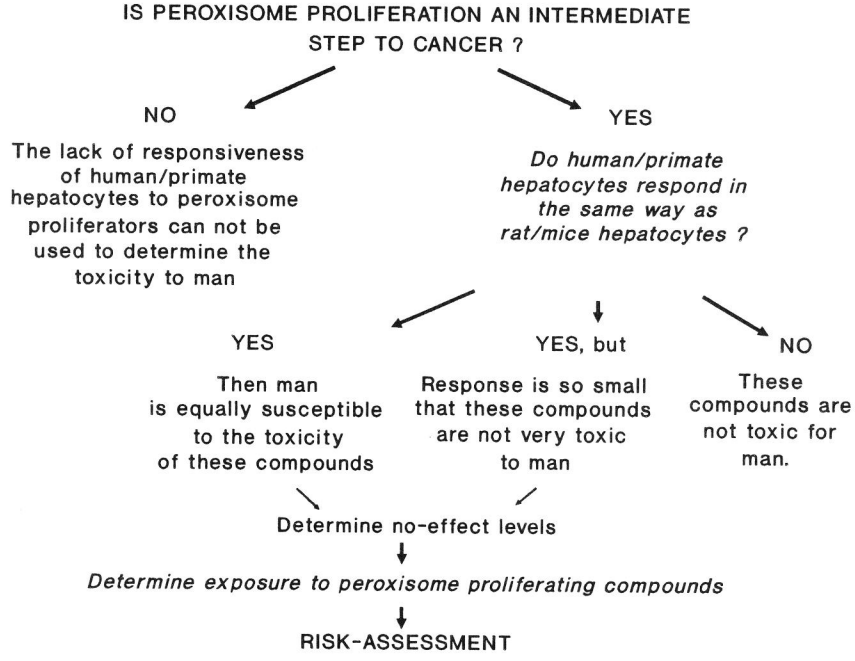

Figure 4 - Scheme to assess the health effects of peroxisome proliferating compounds for humans. The topics we have studied are indicated in italic letters.

available on the exposure to peroxisome proliferating compounds, the risk of human exposure to these agents might be assessed.

Our studies have been mainly focussed on one peroxisome proliferating agent, i.e. the polyvinylchloride plasticizer di(2-ethylhexyl)phthalate. We have chosen this compound since environmental and occupational exposure to DEHP is wide (Gold *et al.*, 1987). According to the scheme outlined in figure 4 our studies covered mainly two topics, i.e. the problem of species differences in peroxisome proliferation caused by DEHP and the development of methods to determine exposure to DEHP.

Our studies were designed to find answers on the following questions:

1. What is the mechanism of action of peroxisome proliferating compounds and how can species differences in sensitivity for peroxisome proliferation be explained?
2. Are there specific biomarkers to assess exposure to peroxisome proliferating compounds?
3. What is the environmental and occupational exposure to DEHP?

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Chapter 2

Effect of di(2-ethylhexyl)phthalate on enzyme activity levels in liver and serum of rats.

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Toxicology ***65****: 199-207, 1990*

Abstract

In order to identify non-invasive, biochemical indicators of di(2-ethylhexyl)phthalate (DEHP) exposure, we have compared the effects in blood serum with biochemical effects in liver in rats fed a diet containing 0, 0.25, 0.75 and 2 % DEHP for 2 weeks. After 3 days of treatment serum arylesterase activity levels and serum triglycerides were decreased to 60% and 20% of control values, respectively. After a 2-week treatment with DEHP the effects were generally stronger. Compared to a control group, serum arylesterase activity levels, serum triglycerides and serum cholesterol were decreased to 40%, 20% and 50%, respectively. Serum cholinesterase activity levels and serum albumin concentrations were increased by the DEHP treatment to 290% and 135% of control values, respectively.

In the livers a hepatomegaly, an induction of cytochrome P-450 IVA1, and induction of the activity of palmitoyl-CoA oxidase and carnitine acetyl-CoA transferase was found to be 180%, 1080%, 1300% and 1700% of control values, respectively. The liver is a more sensitive target for DEHP exposure compared to the biochemical effects in serum, but determination of the serum parameters can be used to determine early biological effects of exposure to DEHP.

Introduction

The phthalate ester di(2-ethylhexyl)phthalate (DEHP) is widely used as a plasticizing agent in numerous consumer plastic products and medical devices. DEHP belongs to the class of agents described as non-genotoxic, hypolipidaemic hepatocarcinogens. These chemicals have in common the

ability to induce peroxisome proliferation in the liver. It has been suggested that the hepatocarcinogenicity of DEHP and other peroxisome proliferating compounds is linked to peroxisome proliferation [1]. Peroxisome proliferating agents induce a number of non-peroxisomal biochemical alterations [2]. The peroxisome proliferator clofibrate has been reported to affect tissue carboxyesterase in liver, kidney and testis in rats and mice [3]. Dietary exposure of rats and mice to the peroxisome proliferating compounds nafenopin and clofibrate decreased serum arylesterase activity levels and increased serum cholinesterase activity levels [4]. Both enzymes are carboxylic ester hydrolases and are involved in the detoxification of organophosphates. Peroxisome proliferation in the liver is associated with the induction of the activity of acyl-CoA oxidase, and carnitine acetyl-CoA transferase [2]. Recently, it was reported that treatment of rats with DEHP results in an induction of cytochrome P-450 IVA1 [5].

In order to identify sensitive, non-invasive biochemical indicators of exposure to DEHP we have investigated in this study the effect of DEHP on several biochemical parameters in serum (arylesterase activity levels, cholinesterase activity levels, triglycerides, cholesterol, albumin) and compared with effects found in liver (mass, cytochrome P450 IVA1 concentration and activities of acyl-CoA oxidase and carnitine acetyl-CoA transferase).

Materials and Methods

Chemicals

DEHP (99.5% pure) was obtained from Janssen Chimica (Beerse, Belgium). Leuko-2,7-dichlorofluorescin diacetate was a product of Eastman Kodak (Rochester N.Y., U.S.A.). Palmitoyl-CoA, acetyl-CoA, peroxidase, rat serum albumin (fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.)

Antibodies against cytochrome P-450 IVA1 were a generous gift of Dr. G.G. Gibson (University of Surrey, U.K.). Triglycerides and cholesterol testkits were from Boehringer Mannheim (FRG). All other chemicals were obtained from commercial sources and were of the highest purity obtainable.

Animals and treatment.

Male random-bred Wistar rats (173 ± 8 g body weight) were individually housed and fed ad libitum a pelleted diet, containing 22% protein, 4.8% fat and 67% carbohydrate. After 7 days of acclimatization four experimental groups were formed. These groups received a diet containing 0%, 0.25%, 0.75% or 2% DEHP (w/w) for 14 days. This diet was custom synthesized RMH-TH by Hope Farms, Woerden, The Netherlands. The actual concentrations of DEHP in the food were determined by means of GC analysis. During the experiment food intake and body weight were

Table 1 - Food consumption during the administration of DEHP (g/day per kg body wt) and the calculated DEHP dose (g DEHP/day per kg body wt).

% DEHP in diet	Before experiment	0-3 days		0-14 days	
	Food consumption	Food consumption	DEHP dose	Food Consumption	DEHP dose
0%	90.3 ± 5.6	91.9 ± 6.9	0	72.2 ± 5.2	0
0.25%	88.1 ± 8.0	94.9 ± 6.9	0.24	71.4 ± 3.8	0.18
0.75%	88.8 ± 4.4	90.9 ± 0.68	0.68	71.4 ± 3.8	0.52
2.0%	89.7 ± 4.9	68.0 ± 7.0*	1.36	68.6 ± 3.1	1.37

All values are means ± SD of 4 rats.
Statistically significant effects are indicated by * $P<0.005$.

monitored daily. One day before the DEHP-treatment started and 3 and 14 days after the start of the treatment blood was collected by orbitae puncture.

Animals were killed by decapitation and livers were perfused with 0.9% NaCl (w/w) for 10 min. Livers were removed and immediately cooled in ice-cold buffer, consisting of 0.25M sucrose/2mM EDTA/10mM Tris-HCl (pH 7.4). Whole liver homogenates (20% w/v) were prepared in the same buffer by manual homogenization. Samples of whole liver homogenates were taken for the determination of peroxisomal and microsomal marker enzymes, the latter fraction prepared by a standard centrifugation method as described [6].

Biochemical assays

Serum arylesterase (E.C. 3.1.1.2) activity was assayed according to the method of Kitchen [7] with phenylacetate as a substrate and in the presence of eserine sulfate, to inhibit cholinesterase. Serum cholinesterase (E.C. 3.1.1.5) activity was measured as described by Butler *et al.* [8] using butyrylthiocholine as a substrate. Cholesterol was determined by the enzymatic method of Siedel [9] and triglycerides by the enzymatic method of Wahlefeld [10].

Acyl-CoA oxidase (with palmitoyl-CoA as a substrate) and carnitine acetyl-CoA transferase were measured in homogenates that were freeze-thawed 3 times. The acyl-CoA oxidase assay was based on the determination of hydrogen peroxide-dependent oxidation of leuko-difluorescin as described [11]. Carnitine acetyl-CoA transferase activity was measured at 25 °C [12]. Cytochrome P-450 was determined by measurement of the CO

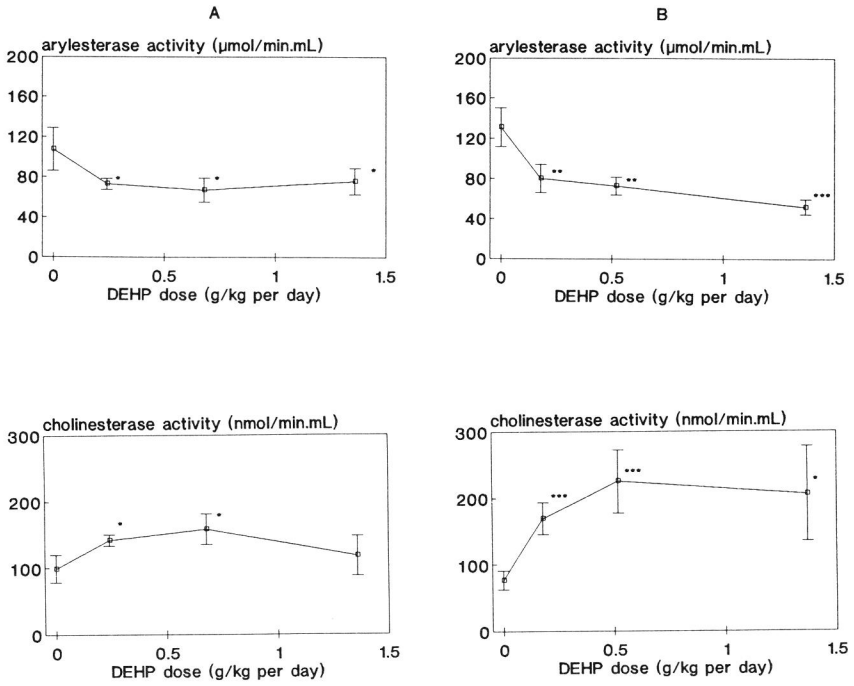

Figure 1 - Effect of consumption of a DEHP-containing diet for 3 (A) and for 14 days (B) on serum arylesterase activity and serum cholinesterase activity levels. Mean ± SD of 4 rats is presented.
Statistically significant effects are indicated by * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.001$.

difference spectrum as described by Omura and Sato [13]. Cytochrome P-450 IVA1 was determined immunochemically with an ELISA method [5].

All assays were performed using saturating substrate concentrations and conditions of linearity with time and enzyme concentration.

Other procedures.

Serum albumin was measured according to Rodkey with crystalline rat serum albumin as standard [14]. Protein concentrations were determined by the method of Lowry *et al.* [15] with crystalline bovine serum albumin as a standard. Differences of the parameters after DEHP treatment compared to control values were tested for statistical significance using the t-test. A level of significance of $p<0.05$ (two tailed) was chosen.

Results

The rats accepted the DEHP containing diet readily. Food consumption of rats receiving DEHP-containing diets was not statistically significantly different from the food consumption of rats using the control diet, except for the 2% DEHP-diet group during the first 3 days. Based on the food intake a DEHP-dose could be calculated (table 1). The dose levels used in this study are lower than in most other published studies [16, 17].

Control serum samples of all 16 rats were collected before starting the experiment. The control values of serum arylesterase- and cholinesterase activity and triglyceride-, cholesterol- and albumin concentrations were 114 ± 15 μmol/min.ml, 102 ± 13 nmol/min.ml, 2.4 ± 0.45 μmol/ml, 1.9 ± 0.3 μmol/ml and 35.2 ± 3.7 mg/ml, respectively.

The effect of DEHP administration for 3 and 14 days on serum arylesterase- and cholinesterase activity levels, triglyceride-, cholesterol- and albumin concentrations are shown in figures 1 and 2, respectively. After DEHP treatment for a period of 3 days arylesterase activity levels were decreased to 60% of control values and the triglyceride concentrations were decreased to 20% of control values. Serum cholinesterase activity levels and serum albumin concentrations were increased whereas no effect on serum cholesterol concentrations was found, except for the 2% dose group.

After DEHP treatment for a period of 14 days the effects were generally stronger. Compared to control values serum arylesterase activity levels (40%), triglyceride concentrations (20%) and cholesterol concentrations (50%) were decreased. On the contrary serum cholinesterase activity levels (290%) and albumin concentrations were increased (135%). The monitoring of consumption of food shows that the decreased serum triglycerides- and cholesterol concentrations were an effect of the DEHP treatment and not from an altered intake of food (table 1).

The effects of DEHP administration on liver parameters are shown in table 2. A dose-related hepatomegaly was found. In liver homogenates, a large increase in hydrogen peroxide production with palmitoyl-CoA as substrate was found, typical for increase in acyl-CoA oxidase activity by the peroxisomal ß-oxidation pathway. Also carnitine acetyl-CoA transferase activities were highly enhanced. Proliferation of the hepatic endoplasmic reticulum results in the induction of cytochrome P-450 IVA1. Our data demonstrate that the initial cytochrome P-450 IVA1 concentration was shown to be 1.5% of total cytochrome P-450. Treatment with DEHP produced a 6-8 fold increase over constitutive levels in the dose groups studied.

It appeared that the effects on the liver were much stronger compared to the effects in serum.

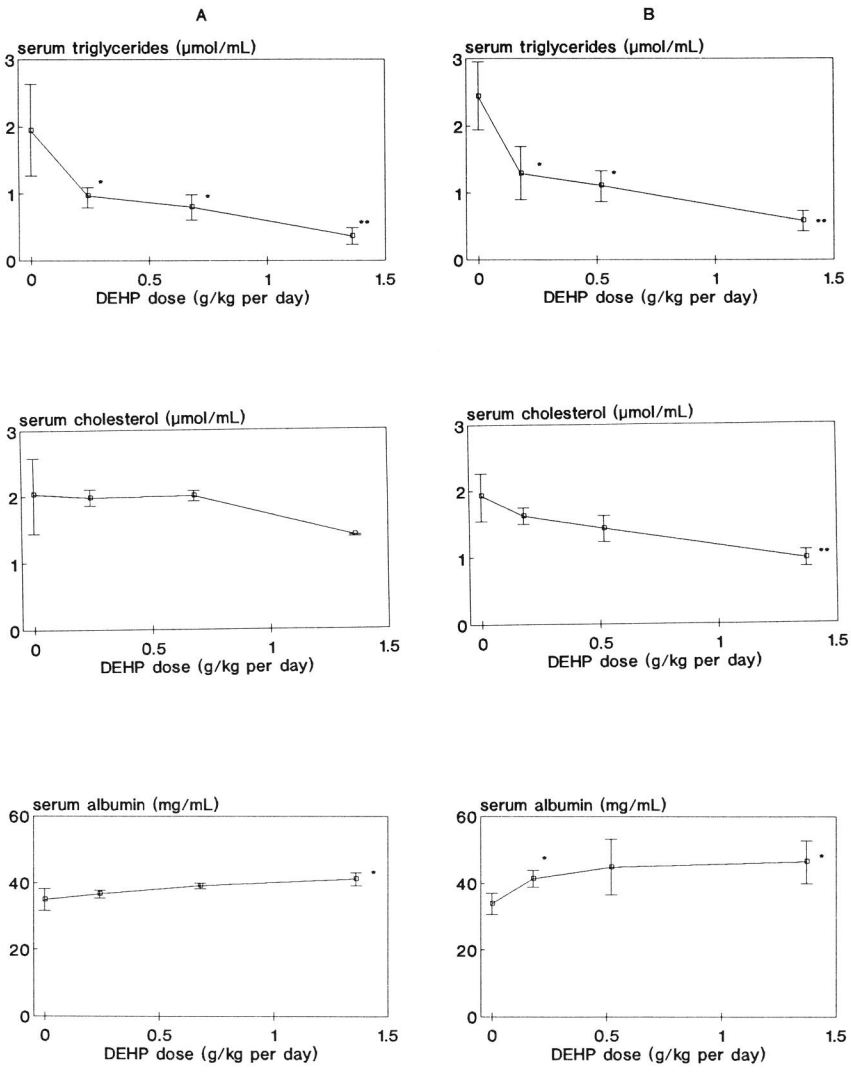

Figure 2 - Effect of consumption of a DEHP-containing diet for 3 (A) and for 14 days (B) on serum triglyceride concentrations, serum cholesterol concentrations and serum albumin concentrations. Mean ± SD of 4 rats is presented. Statistically significant effects are indicated by * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.001$.

Discussion

The findings reported here demonstrate that rats fed a diet with DEHP develop an increased level of serum cholinesterase activity and a decreased level of serum arylesterase activity. The serum arylesterase- and the serum cholinesterase activity level are 40% and 292% of control values, respectively. Similar effects were found by Butler *et al.* [4] after treatment of rats and mice with clofibrate and nafenopin. Rats fed a diet with 0.5% clofibrate for 3 weeks showed increased serum cholinesterase activity (194 to 248% as compared to controls) and decreased serum arylesterase activity (27 to 39% as compared to controls).

Since arylesterase activity is associated with the concentration of high-density lipoprotein [7], Butler *et al.* [4] proposed that the decrease in serum arylesterase activity level found in rodents after treatment with nafenopin and clofibrate is an effect of the lowered serum lipid levels. Our data demonstrate that both serum arylesterase activity level and serum triglycerides are already altered after 3 days of DEHP treatment.

Butler *et al.* [4] suggested that the elevated serum cholinesterase activity level found after treatment of rodents with nafenopin and clofibrate is a result of an increased liver mass, since cholinesterase is synthesized in the liver by parenchymal cells and released promptly after synthesis [4]. Our data show that after treatment with DEHP cholinesterase activity is increased to 290% of control values, but also the albumin is increased to 135% of control values. It is likely that the increase in serum cholinesterase activity and the increase in serum albumin levels are both a result of an increased protein formation capacity of the liver caused by liver enlargement. The hypolipidaemic effects of DEHP were first described by Reddy *et al.* [16] and were confirmed in other studies. Our data demonstrate that the hypolipidaemic effects appear rapidly after exposure and at low dose levels.

The administration of DEHP resulted in a pronounced hepatomegaly. The activities of the hepatic peroxisomal enzymes palmitoyl-CoA oxidase and carnitine acetyl-CoA transferase are increased by DEHP treatment. Tomaszewski *et al.* [18] have shown that acyl-CoA oxidase activity is the most suitable marker for peroxisomal changes caused by DEHP [17]. It has been reported [5, 6] that the increase in the number and size of peroxisomes in rats treated with peroxisome proliferators correlates well with the increased carnitine acetyl-CoA transferase activity, although it is not a specific peroxisomal enzyme. Increases in the activity of both enzymes are generally accepted as indicators for peroxisome proliferation in the liver. Recently, it was shown that treatment of rats with peroxisome proliferating agents induces a specific cytochrome P-450 isoenzyme named cytochrome P-450 IVA1 [5]. This induction shows a very strong correlation with an increased ω-hydroxylation activity of lauric acid. In our study we have also found a strong induction of this cytochrome P-450 IVA1 isoenzyme.

The effect of DEHP on liver parameters is stronger compared to the

Table 2 - Effect of consumption for 14 days of a DEHP-containing diet on liver/body weight, palmitoyl-CoA oxidase activity, carnitine acetyl transferase activity and cytochrome P450 4A1 concentrations in the liver.

DEHP dose (g/day per kg body wt	Liver/body wt (%)	palmitoyl-CoA oxidase (nmol H_2O_2/min.mg protein)	carnitine acetyl-transferase activity (nmol/min. mg protein)	cytochrome P450 4A1	
				% of total cytochrome P450	nmol cytochrome P450 4A1/mg protein
0	3.4 ± 0.2	4 ± 0.4	1.8 ± 0.4	1.4 ± 0.11	0.017 ± 0.01
0.18	4.3 ± 0.4* (126 %)[1]	12.8 ± 8.5 (318 %)	3.8 ± 1.5* (207 %)	9.6 ± 0.6*** (660 %)	0.085 ± 0.02** (500 %)
0.52	4.9 ± 0.5** (144 %)	23.4 ± 10.6* (580 %)	17.5 ± 6.6** (969%)	15.2 ± 0.4*** (1041 %)	0.155 ± 0.02*** (912 %)
1.37	6.1 ± 0.3*** (179 %)	52.3 ± 4.7*** (1297 %)	30.8 ± 9.4*** (1703 %)	15.8 ± 1.7*** (1082 %)	0.158 ± 0.02*** (929 %)

All values are means ± SD of 4 rats.
[1] Percentage of control value in parenthesis
Statistically significant effects are indicated by: * $P < 0.05$; ** $P < 0.005$ and *** $P < 0.001$.

effect on biochemical parameters in serum.

Whether peroxisome proliferating agents are able to affect serum esterase activity levels in humans is not yet known. If indeed the decrease of serum arylesterase activity is caused by the hypolipidaemic activity of DEHP, then this effect can be found in humans, since other compounds of the group of non-genotoxic peroxisome proliferating agents, like clofibrate and nafenopin, are well known hypolipidaemic drugs used in human therapy.

Although the liver is a more sensitive target for DEHP, blood serum indicators can be used to detect early biological changes as a result of DEHP exposure.

Acknowledgments

We thank Dr. J.C.M. Hafkenscheid from the Clinical-Chemical Laboratory of the Radboud Hospital, Nijmegen (NL) for performing the serum triglycerides and -cholesterol determinations.

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Chapter 3

Determination of the cytochrome P450 4 marker, ω-hydroxylauric acid by high performance liquid chromatography and fluorimetric detection.

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Journal of Chromatography, Biomedical Applications ***564****: 266-271, 1991.*

Abstract

The formation of ω-hydroxylauric acid from lauric acid is an indicator of the activity of cytochrome P450 IV family proteins. The two main metabolites of lauric acid, (ω-1)- and ω-hydroxylauric acid, have been completely separated by reversed-phase high-performance liquid chromatography. Measurement of lauric acid hydroxylase activitity in microsomal liver samples, based on derivatization of the substrate and metabolites with the fluorescent agent 4-bromo--methyl-6,7-di- methoxycoumarin, is a precise method (coefficient of variation = 7.6% and 10%, ω- and (ω-1)-metabolites, respectively) with good sensitivity (signal-to-noise ratio in microsomal samples of untreated rats > 20). In microsomal fractions from livers of rats treated with di(2-ethylhexyl)phthalate the extent of the ω-hydroxylation of lauric acid increased dose-dependently (ca. ten fold). The (ω-1)-hydroxylase activity was not altered. A strong correlation between immunochemically determined cytochrome P-450 IVA1 and lauric acid ω-hydroxylase activity was found (r = 0.94, n = 30).

Introduction

The formation of ω-hydroxylauric acid (=12-hydroxylauric acid) from lauric acid is widely acknowledged to be a marker of the activity of the cytochrome P-450 IVA1 isoenzyme [1]. After treatment with hypolipidaemic, peroxisome proliferating compounds such as clofibrate, nafenopin and di(2-ethylhexyl)phthalate (DEHP), ω-hydroxylase activity is induced in liver as well as in kidney [2, 3].

Since the group of hypolipidaemic, peroxisome proliferating compounds is classified as a unique class of epigenetic hepatocarcinogens [4], much attention has been paid to these responses. Recently, Sharma *et al.* [2] postulated that a perturbation of lipid metabolism in the liver results in a proliferation of peroxisomes. Disturbances in lipid metabolism are due to an enhanced ω-hydroxylation of fatty acid by cytochrome P-450 IVA1.

The formation of (ω-) and (ω-1)-hydroxylauric acid (11-hydroxylauric acid) is usually determined by gas chromatography (GC) [5] or high performance liquid chromatography (HPLC) [6]. Since lauric acid and its metabolites have no UV absorption or fluorescence characteristics, detection in the HPLC method is usually achieved by radiochemistry which necessitates the use of radiolabelled lauric acid substrate. Recently, two reports were published in which non-radioactive lauric acid was used as substrate. In one study, lauric acid and its metabolites were converted to a UV-absorbing ester derivative before the HPLC separation. However, co-eluting UV-absorbing peaks in organic solvent extracts of microsomes were reported [7]. Imaoka *et al.* [8] determined lauric acid and its metabolites by HPLC with fluorimetric detection, following derivatization with 9-antryldiazomethane. However, no data on the sensitivity and reproducibility of this method were presented.

The aim of the present study was to develop a reproducible and sensitive method for the determination of the hydroxylase activities towards lauric acid in microsomal preparations without the necessity for radiolabelled substrates. Since 4-bromomethyl-6,7-dimethoxycoumarin (Br-mdmc) can be used as a fluorimetric derivatization agent for carboxylic acid [9], we applied this compound in the determination of the (ω)- and (ω-1)-hydroxy metabolites of lauric acid in microsomal fractions.

Experimental

Reagents and chemicals

Lauric acid, ω-hydroxylauric acid and NADPH were obtained from Sigma (St. Louis, MO, USA). (ω-1)-Hydroxylauric acid was a generous gift of Dr. S. Imaoka (Osaka City University Medical school, Osaka, Japan). 18-Crown-6 ether and 4-(bromo-methyl)-6,7-dimethoxycoumarin were obtained from Aldrich (Milwaukee, WI, USA). DEHP was obtained from Janssen Chimica (Beerse, Belgium). Antibodies against cytochrome P-450 IVA1 were a generous gift of Dr. G. Gordon Gibson, University of Surrey, U.K. All other chemicals were of the highest purity obtainable.

Chromatography

The analyses were performed using the HPLC gradient system model 8800 from Spectra Physics. A 20 μl portion was injected on to a 150 x 4.6 mm i.d. Nucleosil C_{18} (5.0 μm) column (Machery-Nagel, Düren, Germany) (column temperature, 30°C, flow-rate, 1.0 ml/min). The solvent programme started isocratically with 67% (v/v) methanol and 33% water for 25 min, followed by

a 10-min linear gradient to 97% (v/v) methanol and 3% water in 10 min, and a hold for 5 min. The chromatograph was equipped with a fluorescence spectrofotometer (Shimadzu RF-530), interfaced with an Apple II computer. The excitation wavelength was 340 nm, emission wavelength 420 nm. Peak areas were calculated using a Chromatochart program (Interactive Microware).

The rate of the hydroxylase reaction was calculated from the fractional conversion of lauric acid into (ω-1)- and ω-hydroxylauric metabolites. Concentrations of the hydroxylated metabolites formed were determined from their relative peak areas of the total area of the parent compound and metabolites. Since the methanol percentage increases during the run and methanol increases the fluorescence intensity of the Br-mdmc derivatizing agent [9], peak areas of (ω-1)- and ω-hydroxylauric acid were corrected for reduced fluorescence.

Animals and treatment

Male random-bred Wistar rats (150 to 200 g) were pretreated by gastric intubation once a day with DEHP for 3 consecutive days at the following dose levels: 50, 100, 250, 500 and 1000 mg/kg body weight per day. Five or six animals were used in each group. Olive oil was used as a vehicle for DEHP administration. Control animals were given olive oil at 5 ml/kg body weight per day. Animals were killed by decapitation 24h after the last dose. Livers were perfused with 0.9% NaCl (w/w) for 10 min. Whole liver homogenates (20% w/v) were prepared in 0.25M sucrose-2 mM EDTA and 10 mM Tris/HCl (pH 7.4). Microsomal fractions were prepared by a standard centrifugation method [10].

Assay of ω- and (ω-1)-hydroxylase activities towards lauric acid

The activities of the microsomal fraction (1 mg protein) towards lauric acid was measured in a reaction mixture containing 200 nmol lauric acid in 2 ml of 50 mM Tris-HCl (pH 7.5). The test-tubes were incubated for 5 min at 37 °C prior to the addition of 50 μl of 40 mM NADPH to initiate the reaction. After 10 min the reaction was stopped by the addition of 300 μl of 3M HCl. The reaction mixture was extracted twice with 5 ml diethylether. The extracts were evaporated to dryness under a gentle flow of nitrogen at 35 °C.

A 1-ml volume of a solution of 18-crown-6-ether in acetone (250 μg/ml) was added to the dried extract. After the addition of 1 mg dried potassium carbonate and 0.5 ml of acetone with Br-mdmc (2 mg/ml), the derivatization was performed at 70 °C for 45 min in a sealed test tube. The mixture was evaporated to dryness under a gentle flow of nitrogen at 40°C. The residue was dissolved in a fixed volume of methanol, and an aliquot was analysed by HPLC.

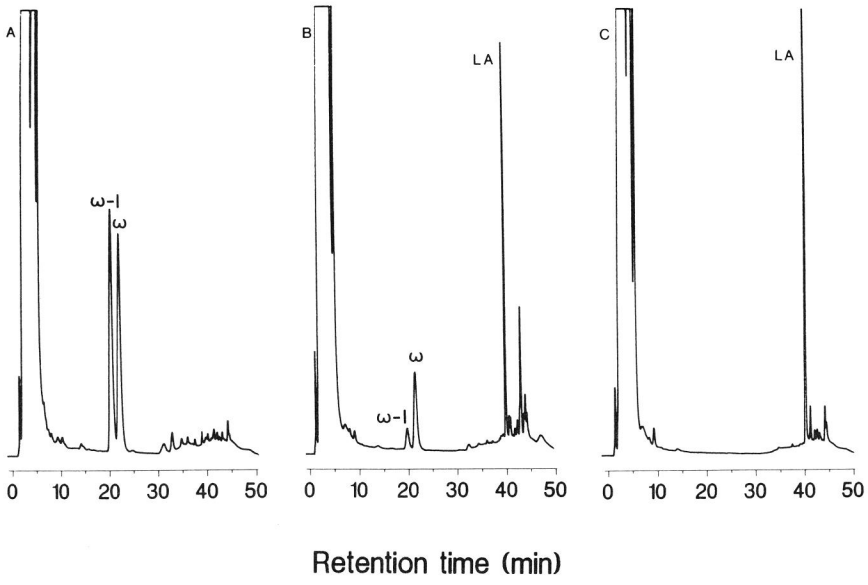

Figure 1 - Chromatograms of diethyl ether extracts of microsomal fractions of rat liver after derivatization with 4-bromomethyl-6,7-dimethoxycoumarin (Br-mdmc). Peaks: ω-1 = (ω-1)-hydroxylauric acid; ω = ω-hydroxylauric acid; LA - Lauric acid. Gradient elution as described in Experimental. (A) Chromatogram of authentic ω- and (ω-1)-hydroxylauric acid standards. (B) Chromatogram of lauric acid metabolites generated by a microsomal fraction of a DEHP-treated rat (1000 mg/kg body weight), after a 10-min incubation of 1 mg protein at 37 °C with NADPH. (C) as B, but without NADPH in the incubation mix.

Other methods

Cytochrome P-450 was determined from the CO-difference spectrum [11]. Cytochrome P-450 IVA1 was determined immunochemically by an enzyme-linked immunosorbent assay (ELISA) method described by Sharma *et al.* [2]. Protein concentrations were determined by the method of Bradford [12].

Results and discussion

Figure 1 shows typical chromatograms obtained from authentic compounds (Figure 1A), and lauric acid metabolites generated by a microsomal fraction of the liver with (Figure 1B) and without NADPH (figure 1C), after derivatization with a fluorescent compound. The retention times of the esters of (ω-1)-hydroxylauric acid, ω-hydroxylauric and lauric acid were 19.5, 22.0 and 40.0 min, respectively. The (ω-1)- and ω-hydroxylauric acid formed in the presence of NADPH by a microsomal fraction of the liver are clearly base-line separated

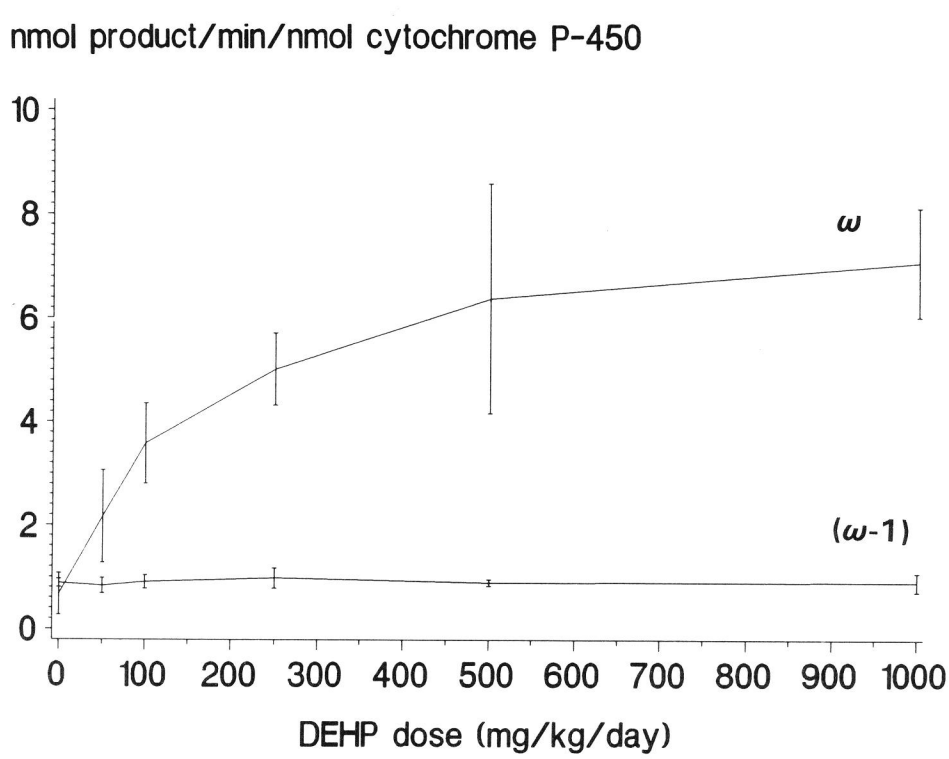

Figure 2 - Effect of DEHP on (ω-1)- and ω-hydroxylase activity towards lauric acid in rat liver microsomes. Mean ± S.D. values are presented. Each group consisted of five or six rats.

and are not detected in the absence of NADPH. Interferences from fatty acids extracted from the microsomal fractions were not observed.

The ω- and (ω-1)-hydroxylase activities towards lauric acid in microsomal fractions isolated from the livers of rats treated with DEHP (doses: 0, 50, 100, 250, 500 and 1000 mg/kg body weight per day) are shown in Fig 2. A dose-dependent increase in the ω-hydroxylase activity was observed, but no alteration in the (ω-1)-hydroxylase activity was found. Similar results have been reported by Sharma *et al.* [13].

The formation of ω-hydroxylauric acid from lauric acid is accepted as a marker of the activity of cytochrome P-450 IVA1 [1]. The cytochrome P-450 IVA1 concentrations in the microsomal fractions have been determined with ELISA. The immunochemically determined cytochrome P-450 IVA1 concentrations correlated strongly with the ω-hydroxylase activity towards lauric acid in these samples ($r = 0.94$, $p = 0.0001$, $n = 30$) (Fig 3) and did not correlate with the (ω-1)-hydroxylase activity ($r = 0.17$, $p = 0.36$, $n = 30$).

Sharma *et al.* [13] used the same ELISA procedure and applied a hydroxylase assay with radiolabelled lauric acid as substrate and found identical results.

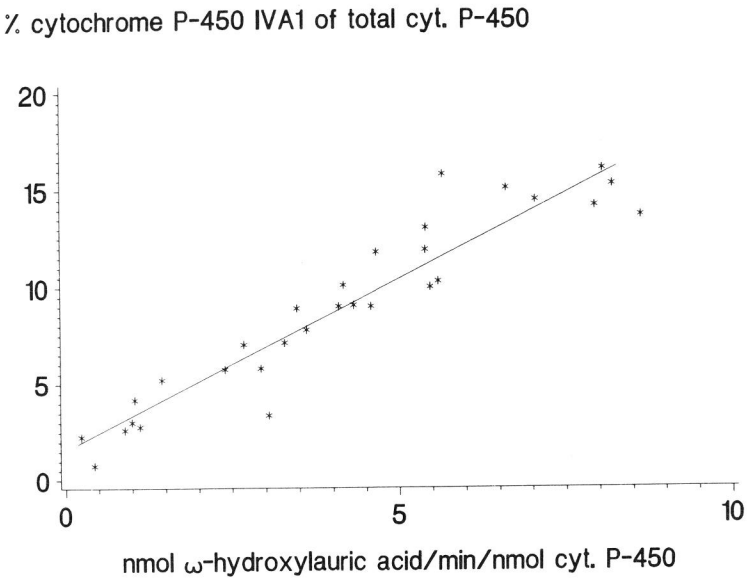

Figure 3 - Plot of immunochemically determined cytochrome P450 4A1 concentrations and ω-hydroxylase activities towards lauric acid in microsomal fractions from rats treated with DEHP ($y = 1.6 + 1.8x$, $r = 0.94$, $p = 0.0001$, $n = 30$)

As the determination of lauric acid metabolites was possible in microsomal liver samples of untreated rats, the sensitivity of the method is sufficient (signal-to-noise ratio for ω- and (ω-1)-hydroxylauric acid in samples of the untreated rats is > 20). The coefficient of variation (C.V.) based on duplicate incubations is 7.6.% for ω-hydroxylauric acid ($n = 40$) and 10% for (ω-1)-hydroxylauric acid ($n = 40$). The Br-mdmc esters of lauric acid and (ω-1)- and ω-hydroxylauric acid in methanol are stable for at least 2 months when stored in the dark at ambient temperature.

Measurement of lauric acid hydroxylase activity based on derivatization of the substrate and metabolites with Br-mdmc provides a precise and sensitive method for the determination of lauric acid hydroxylase activities without the need of using radiolabelled substrates.

Acknowledgments
The authors are indebted to Mrs. P.H.H. van den Broek and Mrs. J.G.P. Peters for technical assistance in parts of this study.

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Chapter 4

Microsomal lauric acid hydroxylase activities after treatment of rats with three classical cytochrome P450 inducers and peroxisome proliferating compounds.

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Biochemical pharmacology ***43****: 2621-2629, 1992.*

Abstract

In order to investigate a proposed relationship between induction of hepatic microsomal lauric acid hydroxylase activity and peroxisome proliferation in the liver, male Wistar rats were treated with peroxisome proliferating compounds, and the lauric acid hydroxylase activity, the immunochemical detectable levels of cytochrome P450 4A1 and the activities of peroxisomal enzymes were determined. In addition, the levels of cytochrome P450 4A1 and lauric acid hydroxylase activities were studied after treatment of rats with three cytochrome P450 inducers. After treatment with aroclor-1254, phenobarbital or 3-methylcholanthrene total cytochrome P450 was 1.7-2.7 times induced. However, no induction of lauric acid ω-hydroxylase activities or P450 4A1 levels were found. After treatment of rats with di(2-ethylhexyl)phthalate (DEHP) a dose-dependent induction of lauric acid ω-hydroxylase activities, levels of cytochrome P450 4A1 and peroxisomal fatty acid β-oxidation was found. Even at a dose-level of 100 mg DEHP/kg body weight per day a significant induction of these activities was observed. The main metabolites of DEHP, mono(2-ethylhexyl)phthalate and 2-ethyl-1-hexanol also caused an induction of levels of P450 4A1, lauric acid ω-hydroxylase activities and the activity of peroxisomal palmitoyl-CoA oxidase. 2-Ethyl-1-hexanoic acid did not influence lauric acid ω-hydroxylase activities, but did induce levels of P450 4A1 and palmitoyl-CoA oxidase activities. Three other compounds (perfluoro-octanoic acid, valproate and nafenopin) induced both lauric acid ω-hydroxylase activity and peroxisomal palmitoyl-CoA oxidase activity. The plasticizer di(2-ethylhexyl)adipate, did not induce levels of P450 4A1, lauric acid ω-hydroxylase activities or palmitoyl-CoA oxidase activities. With the compounds tested a close association between the induction of lauric acid ω-hydroxylase activities and peroxisomal palmitoyl-CoA oxidase activity was found. These

data support the theory that peroxisome proliferating compounds do induce lauric acid ω-hydroxylase activities and that there might be a mechanistic inter-relationship between peroxisome proliferation and induction of lauric acid ω-hydroxylase activities.

Introduction

A large number of chemicals that induce hepatic peroxisome proliferation cause the emergence of liver cell carcinoma in lifetime animal assays. This group includes highly used hypolipidaemic drugs, like clofibrate and nafenopin, chlorophenoxyacid herbicides and industrial plasticisers, like di(2-ethylhexyl)phthalate (DEHP). Chemically, these compounds seem to be a heterogenous group, however many of them can be metabolized to a hydrophobic backbone with a carboxylic function. Administration of these compounds to rodents results in an increase in the number and volume of peroxisomes in hepatocytes and also in an increase in the number of cells undergoing DNA synthesis (for review: [1, 2]). Peroxisome proliferators are called non-genotoxic hepatocarcinogens since they cause liver cell carcinoma but fail to cause DNA damage directly [3]. The exact mechanistic relationship of peroxisome proliferation and tumour formation remains unknown.

Proliferation of peroxisomes is proposed to be mediated by an increased formation of ω-hydroxy fatty acids due to cytochrome P450 4A1 activity, which are converted to dicarboxylic acids in the cytoplasm of hepatocytes [4]. Dicarboxylic acids are mainly metabolized by the peroxisomal β-oxidation system [5,6]. In the metabolism of fatty acids by the peroxisomal β-oxidation system hydrogen peroxide is produced. It has been proposed that the imbalance between hydrogen peroxide production and degradation, results in an oxidative stress and ultimately in DNA damage. Propagation of this leasion by the well-documented hyperplastic response to peroxisome proliferators might result in liver cell carcinoma [7].

After treatment of rats *in vivo* or hepatocytes of rats *in vitro* with peroxisome proliferators it was shown that RNA encoding cytochrome P450 4A1 appears prior to RNA encoding peroxisomal enzymes [8,9,10]. Bell *et al.* [9] showed by immunohistochemical staining that cytochrome P450 4A and peroxisomal acyl-CoA oxidase were induced in the same region of the liver by the peroxisome proliferator methylclofenapate. Cytochrome P450 4A1, formerly termed cytochrome P452, is a fatty acid hydroxylating enzyme (for review: [11]). Medium-chain length (C6-C12) fatty acids are oxidized by either of two microsomal pathways, ω- or (ω-1)-hydroxylation. Tamburini *et al.* [12] reported that purified cytochrome P450 4A1 in a reconstituted enzyme system exhibits a lauric acid ω- and (ω-1)-hydroxylase activity in a 6:1 ratio. However, Thomas *et al.* [13] found that purified cytochrome P450 4A1 in a reconstituted system only catalyses the ω-hydroxylase activity, while an equimolar mixture of 4A2 and 4A3

catalyze both ω- and (ω-1)-hydroxylase activity in a 3:1 ratio. Tanaka *et al.* [14] found that multiple purified cytochrome P450 enzymes have lauric acid (ω-1)-hydroxylase activities but not ω-hydroxylase activities, so lauric acid (ω-1) hydroxylase activity is not specific for cytochrome P450 4A1.

It was demonstrated that ω-hydroxylase activity towards lauric acid and cytochrome P450 4A1 can be induced by xenobiotics like hypolipidaemic agents [4], di-(2-ethylhexyl)phthalate (DEHP) [15], chlorophenoxyacid herbicides [16] and perfluorinated compounds [17]. DEHP is widely used as plasticizer in PVC plastics and is an important environmental pollutant [18].

In the body, DEHP is rapidly hydrolysed to mono-(2-ethylhexyl)phthalate (MEHP) and 2-ethyl-1-hexanol. 2-Ethyl-1-hexanol can be further oxidized to 2-ethyl-1-hexanoic acid [19]. 2-Ethylhexanoic acid is also used as a wood preservative agent. 2-Ethyl-1-hexanol, 2-ethyl-1-hexanoic acid, the plasticizer di(2-ethylhexyl)adipate (DEHA), the epileptic drug valproate and perfluorooctanoic acid enhance peroxisomal β-oxidation enzymes but the effects on cytochrome P450 4A1 and its associated lauric acid ω-hydroxylase activity have not been reported yet [20-23]. Studies on the effects of other cytochrome P450 inducers on lauric acid hydroxylase activities have been limited to phenobarbital, 3-methylcholanthrene and aroclor 1254. Since different effects were found we have repeated these studies and in addition we have quantified the levels of immunochemical detectable P450 4A1.

In this paper we further studied the relationship between induction of microsomal lauric acid ω-hydroxylase activity and peroxisomal palmitoyl-CoA oxidase activity by testing a number of chemicals, known to induce peroxisomal ß-oxidation, on their ability to induce lauric acid ω-hydroxylase activities and cytochrome P450 4A1.

Materials and methods

Chemicals and reagents

Nafenopin was a gift of Ciba Geigy (Basle, Switzerland). Valproate (2-propylpentanoic acid), 3-methylcholanthrene, lauric acid, ω-hydroxylauric acid, NADPH, palmitoyl-CoA, acetyl-CoA and aminotriazole were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2,7-Dichlorofluorescin diacetate was purchased from Eastman Kodak Co. (Rochester, NY, U.S.A.). DEHP, DEHA and 2-ethyl-1-hexanol were obtained from Janssen Chimica (Beerse, Belgium). 2-Ethyl-1-hexanoic acid, 18-crown-6 ether and 4-(bromo-methyl)-6,7-dimethoxycoumarin (Br-mdmc) were obtained from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Perfluoro-octanoic acid was from Lancaster Synthesis Ltd. (Morecambe, UK). Aroclor-1254 was from Alltech Associated, Inc. (Deerfield, Ill, U.S.A.) and phenobarbital was obtained from OPG (Utrecht, The Netherlands).

MEHP was synthesised by refluxing phthalic anhydride (14.8 g) and 2-ethyl-1-hexanol (13 g) in 20 mL toluene for 2 hr in the presence of 15 mg 4-dimethylaminopyridine. The toluene was distilled off and the product

Table 1 - The effect of aroclor 1254, phenobarbital and 3-methylcholanthrene on cytochrome P450 content, P450 4A1 content and ω- and (ω-1)-lauric acid hydroxylase activities.

Treatment	total cytochrome P450 (nmol/mg protein)	specific cytochrome P450 4A1		lauric acid hydroxylase activity			
		nmol/ mg protein	% of total cyt. P450	(ω-1)-hydroxylase		ω-hydroxylase	
				nmol/ min.nmol P450	nmol/ min.mg protein	nmol/ min.nmol P450	nmol/ min.mg protein
aroclor 1254	2.25 ± 0.3*	0.017 ± 0.01	0.74 ± 0.3°	0.54 ± 0.1	1.21 ± 0.3*	0.18 ± 0.05*	0.40 ± 0.1
olive oil control	0.82 ± 0.1	0.015 ± 0.004	1.78 ± 0.4	0.67 ± 0.07	0.55 ± 0.1	0.72 ± 0.1	0.60 ± 0.2
phenobarbital	1.93 ± 0.3*	0.010 ± 0.005	0.58 ± 0.3°	0.64 ± 0.05	1.21 ± 0.1*	0.18 ± 0.08*	0.36 ± 0.1
saline control	0.75 ± 0.03	0.014 ± 0.004	1.93 ± 0.5	0.69 ± 0.1	0.52 ± 0.1	0.80 ± 0.3	0.60 ± 0.3
3-methylcholanthrene	1.71 ± 0.08*	0.015 ± 0.008	0.87 ± 0.4°	0.69 ± 0.2*	1.18 ± 0.3	0.47 ± 0.1*	0.81 ± 0.2
olive oil control	0.98 ± 0.09	0.021 ± 0.008	2.2 ± 0.9	1.1 ± 0.2	1.07 ± 0.1	1.21 ± 0.4	1.18 ± 0.4

values are means ± SD of 4 rats.

* = statistically different from control $p \leq 0.05$ (Student's t-test for unpaired samples (with correction for unsufficient homogeneity of variance)).

° = statistically different from control $p \leq 0.05$ (Mann-Whitney U test)

was dissolved in diethylether and extracted into 0.5M K_2CO_3. The product was released for extraction into diethylether by acidification with 2M $KHSO_4$. The product was 99% pure and the structure was confirmed by NMR and Mass Spectrometry.

Antibodies against cytochrome P450 4A1 were a generous gift of Dr. G. Gordon Gibson, University of Surrey, Guildford, U.K. All other chemicals were of the highest purity obtainable.

Animals and treatment

Male random-bred Wistar rats (Cpb:WU (SPF)), body wt 175-225 g) were maintained at 23-25 °C with an alternating 12-hr light and dark cycle and with free access to RMH food pellets (Hope Farms, Woerden, The Netherlands) and water. For the treatment with 3-methylcholanthrene male random bred Wistar rats (Bor:WIS (SPF)), body wt 350 g) were used .

Rats were treated i.p. with aroclor-1254 in olive oil (500 mg/kg body wt) or with olive oil (2 mL/kg body wt). Five days after the aroclor-1254 treatment the rats were killed by decapitation. Rats were treated i.p for 4 days with phenobarbital in saline (75 mg/kg body wt) or with saline (1 mL/kg body wt) and with 3-methylcholanthrene in olive oil (25 mg/kg body wt) or with olive oil (0.6 mL/kg body wt). Twenty four hours after the last dose rats were killed. All the next doses are expressed as mg/kg body wt per day. In brackets is the dose expressed as mmol/kg body wt per day. Rats were pretreated by gastric intubation once a day for 3 days with DEHP (dose levels: 50 (0.13), 100 (0.26), 250 (0.64), 500 (1.28) and 1000 (2.6) mg/kg; MEHP (250 (0.9) mg/kg); DEHA (475 (1.3) mg/kg); 2-ethyl-1-hexanol (500 (3.8) mg/kg); 2-ethyl-1-hexanoic acid (550 (3.8) mg/kg); valproate (550 (3.8) mg/kg); nafenopin (100 (0.3) mg/kg) and perfluoro-octanoic acid (50 (0.1) mg/kg). Five to seven animals were used per group. Olive oil was used as a vehicle for administration. Control animals were given olive oil at 5 mL/kg body wt per day. One group of five rats was pretreated by gastric intubation with saline. Twenty four hours after the last dose, animals were weighted and then killed by decapitation prior to perfusion of the livers.

Preparation of liver fractions

Livers were perfused with 0.9% NaCl (w/w) for 10 min. Entire livers were removed and weighed. Whole liver homogenates (20% w/v) were prepared in 0.25M sucrose, 2 mM EDTA and 10 mM Tris-HCl (pH 7.4) (SETH buffer) using a teflon-glass homogeniser. A part of this homogenate was freeze/thawed three times, followed by centrifugation at 9,000g for 5 min. The supernatants were flash frozen in liquid nitrogen and stored at -80°C until they were thawed for enzymatic analysis of palmitoyl-CoA oxidase- and carnitine acetyltransferase activity.

The remaining part of the whole liver homogenate was centrifuged at 9,000g for 20 minutes and the supernatant was centrifuged at 105,000g for 1 hr. The pellet (microsomal fraction) was homogenized in SETH buffer and aliquots were flash frozen in liquid nitrogen and stored at -80°C until

Table 2 - The effect of DEHP on liver size, cytochrome P450 and cytochrome P450 4A1.

dose	liver/ body weight ratio	total cytochrome P450 (nmol/mg protein)	specific cytochrome P450 4A1	
			nmol/mg protein	% of total cyt. P450
0	4.8 ± 0.4	0.55 ± 0.08	0.013 ± 0.005	2.4 ± 0.9
50	5.1 ± 0.3	0.64 ± 0.1	0.032 ± 0.01*	4.9 ± 0.9°
100	5.2 ± 0.4	0.64 ± 0.1	0.049 ± 0.01*	7.7 ± 1.4°
250	5.6 ± 0.5°	0.59 ± 0.06	0.069 ± 0.01*	10.9 ± 1.4°
500	5.7 ± 0.4°	0.69 ± 0.1	0.094 ± 0.03*	13.5 ± 2.4°
1000	5.8 ± 0.2°	0.71 ± 0.1	0.109 ± 0.03*	15.4 ± 0.6°

values are means ± SD of 5-7 rats.
* = statistically different from control $p \leq 0.05$ (ANOVA and Dunnett's test)
° = statistically different from control $p \leq 0.05$ (Mann-Whitney U test with Bonferroni correction)

they were thawed for determination of cytochrome P450 content, cytochrome P450 4A1 concentration and lauric acid hydroxylase activity.

Other methods

Palmitoyl-CoA oxidase activity was determined according to Reubsaet *et al.* [24]. Carnitine acetyltransferase was determined at 25°C according to Gray *et al.* [25]. Lauric acid hydroxylase activities were determined by reverse phase HPLC and fluorimetric detection based on the derivatization of lauric acid and its metabolites with the fluorescent compound Br-mdmc as previously presented elsewhere [26]. Total cytochrome P450 was determined from the CO-difference spectrum by the method of Omura and Sato [27]. Cytochrome P450 4A1 was determined immunochemically by an ELISA method described by Sharma *et al.* [4]. On immunoblots the antibodies react with two protein bands (51.5 and 52 kDa). The 51.5 kDa protein co-migrates with authentic rat cytochrome P450 4A1, the 52 kDa band is probably 4A2 or 4A3 [28]. Protein concentrations were determined by the method of Bradford [29] using crystalline bovine serum albumin as standard.

Statistical analysis

Data were analysed using SAS-software, version 6. All tests performed are

two tailed.

Results

Studies with classical cytochrome P450 inducers

The effects of phenobarbital, 3-methylcholanthrene and aroclor-1254 administration on total cytochrome P450 content, the specific cytochrome P450 4A1 levels and lauric acid (ω-1)- and ω-hydroxylase activities are summarized in table 1.

All compounds tested increased the total cytochrome P450 content of liver microsomes (1.7-2.7 times). Specific cytochrome P450 4A1 levels in control animals as determined by ELISA were shown to be 1.8 to 2.2% of total cytochrome P450, and no induction of cytochrome P450 4A1 by phenobarbital, 3-methylcholanthrene or aroclor-1254 was observed. When the levels of P450 4A1 were expressed as percentage of total cytochrome P450 all 3 compounds decreased the P450 4A1 content. Furthermore no induction was found of the ω-hydroxylase activity towards lauric acid, the marker substrate for cytochrome P450 4A1, when this activity was expressed per mg protein. When this activity was expressed per nmol P450 a decrease was found. A 2-fold increase in the (ω-1)-hydroxylase activity was found after aroclor-1254 and phenobarbital treatment when this activity was expressed per mg protein. No increases were found when the (ω-1)-hydroxylase activity was expressed per nmol P450. We prefer this latter expression because it is possible that total cytochrome P450, including P450 4A1 levels and lauric acid hydroxylase activities, is induced by the test compound, which can obscure the specific induction of lauric acid hydroxylase activities.

Our results clearly show that classical enzyme inducers used in toxicological studies such as phenobarbital, 3-methylcholanthrene or aroclor-1254 do not induce lauric acid hydroxylase activities or cytochrome P450 4A1.

Dose-response studies with DEHP

The effect of DEHP administration on liver/body weight ratio, total cytochrome P450 content and specific cytochrome P450 4A1 isoenzyme levels are shown in table 2. In rats receiving DEHP doses of more than 250 mg/kg body weight per day a significant hepatomegaly was found. Total cytochrome P450 levels were not altered by the DEHP treatment. Following DEHP treatment immunochemically determined cytochrome P450 4A1 concentrations were strongly increased in a dose-dependent manner. Even at the lowest DEHP dose (50 mg/day per kg body weight) a 2-fold induction of cytochrome P450 4A1 was found. At dose levels of 100 mg DEHP/kg body weight per day or higher, the associated cytochrome P450 4A1-driven lauric acid ω-hydroxylase activity was induced in a dose-dependent manner (figure 1a). At dose-levels of 500 or 1000 mg DEHP/kg body weight per day this activity was increased 8-9 fold. No increase in the lauric

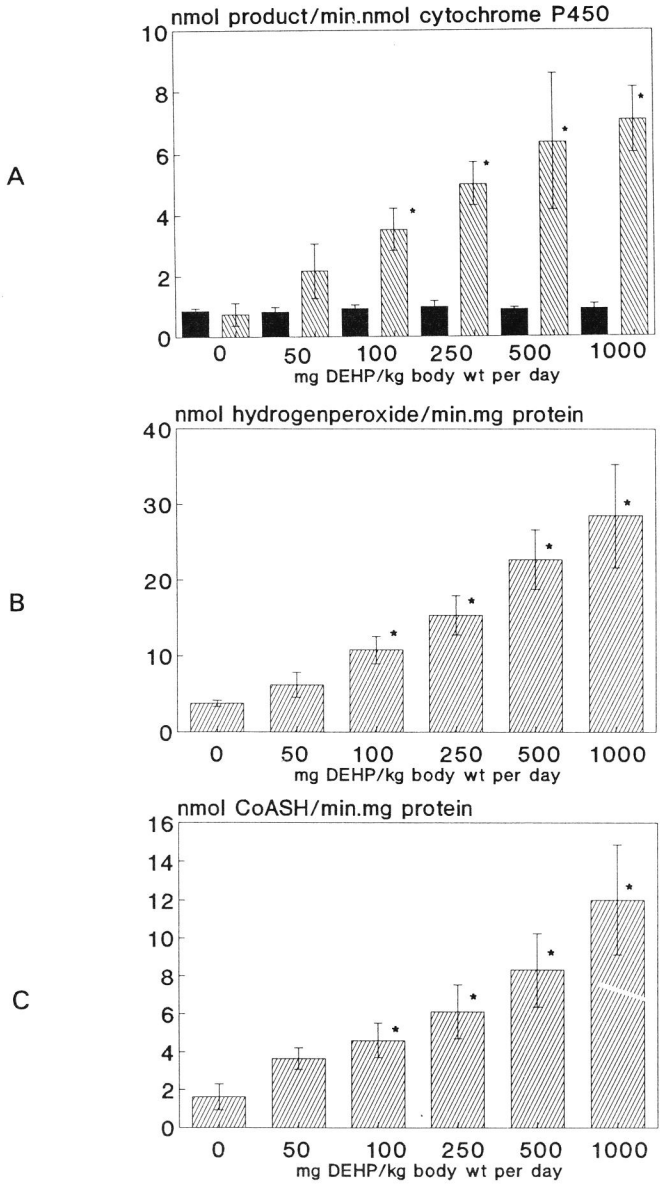

Figure 1 - Effect of DEHP on lauric acid hydroxylase activities (A), peroxisomal palmitoyl-CoA oxidase activities (B) and carnitine acetyltransferase activities (C). Each bar represents the mean ± SD of 5-7 rats.
■ = (ω-1)-hydroxylauric acid and ▨ = ω-hydroxylauric acid

* Statistically different from control $p \leq 0.05$ (ANOVA and Dunnett's test)

Table 3 - The effect of DEHP and metabolites of DEHP on liver size, cytochrome P450 and cytochrome P450 4A1.

treatment	dose (mmol/kg body weight)	liver/ body weight ratio	total cytochrome P450 (nmol/mg protein)	specific cytochrome P450 4A1	
				nmol/mg protein	% of total cyt. P450
Control	-	5.0 ± 0.2	0.69 ± 0.1	0.011 ± 0.005	1.6 ± 0.7
DEHP	1.3	5.9 ± 0.4°	0.96 ± 0.16*	0.129 ± 0.03*	13.5 ± 1.8°
MEHP	0.9	5.6 ± 0.8	0.76 ± 0.05	0.084 ± 0.1*	11.0 ± 1.6°
2-ethyl-1-hexanol	3.8	5.4 ± 0.3	0.73 ± 0.07	0.037 ± 0.01*	5.1 ± 1.2°
2-ethyl-1-hexanoic acid	3.8	5.8 ± 0.4	0.77 ± 0.09	0.033 ± 0.005*	4.3 ± 0.7°

values are means ± SD of 5-7 rats.
* = statistically different from control $p \leq 0.05$ (ANOVA and Dunnett's test)
° = statistically different from control $p \leq 0.05$ (Mann-Whitney U test with Bonferroni correction)

acid (ω-1)-hydroxylase activity was found. Acyl-CoA oxidase activity is rate-limiting in the peroxisomal ß-oxidation and it was shown that palmitoyl-CoA oxidase was the most sensitive marker for peroxisome proliferation in the liver [30]. At dose levels of 100 mg DEHP/kg body wt per day or higher palmitoyl-CoA oxidase activities were dose-relatedly induced by the DEHP treatment (figure 1b). It has been reported that the increase in number and size of peroxisomes in rats treated with peroxisome proliferators correlates well with increased carnitine acetyl-CoA oxidase activity, although it is not a specific peroxisomal enzyme [4]. At dose levels of 100 mg DEHP/kg body wt per day or higher carnitine acetyl transferase activity also exhibits a dose-dependent increase following the administration of DEHP (figure 1c). A dose of 100 mg DEHP/kg body wt per day gives an increase in both peroxisomal enzyme activities and of lauric acid ω-hydroxylase activities. This dose-level is 2.5 times lower than in an another study [15].

Studies on DEHP metabolites

The effects of the metabolites MEHP, 2-ethyl-1-hexanol and 2-ethyl-1-hexanoic acid were compared with the effects of DEHP. Administration of DEHP did result in a liver enlargement and in an increase in the total cytochrome P450 content of the liver microsomes (table 3). DEHP, MEHP, 2-ethyl-1-hexanol and 2-ethyl-1-hexanoic acid increased cytochrome P450 4A1 levels (table 3). None of the compounds altered lauric acid (ω-1)-hydroxylase activities, while DEHP, MEHP and 2-ethylhexanol resulted in a significant induction of lauric acid ω-hydroxylase activities (figure 2a).

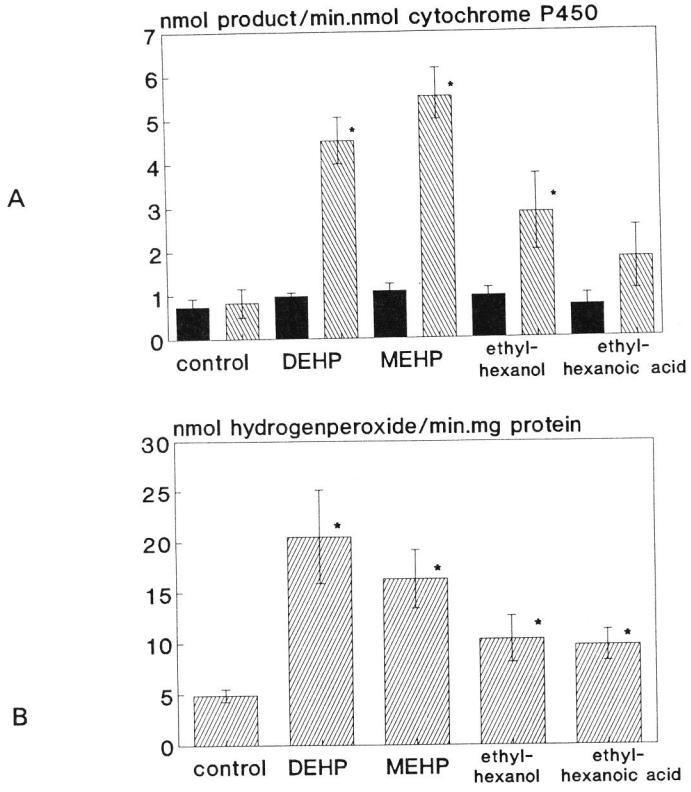

Figure 2 - Effect of DEHP, MEHP, 2-ethyl-1-hexanol and 2-ethyl-1-hexanoic acid on lauric acid hydroxylase activities (A) and peroxisomal palmitoyl-CoA oxidase activities (B).
Each bar represent the mean ± SD of 5-7 rats.
■ = (ω-1)-hydroxylauric acid and ▨ = ω-hydroxylauric acid

* Statistically different from control $p \leq 0.05$ (ANOVA and Dunnett's test).

Palmitoyl-CoA oxidase activitity, a marker for peroxisome proliferation was increased by all four compounds tested (figure 2b).

Studies on other compounds possible having a peroxisome proliferating action

Effects of administration of valproate, perfluoro-octanoic acid and DEHA to rats were compared with the well known effects of the peroxisome proliferator nafenopin. Administration of perfluoro-octanoic acid and nafenopin resulted in a hepatomegaly (table 4). Perfluoro-octanoic acid was

Table 4 - The effect of olive oil, saline, DEHA, valproate, perfluoro-octanoic acid and nafenopin on liver size, cytochrome P450 and cytochrome 4A1.

Treatment	dose (mmol/kg body weight)	liver/ body weight ratio	total cytochrome P450 (nmol/mg protein)	specific cytochrome P450 4A1	
				nmol/mg protein	% of total cyt. P450
olive oil	-	5.0 ± 0.2	0.69 ± 0.1	0.011 ± 0.005	1.63 ± 0.7
saline	-	4.9 ± 0.3	0.74 ± 0.1	0.009 ± 0.002	1.21 ± 0.4
DEHA	1.3	5.0 ± 0.4	0.67 ± 0.1	0.014 ± 0.003	2.14 ± 0.4
valproate	3.8	5.5 ± 0.5	0.74 ± 0.1	0.039 ± 0.007*	5.29 ± 0.4°
perfluoro-octanoic acid	0.1	7.1 ± 0.5°	1.69 ± 0.3*	0.199 ± 0.03*	11.86 ± 1.7°
nafenopin	0.3	6.8 ± 0.8°	0.71 ± 0.07	0.133 ± 0.02*	18.76 ± 1.6°

values are means ± SD of 5-7 rats.

* = statistically different from control $p \leq 0.05$ (ANOVA and Dunnett's test)
° = statistically different from control $p \leq 0.05$ (Mann-Whitney U test with Bonferroni correction)

also able to increase the total cytochrome P450 level 2.5 times. Perfluoro-octanoic acid, valproate and nafenopin induced levels of cytochrome P450 4A1 (3-11 fold). These three compounds also increased the ω-hydroxylase activity towards lauric acid 3-11 fold, but only nafenopin increased the lauric acid (ω-1)-hydroxylase activity (figure 3a). Palmitoyl-CoA oxidase activities were induced in the liver of rats treated with these compounds. No (significant) increase in cytochrome P450 4A1 levels, in the ω-hydroxylase activity towards lauric acid or palmitoyl-CoA oxidase activities were found after the DEHA treatment (figure 3b).

It was reported that high fat diets also produce lauric acid ω-hydroxylase activity induction [31]. Therefore, we studied a possible bias in our experiments of olive oil, which was the vehicle in most of our experiments. A group of rats treated once a day by gastric intubation for 3 days with olive oil was compared with a group of rats treated with saline. No differences were found in total cytochrome P450 concentrations, cytochrome P450 4A1 levels (table 4), or in hydroxylase activities towards lauric acid and palmitoyl-CoA oxidase activities (figure 3a and 3b) between the olive oil or the saline treated group. The vehicle used in our experiments appeared to have no effect on the parameters studied.

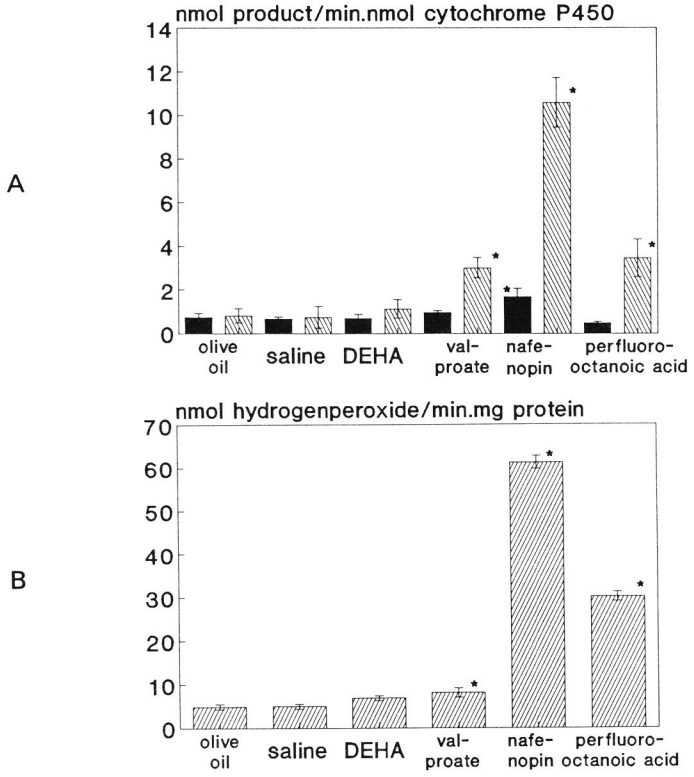

Figure 3 - Effect of olive oil, saline, DEHA, valproate, nafenopin and perfluoro-octanoic acid on lauric acid hydroxylase activities (A) and peroxisomal palmitoyl-CoA oxidase activities (B). Each bar represents the mean of 5-7 rats.
■ = (ω-1)-hydroxylauric acid and ▨ = ω-hydroxylauric acid.

* Statistically different from control $p \leq 0.05$ (ANOVA and Dunnett's test)

Discussion

None of the classical P450 inducers tested increased cytochrome P450 4A1 levels or the ω-hydroxylase activity towards lauric acid. The results obtained with aroclor-1254 are in contrast with the results of Borlakoglu *et al.* [32] who reported a 5-fold increase in the formation of ω-hydroxylauric acid, expressed per mg protein, in the liver of female rats treated with aroclor-1254. Since we have used male rats in our experiments, the observed differences in inducibility of the ω-hydroxylase activity towards lauric acid after aroclor-1254 treatment might be due to sex differences.

Comparisons of our data with the data of others is somewhat complicated because lauric acid hydroxylase activities are by some authors expres-

sed per mg protein and by others per nmol cytochrome P450. We found a 2-fold increase of (ω-1)-hydroxylase activity in the livers of rats treated with aroclor-1254 and phenobarbital, when the activity is presented per mg protein, but no increase was found when the activity was expressed per nmol cytochrome P450. Similar increases in lauric acid (ω-1)-hydroxylase activities (when expressed per mg protein) after phenobarbital treatment were also found by Okita and Masters [33] and Cresteil *et al.* [34]. Tanaka *et al.* [14] found that both activities are decreased after phenobarbital treatment when these activities were expressed per nmol P450. After 3-methylcholanthrene treatment we found a decrease in both ω- and (ω-1)-hydroxylase activities when this activity was expressed per nmol P450. Similar findings were reported by Tanaka *et al.* [14]. We found no differences between the two activities when the activity was expressed per mg protein, but Cresteil *et al.* [34] found a decrease. Taken collectively our data and the data of others [14, 33, 34] make clear that phenobarbital and 3-methylcholanthrene do not induce lauric acid ω-hydroxylase activities or levels of immunochemical detectable P450 4A1. These findings are supported by the recent finding that levels of RNA levels encoding two other members of the cytochrome P450 4A family (cytochrome P450 4A2 and 4A3) were increased in rats after treatment with clofibrate, but not after treatment with the P450 inducers phenobarbital, 3-methylcholanthrene and dexamethasone [35].

Recent studies with hypolipidaemic peroxisome proliferators, with chlorinated phenoxy acid herbicides and with perfluorinated compounds have demonstrated an excellent correlation between microsomal cytochrome P450 4A1 and its associated lauric acid ω-hydroxylase activity on the one hand, and peroxisomal volume and peroxisomal ß-oxidation activity on the other in the liver of rats [4, 16, 17]. After treatment of rats with different doses of DEHP we found a dose-related increase in cytochrome P450 4A1 levels and its associated lauric acid ω-hydroxylase activity, as well as peroxisomal enzymes like palmitoyl-CoA oxidase and carnitine acetyl transferase. This is in close agreement with data presented by Sharma *et al.* [15], although we have used 2.5 times lower dose levels. In most studies DEHP is administered to rats as part of the diet. The lowest dose of DEHP used in our study which induced both lauric acid ω-hydroxylase- and palmitoyl-CoA activity is equal to a diet containing 0.1% DEHP (calculated on basis of previously published data [36]) and this dose is one of the lowest doses which has been reported to induce palmitoyl-CoA oxidase activities and lauric acid ω-hydroxylase activities.

When the DEHP metabolites MEHP, 2-ethyl-1-hexanol and 2-ethyl-1-hexanoic acid were administrated orally to rats, an induction of the levels of P450 4A1 was found. MEHP and 2-ethyl-1-hexanol, but not 2-ethyl-1-hexanoic acid increased the lauric acid ω-hydroxylase activity. All three compounds induced peroxisomal palmitoyl-CoA oxidase.

Another plasticizer, DEHA was also reported to enhance peroxisomal ß-oxidation activity [20]. With the dose level used in our study, DEHA gave no (significant) induction of palmitoyl-CoA oxidase activity or lauric acid ω-

Table 5 - Matrix of Pearson correlation coefficients of several morphological and biochemical effects on rat liver after administration of peroxisome proliferating compounds[1].

	total cyt. P450 (nmol/mg protein)	P450 4A1 (nmol/ mg protein)	lauric acid (ω-1)-hydroxy-lase activity (nmol/min. mg protein)	lauric acid ω-hydro-xylase activity (nmol/min. mg protein)	palmitoyl-CoA oxidase activity
liver/body weight	0.539***	0.745***	0.450**	0.779***	0.765***
total cyt. P450		0.776***	0.274	0.448**	0.282
P450 4A1			0.633***	0.852***	0.727***
(ω-1)-hydroxylase activity				0.808***	0.665***
ω-hydroxylase activity					0.895***

Statistical significant effects are indicated by **$p < 0.005$ and ***$p < 0.001$.

[1] Data from the following compounds were used: DEHP (1.3 mmol/kg body wt), MEHP, 2-ethyl-1-hexanol, 2-ethyl-1-hexanoic acid, DEHA, valproate, perfluorooctanoic acid, nafenopin and olive oil (N = 46).

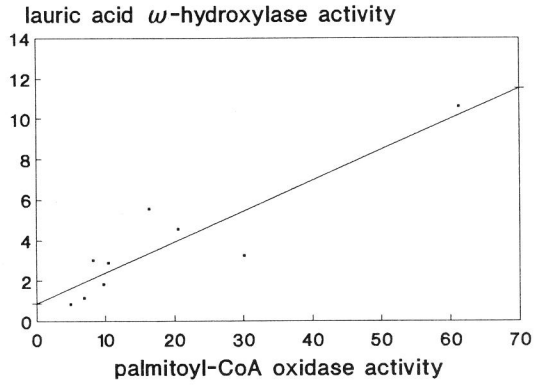

Figure 4 - Association between palmitoyl-CoA oxidase activities and lauric acid ω-hydroxylase activities after treatment of rats with DEHP (500 mg/kg per day), MEHP, 2-ethyl-1-hexanol, 2-ethyl-1-hexanoic acid, nafenopin, valproate, perfluoro-octanoic acid, DEHA and olive oil. Of each group mean values were used. ($y = 0.88 + 0.15x$, $r = 0.91$, $n = 9$).

hydroxylase activity.

Our results also indicate that the anti-epileptic drug valproic acid, a chemical analogue of the DEHP metabolite 2-ethylhexanoic acid, and perfluoro-octanoic acid are inducers of cytochrome P450 4A1 and lauric acid ω-hydroxylase activities. The strong inducibility of peroxisome proliferation by perfluorinated fatty acids is unexpected because the structures of these chemicals are dissimilar from any known peroxisome proliferating compounds. Owing to their metabolically inert properties these compounds may be proximate inducers themselves.

The data obtained in the experiments with DEHP (500 mg/kg per day), 2-ethyl-1-hexanoic acid, 2-ethyl-1-hexanol, MEHP, DEHA, nafenopin, valproate and perfluoro-octanoic acid were used to study the correlation of biological effects of peroxisome proliferating compounds on the liver. A correlation matrix is shown in table 5. Total cytochrome P450 did not correlate well with palmitoyl-CoA oxidase activities. A high correlation was found between P450 4A1 levels, and its associated lauric acid ω-hydroxylase activities and peroxisomal palmitoyl-CoA oxidase activities. The high degree of association between palmitoyl-CoA oxidase activity and lauric acid ω-hydroxylase activity is also shown in figure 4. Although cause and effect can not be separated by a correlation analysis, our data and data from others [4, 17] are suggestive of a mechanistic interrelationship between induction of lauric acid ω-hydroxylase activity by P450 4A1 and peroxisome proliferation.

In conclusion: the induction of cytochrome P450 4A1 and its associated lauric acid ω-hydroxylase activity seems to be specific for peroxisome proliferating compounds. Determination of levels of P450 4A1 and lauric

acid ω-hydroxylase activities in identifying peroxisome proliferators is very useful since these parameters are very sensitive. The demonstration of the ability of several peroxisome proliferating compounds to induce cytochrome P450 4A1 further support the hypothesis that there might be a mechanistic interrelationship between peroxisome proliferation and induction of lauric acid ω-hydroxylase activities.

Acknowledgments
The authors wish to thank Nicole Palmen (University of Limburg, The Netherlands) for the treatment of the rats with 3-methylcholanthrene. We also thank prof. P.Th. Henderson (University of Limburg, The Netherlands) for critical reading of the manuscript.

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Chapter 5

Lauric acid hydroxylase activity and cytochrome P-450 IV family proteins in human liver microsomes

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Biochemical Pharmacology **42**: *1841-1844, 1991*

Introduction

Cytochrome P-450 enzymes are important in the metabolism of many endogenous substrates, as well as a large variety of drugs, chemical carcinogens, and environmental pollutants. Ten families of cytochrome P-450 enzymes have been identified so far in mammals [1]. Six of these are small families of one or two members and are involved in pathways of steroidogenesis or bile acid synthesis in highly specialized tissues [2]. One family, cytochrome P-450 IV, consists of enzymes that are involved in hydroxylating fatty acids [2]. The remaining three families encode xenobiotic metabolizing enzymes. In these latter four families, it appears that a tremendous variability exists between species in the number of P450 genes and the substrate specificities of individual P450 forms [2]. It is therefore difficult to extrapolate toxicology and carcinogenicity studies from rodents to man. These species differences emphasize the necessity to study human cytochrome P450 enzymes.

One of the most active cytochrome P450 IV fatty acid-metabolizing enzymes is P450 IVA1, formely termed cytochrome P452 [3-5]. The ω-hydroxylase activity towards lauric acid is suggested to be an indicator of P-450 IV activity [2,3,5]. After treatment of rats with hypolipidaemic, peroxisome proliferating compounds like clofibrate, nafenopin or di(2-ethylhexyl)phthalate (DEHP) both the ω-hydroxylase activity towards lauric acid, and cytochrome P-450 IVA1 protein content are substantially induced in liver and kidney, whereas the (ω-1)-hydroxylase activity is much less induced [5-8].

Since the hypolipidaemic, peroxisome proliferating compounds are classified as epigenetic hepatocarcinogens [9, 10], attention has been focussed to elucidate the possible mechanism(s) of carcinogenesis. It has been suggested that the carcinogenicity might result from an increased hydrogen peroxide production generated via the peroxisomal fatty acid β-oxidation in the liver [10]. Sharma *et al.* (1988) proposed that the enhanced peroxisomal β-oxidation is a result of the perturbation of lipid metabolism, which in turn might be the result of an enhanced ω-hydroxylation of fatty acids by cytochrome P450 IVA1.

Species differences in peroxisome proliferation have been reported. Non-rodent species are reported to be less sensitive to peroxisome proliferators than rodents [11-13]. Since peroxisome proliferation is causally linked to hepatocarcinogenicity there is considerable debate about the significance of peroxisome proliferators as hepatocarcinogens in humans. More information regarding the cause of the interspecies differences of peroxisome proliferation is needed.

It seems possible that species differences in peroxisome proliferation are the result of differences in the presence or inducibility of cytochrome P450 IVA1 [11-13]. In this paper we have studied the presence of cytochrome P450 IV family enzymes in hepatic microsomes derived from human samples, as a preliminary step in determining the potential human risk posed by peroxisome proliferators.

Materials and Methods

Chemicals and reagents

Nafenopin was a gift of Ciba Geigy (Basle, Switzerland). Lauric acid, ω-hydroxylauric acid and NADPH were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). 18-Crown-6 ether and 4-(bromomethyl)-6,7-dimethoxycoumarin were obtained from the Aldrich Chemical Co. (Milwaukee, WI, USA). DEHP was purchased from Janssen Chimica (Beerse, Belgium).

Antibodies against a highly purified, electrophoretically homogenous preparation of rat hepatic cytochrome P450 IVA1 were raised in sheep and the specificity of the antibodies is described elsewhere [6,7]. All other chemicals were of the highest purity obtainable.

Liver samples

Human liver samples were obtained from kidney transplant donors (n = 13). The investigations were approved by the local ethical committee on human experimentation.

Rat liver samples were from male random-bred Wistar rats (Cbp:WU (SPF)), body wt 150-200 g). Rats were pretreated by gastric intubation once daily for 3 days with either di(2-ethylhexyl)phthalate (500 mg/kg body wt), nafenopin (100 mg/kg body wt), olive oil (5 mL/kg body wt) or saline. Twenty-four hours after the last dose rats were killed by decapitation. Livers were perfused with

0.9% (w/w) NaCl; for 10 min.

Whole liver homogenates were prepared in 0.25M sucrose/2 mM EDTA and 10 mM Tris/HCl (pH 7.4). Homogenate was centrifuged at 10,000g for 20 min. Microsomal fractions were prepared from the 10,000g supernatant by centrifugation at 105,000g for 60 min.

Assays

The ω- and (ω-1)-hydroxy metabolites of lauric acid were separated by reverse phase HPLC after derivatization with the fluorescent compound 4-(bromomethyl)-6,7-dimethoxycoumarin, as described by Dirven *et al.* [8]. Total cytochrome P-450 content was determined by the method of Omura and Sato [14].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with a polyclonal antibody against rat hepatic cytochrome P-450 IVA1 was performed according to Peters and Jansen [15]. A semi-quantitative estimate of the intensity of the cytochrome P-450 IV bands on the immunoblot was made using a laser densitometer (Ultrascan XL, LKB, Bromma, Sweden). Protein concentrations were determined by the method of Bradford [16].

Results and discussion

Table 1 gives the lauric ω- and (ω-1)-hydroxylase activities in microsomal preparations derived from either human or saline-treated rat liver. The activities determined in human liver are 2-4 higher than those measured in rat liver. Also the ratios between ω- and (ω-1)-hydroxylase activities are different in rat as compared to man: in man, the ω-hydroxylase activity is twice as large as the (ω-1)-hydroxylase activity, whereas in rat both activities are comparable.

Immunoblot analysis of human and rat microsomal fractions was performed using a sheep polyclonal antibody raised against purified rat hepatic cytochrome P450 IVA1. Figure 1 shows that this antibody reacts with two protein bands (molecular weights 51.5 and 52 KDa) for olive oil-, DEHP- or nafenopin-treated rat liver microsomes. This pattern is identical as described by Makowska *et al.* [7] and Hardwick et al. [4]. Both bands are induced by the treatment with either DEHP or nafenopin. The lower band (51.5 KDa) co-migrates with rat hepatic P450 IVA1. The additional band of approximately 52 KDa might be another member of the P450 IV subfamily [7], like cytochrome P450 IVA2 or P450 IVA3 [17], but this has to be verified.

In all human liver samples a strong and a weak reacting band was detected. The strong reacting band has a slightly lower molecular weight (approximately 51 KDa) than rat hepatic cytochrome P450 IVA1. The weak reacting band has a molecular weight of approximately 56.5 kDa. These findings suggest the existence of proteins in human liver that are immunochemically related to rat hepatic cytochrome P-450 IVA1. A correlation was found between the

Table 1 - ω- and (ω-1)-hydroxylase activities towards lauric acid in human and rat liver microsomes.

Patients No.	Sex	Age	Total cytochrome P450 (nmol/mg protein)	Hydroxylase acitivity (nmol product/min. nmol total cyt. P450)		Intensity of 51 kDa band (absorbance units/pmol total cyt. P450)
				(ω-1)-hydroxy-lauric acid	ω-hydroxy-lauric acid	
1	F	30	0.24	1.83	2.53	ND
2	F	18	0.78	1.15	1.85	0.15
3	-	-	0.51	0.70	2.14	0.15
4	M	27	0.26	1.24	2.87	0.13
5	M	15	0.33	1.55	1.82	0.10
6	M	20	0.34	0.33	2.39	0.16
7	M	46	0.84	1.38	2.16	0.07
8	-	-	0.27	1.89	4.08	0.08
9	M	18	0.39	2.25	4.99	0.06
10	-	-	0.50	1.15	2.8	0.06
11	-	-	0.42	2.32	3.79	0.12
12	M	47	0.20	3.29	4.5	ND
13	F	18	0.26	2.25	4.57	0.08
Mean ± SD			0.41 ± 0.20	1.64 ± 0.78	3.11 ± 1.12	
Rats Mean ± SD[1]			0.74 ± 0.16	0.66 ± 0.11	0.74 ± 0.49	

ND, not determined.
-, Sex and age of these donors are not known.
[1] Mean ± SD of five rats treated with saline

staining intensities of the 51.5 kDa band and the rate of lauric acid ω-hydroxylation for the treated and untreated rat samples (see text figure 1). However, an observable correlation was not found between the staining intensities of the 51 kDa band and the rate of lauric acid ω-hydroxylation for the human samples, suggesting that the 51 kDa protein has hardly any lauric acid ω-hydroxylase activity.

Apart from cytochrome P450 IVA1 other cytochrome P450 IV families may have lauric acid ω-hydroxylase activity [2,5], and it is possible that that these enzymes catalyse the pronounced lauric acid hydroxylation activities, as determined in the human liver samples. In addition it should be notified, when

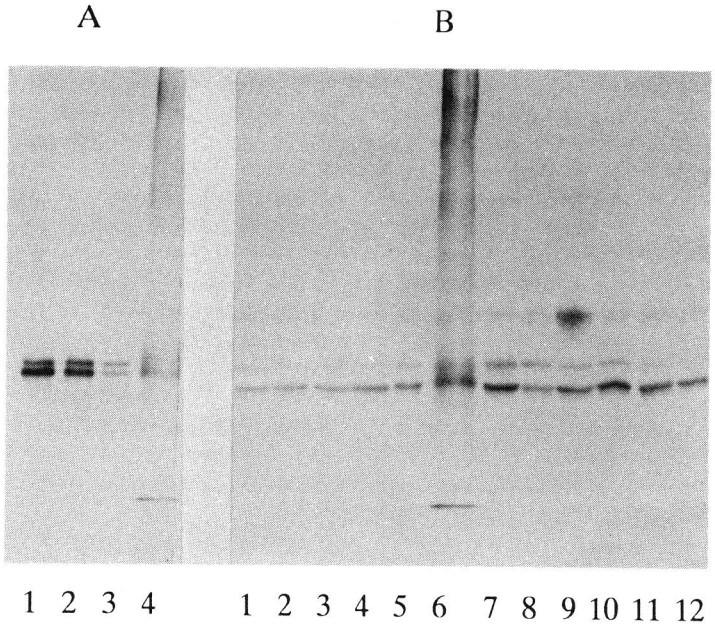

Figure 1 - Immunodetection of hepatic cytochrome P450 IV proteins in rat and human liver microsomes. Microsomal fractions were subjected to SDS-polyacrylamide gel electrophoresis (7% acrylamide) and immunoblotted with an antibody to rat hepatic cytochrome P450 IVA1.
A: Track 1, nafenopin-treated rat liver microsomes [2 pmol][1]; track 2, DEHP-treated rat liver microsomes [2 pmol][2]; track 3, olive oil-treated rat liver microsomes [5 pmol][3]; track 4, authentic rat hepatic cytochrome P450 IVA1 standard [6 pmol].
B: Track 1-5, human liver microsomes corresponding to patients Nos. 13, 10, 9, 8 and 7, respectively [5 pmol]; track 6, authentic rat cytochrome P450 IVA1 [6 pmol]; track 7-12, human liver microsomes corresponding with patients Nos. 6, 5 ,4 ,3, 2 and 11, respectively [5 pmol]. Amounts of microsomes loaded onto the gel are expressed as pmol total cytochrome P450, and is given in brackets.
[1] Lauric acid ω-hydroxylase activity is 8.35 nmol/min.nmol cytochrome P450 and intensity of 51.5 KDa band is 0.55 absorbance units/pmol. [2] Lauric acid ω-hydroxylase activity is 4.07 nmol/min.nmol cytochrome P450 and intensity of 51.5 KDa band is 0.32 absorbance units/pmol. [3] Lauric acid ω-hydroxylase activity is 0.70 nmol/min.nmol cytochrome P450 and intensity of 51.5 KDa band is 0.05 absorbance units/pmol.

analysing human cytochrome P450 IV enzymes by using an antibody raised to rat hepatic cytochrome P450 IVA1, the human cytochrome P450 IV levels may be underestimated due to lack of specificity of the antibody for the human cytochrome P-450 IV proteins.

We conclude that in human liver cytochrome P450 IV proteins are present, which are related to rat hepatic cytochrome P450 IVA1, but have different

physicochemical properties. As a consequence, the reported species differences in peroxisome proliferation between rodents and primates [11-13] might be due to differences in the inducibility of these proteins.

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Chapter 6

Effects of the peroxisome proliferator mono(2-ethylhexyl)phthalate in primary hepatocyte cultures derived from rat, guinea pig, rabbit and monkey: relationship between interspecies differences in biotransformation and peroxisome proliferating potencies.

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Biochemical Pharmacology, in press.

Abstract

Primary hepatocyte cultures derived from rat, rabbit, guinea pig and monkey, have been treated in vitro with metabolites of di(2-ethylhexyl)phthalate (DEHP), i.e. mono(2-ethylhexyl)phthalate (MEHP), mono(5-carboxy-2-ethyl)pentylphthalate (metabolite V) and mono(2-ethyl-5-oxo-hexyl)-phthalate (metabolite VI). In rat hepatocyte cultures MEHP and metabolite VI were equally potent in inducing peroxisome proliferation, while metabolite V was much less potent. In rat hepatocytes a 50% increase in both peroxisomal palmitoyl-CoA oxidase activity and microsomal lauric acid ω-hydroxylation activity, was found after treatment with 5-15 μM MEHP. In guinea pig, rabbit and monkey hepatocyte cultures, a 50% increase in peroxisomal palmitoyl-CoA oxidase activity was found after treatment with 408-485 μM MEHP. No induction of lauric acid ω-hydroxylation activity was found. These results indicate that peroxisome proliferation can be induced by MEHP in rabbit, guinea pig and monkey hepatocytes, but that these species are at least 30-fold less sensitive for peroxisome proliferation than rats. The proposed mechanistic inter-relationship between induction of lauric acid ω-hydroxylation activity and peroxisome proliferation, is found in rat hepatocytes, but not in hepatocytes of the other three species.
Treatment of guinea pig hepatocyte cultures with MEHP resulted in an increase in triglyceride concentrations in the hepatocytes. In rat and rabbit hepatocyte cultures, triglyceride concentrations were much less altered by

MEHP. In monkey hepatocytes a decrease in hepatic triglyceride concentration was found after treatment with MEHP. These effects are in agreement with in vivo effects observed before.
After treatment of primary hepatocyte cultures with MEHP, high concentrations of ω- and (ω-1)-hydroxylated metabolites of MEHP were found in media from rat, rabbit and guinea pig cultures. The formation of these metabolites did not decline in time. During treatment the metabolite profile in media from rat hepatocytes moved towards ω-hydroxy metabolites of MEHP. In media from monkey hepatocytes the lowest concentrations of hydroxylated metabolites were determined. No major species differences were found in the potency to form oxidized MEHP metabolites, and thus no unique metabolite differences were found, which could explain the species differences in sensitivity for peroxisome proliferation.

Introduction

The current interest in peroxisome proliferation by xenobiotics, is the result of the finding that chronic exposure of rats and mice to peroxisome proliferators, eventually results in the development of hepatic tumours. The basic mechanism(s) by which peroxisome proliferators induce tumours in rats and mice is unknown. Tumour formation might be due to promotion of preneoplastic cells to cancer and/or might be the result of an increase in number and volume of peroxisomes [1]. In this latter model it is proposed that hydrogen peroxide, produced by the increased peroxisomal fatty acid ß-oxidation system, results in oxidative damage of the genome [2]. Peroxisome proliferation might be the result of a disturbed fatty acid homeostasis in liver cells, probably related to an increased formation of ω-hydroxy fatty acids by cytochrome P450 4A1 [3]. If indeed peroxisome proliferation is an intermediate step in tumour formation, it remains uncertain whether peroxisome proliferating compounds are a health hazard for humans, since humans are considered to be less sensitive for peroxisome proliferation than rats.

Peroxisome proliferation has been demonstrated in most mammalian species. Rats and mice are considered to be very sensitive, hamsters and possible rabbits are intermediate sensitive, while dogs, cats, and monkeys are weak reactors [4-11]. Mechanisms underlying these species differences in sensitivity for peroxisome proliferation, are not very well understood. Species differences in the presence of specific receptors [12] and species differences in the metabolism or pharmacokinetics of peroxisome proliferating compounds, have been proposed [13, 14].

For the polyvinylchloride (PVC) plasticizer di(2-ethylhexyl)phthalate (DEHP), formation of a specific metabolite (e.g. metabolite VI (see figure 1) is proposed to be essential for induction of peroxisome proliferation [14]. DEHP is a relatively weak peroxisome proliferator, but since this compound is used in large quantities and since DEHP is also an important environmental pollutant [15],

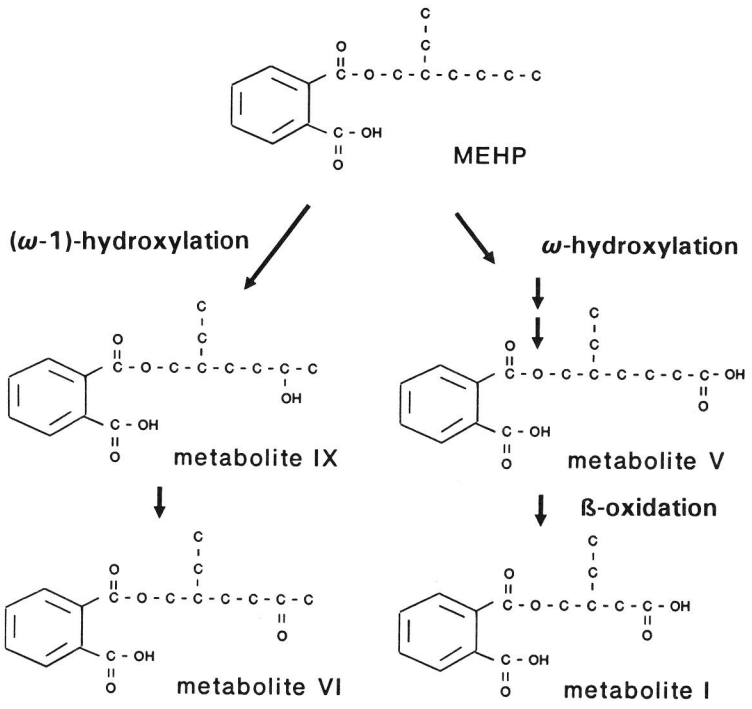

Figure 1 - The metabolism of MEHP. MEHP can undergo ω- and (ω-1)-hydroxylation reactions, with as (final) products metabolite IX, VI, V and I. Numbering of the metabolites is according to Albro *et al.* [38].

it is believed that a large number of people are exposed to this compound.

Primary cultures of rat, monkey, human, dog, hamster and guinea pig hepatocytes have been treated in vitro with peroxisome proliferating compounds [9, 11, 14-18]. Primary cultures of rabbit hepatocytes have not been tested, yet. Several markers for the response have been determined including peroxisome morphometrics, increases in the activity of peroxisomal enzymes, lauric acid ω-hydroxylation activity and levels of RNA for peroxisomal enzymes and P450 4A1. Other parameters associated with peroxisome proliferation, like hepatic lipid levels (triglyceride and cholesterol concentrations) [7], have not been determined after in vitro treatment of hepatocytes with peroxisome proliferators. These parameters are of interest since peroxisome proliferators alter the fatty acid homeostasis in liver cells, indicating that both the fatty acid catabolic reactions (ß-oxidation activities), as well as anabolic reactions (triglycerides synthesis) might be altered.

Studies on species differences in peroxisome proliferation should include studies with human hepatocytes. However, practical considerations (i.e. the poor availability of human livers, technical difficulties in establishing good

monolayer cultures and the relatively large numbers of cells required for analysis of microsomal enzyme activity) prompted us to use monkey hepatocytes in our experiments.

In the experiments described in this paper, we have compared the effects of metabolites of DEHP (see figure 1) in primary hepatocyte cultures of rat, rabbit, guinea pig and on Cynomolgus monkey hepatocytes. Parameters determined (peroxisomal palmitoyl-CoA oxidase activity, lauric acid hydroxylation activities, RNA levels for acyl-CoA oxidase and P450 4A1, triglycerides- and cholesterol concentrations) were studied in relation to the potency to form metabolites.

Materials and methods

Chemicals

Collagenase B, cholesterol and triglycerides test kits were obtained from Boehringer Mannheim GmbH, Germany. Palmitoyl-CoA, hydrocortisone-21-hemisuccinate, insulin, gentamycin sulfate, BSA, aminotriazole, NADPH, lauric acid were obtained from Sigma Chemical Co. (St Louis, MO, USA). 2,7-Dichlorofluorescin diacetate was purchased from Eastman Kodak Co. (Rochester, NY, U.S.A.). 18-Crown-6-ether and 4-(bromo-methyl)-6,7-dimethoxycoumarin were from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Triethyloxonium tetrafluoroborate was from Lancaster Synthesis Ltd (Morecamb, UK). Newborn Calf Serum, William's E powder and L-glutamine were obtained from Flow Laboratories.

A cDNA probe for rat cytochrome P450 4A1 was provided by Dr. G.G. Gibson, University of Surrey, UK. A cDNA probe for rat acyl-CoA oxidase was provided by Dr. Hashimoto, Shinshu University School of Medicine, Japan. MEHP (99% pure) was synthesized as described elsewhere [19]. Mono-hexylphthalate was synthesized according to the same procedure. Mono-(2-ethyl-5-oxohexyl)phthalate (metabolite VI) (99 % pure) was synthesized by Drs. Nefkens and van Zeist (Department of Organic chemistry, University of Nijmegen). Mono-(5-carboxy-2-ethyl-pentyl)phthalate (metabolite V) (96 % pure) was a kind gift of Dr. Sjöberg (Department of Drugs, National Board of Health and Welfare, Uppsala. Sweden). The identity of all metabolites was confirmed with NMR and MS.

Isolation of hepatocytes

Male Wistar rats (Cpb:WU) (Rattus norvegicus) (200 g), Male Dunkin Hartley guinea pigs (Cavia porcellus) (350g) and male New Zealander rabbits (Oryctolagus cuniculus) (2500 g) were purchased from the Central Animal Laboratory, University of Nijmegen.

Cynomolgus monkeys (Macaca fascicularis) were bred at the National Institute of Public Health and Environmental Protection (RIVM, Bilthoven, The Netherlands). Young monkeys (2 to 3 years old) served as donors for kidneys, used in the production of poliomyelitis vaccine at that institute.

Rats, rabbits and guinea pigs were anesthetized by i.p. injection of sodium pentobarbital. In Rabbits and guinea pigs, 2% lidocaine was injected subcutaneously in the abdominal wall as well. Monkeys were premedicated i.m. with atropine and xylazine and anesthetized i.m. with ketamine. Monkey livers were perfused with ice-cold saline in situ and transported on ice to our laboratory within 60 minutes after hepatectomy.

Isolation of hepatocytes was based on methods described by Berry and Friend [20] and by Seglen [21]. Monkey liver was perfused with 500 ml of a Ca^{2+}-free HEPES buffer containing 0.25 mM EGTA and 1000 ml of this buffer without EGTA, followed by a recirculating perfusion with a 0.05% (w/v) collagenase containing HEPES buffer for 30 minutes. Guinea pig and rabbit liver were perfused with 500 ml Ca^{2+}-free HEPES buffer containing 0.25 mM EGTA and 500 ml buffer of this buffer without EGTA, followed by a recirculated perfusion with 0.05% (w/v) collagenase for 10 minutes. Rat liver was perfused with 500 ml buffer of the Ca^{2+}-free buffer, followed by a recirculating perfusion with 0.05% (w/v) collagenase for 10 minutes. The perfusion rate was 50 mL/min and the temperature 37 °C.

The liver was gently torn apart and cells were dispersed in a Ca^{2+}-containing HEPES-TES buffer with 2% bovine serum albumin. For all four species, hepatocytes were obtained by a similar purification procedure of repeated filtering, washing and centrifugation at 50g (rabbit, rat and guinea pig) and 100g (monkey). Cell yields and viability were assessed using the trypan blue exclusion test. Cell yield was for rabbits 1280 x 10^6 cells (80%), monkeys 1100 x 10^6 cells (89%), guinea pigs 700 x 10^6 cells (90%) and rats 400 x 10^6 cells (90%). Numbers in parenthesis are the viability of the cells.

Cell culture and treatment

Hepatocytes were plated at a density of 9 x 10^6 /10 mL medium in a 9 cm tissue culture dish (Nunc) in Williams' medium E supplemented with 5% (v/v) newborn calf serum, 2 mM glutamine, 1 μM insulin, 10 μM hydrocortison and gentamycin (50 mg/L). Cells were incubated in a humidified atmosphere in air (95%) and CO_2 (5%) at 37 °C. During the first 4 h 4 mM Ca^{2+} and 4 mM Mg^{2+} was added to the medium. After 4 h the medium was changed.

After 24 h medium was replaced by medium containing various concentrations of mono(2-ethylhexyl)phthalate, metabolite V or metabolite VI dissolved in dimethylformamide (DMF) (final concentration DMF in medium was 0.1% (v/v)). The culture media, containing the appropriate concentration of test compounds, were renewed every 24 h.

The appearance of monolayers derived from all 4 species was very similar: cells attached and formed confluent monolayers within 24 hours after seeding. Cytotoxicity of the DEHP metabolites tested, was studied in parallel experiments. In all four species, there was no decrease observed in the activity of the mitochondrial enzyme succinate-dehydrogenase towards 3-4,5-dimethylthiazpol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (assayed according to Mossman [22] and Denizoit and Lang [23]), neither did the concentrations tested influence the lysosomal accumulation of neutral red

(assayed as described by Borenfreund and Puerner [24]). Ethoxyresorfurin O-dealkylation (EROD) activities were determined after treatment for 72 h with 50 μM B-naphthoflavone (BNF) according to Wortelboer *et al.* [25].

Cell harvesting and preparation of cell homogenates

After 96 h cells were harvested by removing the medium, washing the monolayer twice with ice-cold saline and scraping with a rubber policeman in cold sucrose-EDTA-Tris buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris/HCl, pH 7.4). Cells were centrifuged and the pellet was flash frozen in liquid nitrogen and stored at -80 °C. Cells were sonicated as described by Wortelboer *et al.* [24] using an MSE 100 Watt Ultrasonic Desintegrator. Part of the homogenate was flash frozen in liquid nitrogen and stored at -80 °C and it was used for determining lauric acid hydroxylation activities, triglyceride and cholesterol concentrations. Part of the homogenate was freeze/thawed 3 times, followed by centrifugation at 9,000g for 10 min. Supernatants were quickly frozen in liquid nitrogen and stored at -80 °C until they were thawed for determining of palmitoyl-CoA oxidase activity.

For the extraction of total RNA cells were harvested in 4M guanidium isothiocyanate solution (pH 7), containing 0.1 M 2-mercaptoethanol, 0.01 M TRIS (pH 7.4), and 0.5% N-lauroyl sarcosine. Total RNA was extracted according to the method of Cathala *et al.* [26]. Typical extractions yielded 80 μg RNA per 90 mm dish.

Biochemical determinations

Palmitoyl-CoA oxidase was measured as described by Reubsaet *et al.* [27]. Lauric acid hydroxylation activities were determined as previously described [28]. Protein concentration was determined by the Coomassie-brilliant blue method [29] using crystalline bovine serum albumin as standard. Triglyceride and cholesterol concentrations were determined enzymatically using test kits from Boehringer Mannheim.

Northern blotting

Total RNA fractions were submitted to electrophoresis and transferred by capillary blotting to Hybond-N filters (Amersham International, UK). Filters were then hybridized to ^{32}P randomly labelled full length cDNA probes. Filters were washed with high stringency (0.5xSSC, 55 °C). Filters were autoradiographed for 72 h.

Analysis of metabolites in culture media

After 48 (24-48) and 96 (72-96) h medium was collected and stored at -20 °C. A part of the medium was hydrolysed overnight with ß-glucuronidase/arylsulfatase and the other part was left untreated. After the addition of the internal standard monohexylphthalate, samples were acidified and extracted with diethylether. After derivatization with triethyloxonium tetrafluoroborate samples were analyzed by GC-MS (Varian Saturn, GC-MS) as described elsewhere [30]. Identification of the metabolites was carried out by the combination of full scan spectra, selected ion fragments (m/z 149 and

m/z 177) and retention times of pure standards.

Quantification was carried out by reference to calibration curves constructed from (media) samples containing MEHP, metabolite IX, metabolite VI and metabolite V (see figure 1). Knowing that the slope of the calibration curves of all 4 metabolites was comparable, metabolite I was quantified using the calibration curve of metabolite V.

Results

Effects of metabolites of DEHP in rat hepatocytes

The effect of treatment of rat hepatocytes with MEHP, metabolite VI and metabolite V on peroxisomal palmitoyl-CoA oxidase activity, is shown in figure 2A. MEHP and metabolite VI induced this activity dose-dependently. At the highest concentration tested (300 μM), this activity was 6-9-fold induced. MEHP and metabolite VI appeared to have the same potency to induce peroxisomal ß-oxidation. Metabolite V did not induce palmitoyl-CoA oxidase activity.

Lauric acid ω-hydroxylation activity was dose-dependently induced by both MEHP and metabolite VI (figure 2B). At 300 μM this activity was 20-fold increased by MEHP, 55-fold by metabolite VI and 3-fold by metabolite V. Lauric acid (ω-1)-hydroxylation activity was 6-9 fold increased by both MEHP and metabolite VI (results not shown), but this activity is supposed to be not characteristic for P450 4A1 [31].

In figure 3 it is shown that after 96 hours in culture, the RNA levels for both cytochrome P450 4A1 and acyl-CoA oxidase are dose-relatedly induced by treatment with MEHP.

The lowest concentration tested which significantly induced both palmitoyl-CoA oxidase activity and lauric acid ω-hydroxylation activity, was for MEHP 60 μM and for metabolite VI 100 μM. From this experiment we concluded that metabolite VI and MEHP are equally potent in inducing peroxisome proliferation. In the further experiments we have limited the testing in hepatocytes of several species to only one metabolite, i.e. MEHP.

Effects of MEHP in hepatocytes of rabbits, guinea pig and monkey compared to rat

In parallel experiments it was shown that primary cultures of rat, monkey and rabbit hepatocytes, did respond to the known P450 inducer ß-naphtoflavone (BNF). EROD activities from BNF treated hepatocytes were 8-44 fold increased in rats, 70-140 fold in rabbits and 30-60 fold in monkeys. No induction of the EROD activity by BNF was found in guinea pig hepatocytes.

Only at the highest concentration MEHP tested (600 μm), a 1.8-fold induction of palmitoyl-CoA oxidase activity was found in primary cultures of rabbit, guinea pig and male monkey hepatocytes (figure 4a). No induction of lauric acid ω-hydroxylation (figure 4b) or (ω-1)-hydroxylation activities (results not shown) was found in these 3 species. A considerable inter-individual

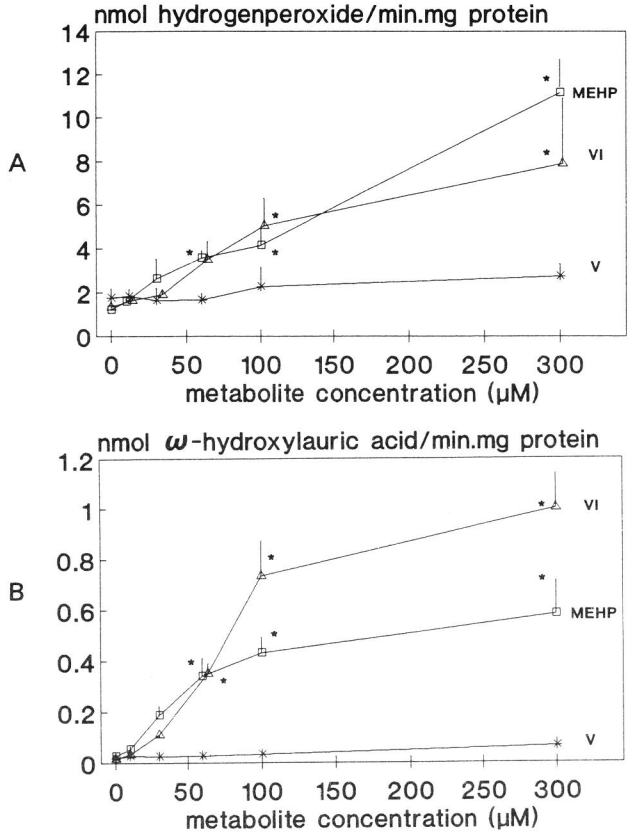

Figure 2 - Effect of MEHP, metabolite VI and metabolite V on palmitoyl-CoA oxidase activities (A) and lauric acid ω-hydroxylation activities (B) in primary cultures of rat hepatocytes.
Hepatocytes were preincubated for 24 hr, followed by 72 hr treatment. Each metabolite was tested in 3-4 experiments, in each experiment hepatocytes of different rats were used. Mean values ± SD of these 3-4 experiments are presented.
* significantly different ($p \leq 0.05$) from 0 μM (ANOVA and Dunnett's test).

variation was observed in the parameters studied. In hepatocytes of 3 female monkeys a significant (1.9 fold) induction of palmitoyl-CoA oxidase activities, after treatment with 600 μM MEHP, was determined. No increase in lauric acid ω-hydroxylation activity was observed (results not shown).

With the P450 4A1 and acyl-CoA oxidase cDNA probes used in the rat studies, no signals could be detected after Northern blot analysis of RNA samples of hepatocytes of rabbit, guinea pig or monkey treated in vitro with MEHP.

After treatment of guinea pig hepatocytes with MEHP, an increase in

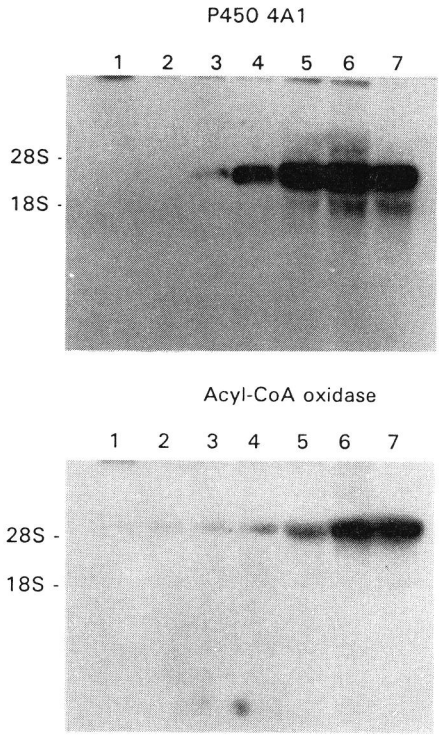

Figure 3 - Northern analysis of RNA isolated from rat hepatocytes treated in vitro with MEHP. RNA samples (20 μg) were hybridized with probes for peroxisomal acyl-CoA oxidase and cytochrome P450 4A1
lane 1: 24 h after hepatocyte isolation
lane 2-7: 96 h after hepatocyte isolation and after treatment for 72 h with 0 , 10, 30, 60, 100 and 300 μM MEHP.

triglyceride level in the hepatocytes was observed (figure 5A), while in (male) monkey hepatocytes triglyceride level was decreased. In hepatocytes of rabbits, triglyceride levels were slightly decreased after treatment with 60 and 100 μM MEHP, but a small increase was found after treatment with 600 μM MEHP. In rat hepatocytes no significant alteration in triglyceride levels upon treatment with MEHP was found. Cholesterol concentration in the liver cells of rat, guinea pig and monkey, were not influenced after treatment with MEHP, while in hepatocytes of rabbits, a small decrease in cholesterol concentration was found after treatment with high concentrations of MEHP (figure 5B).

Summarizing, the effects of MEHP in rat hepatocytes are much stronger than in hepatocytes of the other three species.

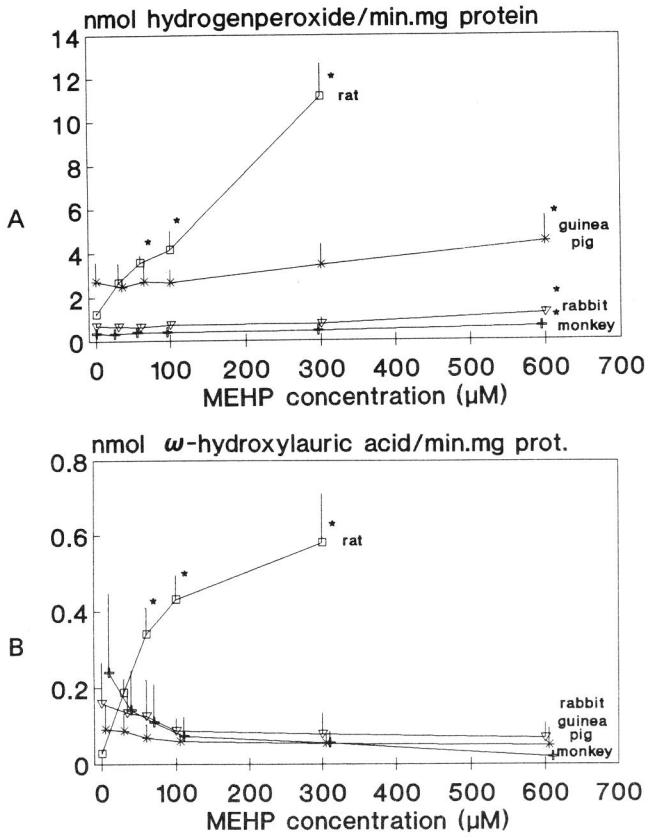

Figure 4 - Effect of MEHP on palmitoyl-CoA oxidase activities (A) and on lauric acid ω-hydroxylation activities (B) in primary cultures of rat, rabbit, guinea pig and monkey hepatocytes.
Hepatocytes were preincubated for 24 hr, followed by 72 hr of treatment. Each metabolite was tested in 3-5 experiments, in each experiment hepatocytes of different animals were used. Mean values ± SD of these 3-5 experiments are presented.
* Significantly different ($p \leq 0.05$) from 0 μM (ANOVA and Dunnett's test).

Biotransformation of DEHP metabolites in primary cultures of rats rabbits, guinea pigs and monkeys

Rats: media samples were collected after treatment of hepatocytes with 100 μM MEHP. In the 24-48 h samples at least 4 metabolites were identified. Due to the availability of reference compounds for the metabolites VI, IX and V, we could positively identify these metabolites in our samples. The unknown metabolite is probably metabolite I, since the mass spectrum of this metabolite had a characteristic fragment of m/z 143. Almost all metabolites and the

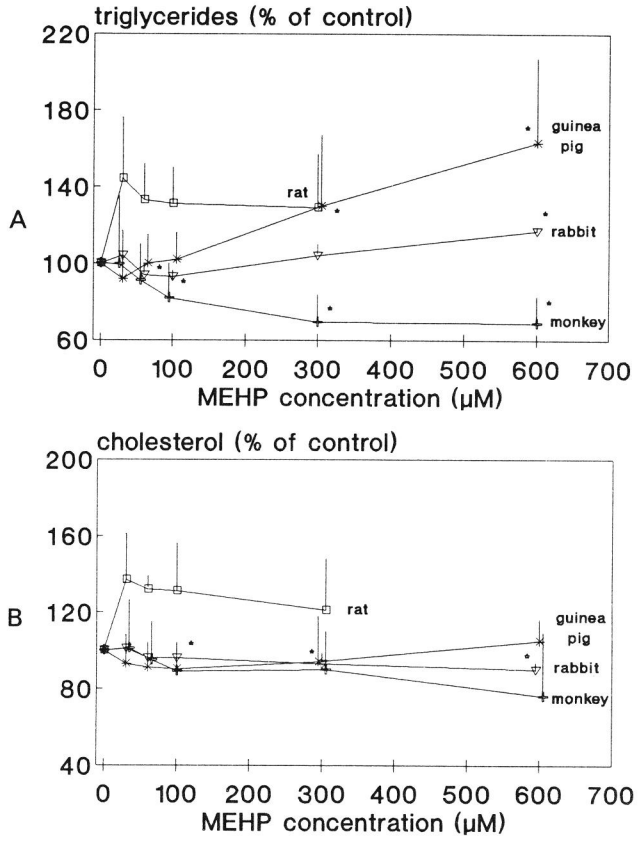

Figure 5 - Effect of MEHP on triglyceride concentration (A) and cholesterol concentration (B) in hepatocytes of rat, rabbit, guinea pig and monkey. All values are expressed as percentage of control values. Control triglyceride values are for rat 135 nmol/mg protein; for guinea pig 72 nmol/mg protein; for rabbit 148 nmol/mg protein and for monkey 172 nmol/mg protein. Control cholesterol values are for rat 145 nmol/mg protein; for guinea pig 82 nmol/mg protein; for rabbit 148 nmol/mg protein and for monkey 196 nmol/mg protein.
Hepatocytes were preincubated for 24 hr, followed by 72 hr of treatment. Each metabolite was tested in 3-5 experiments, in each experiment hepatocytes of different animals were used. Means ± SD of these 3-5 experiments are presented. * statistically significant ($p \leq 0.05$) from 0 μM (Kruskal-Wallis and Mann-Whitney test).

parent compound were present in non-conjugated form. In the 72-96 h samples the same 4 metabolites were determined, but now metabolite V and metabolite I were the major metabolites formed (figure 6).
In media of primary cultures of rat hepatocytes treated with metabolite V, 20% was metabolized to metabolite I. Neither metabolite V nor metabolite I

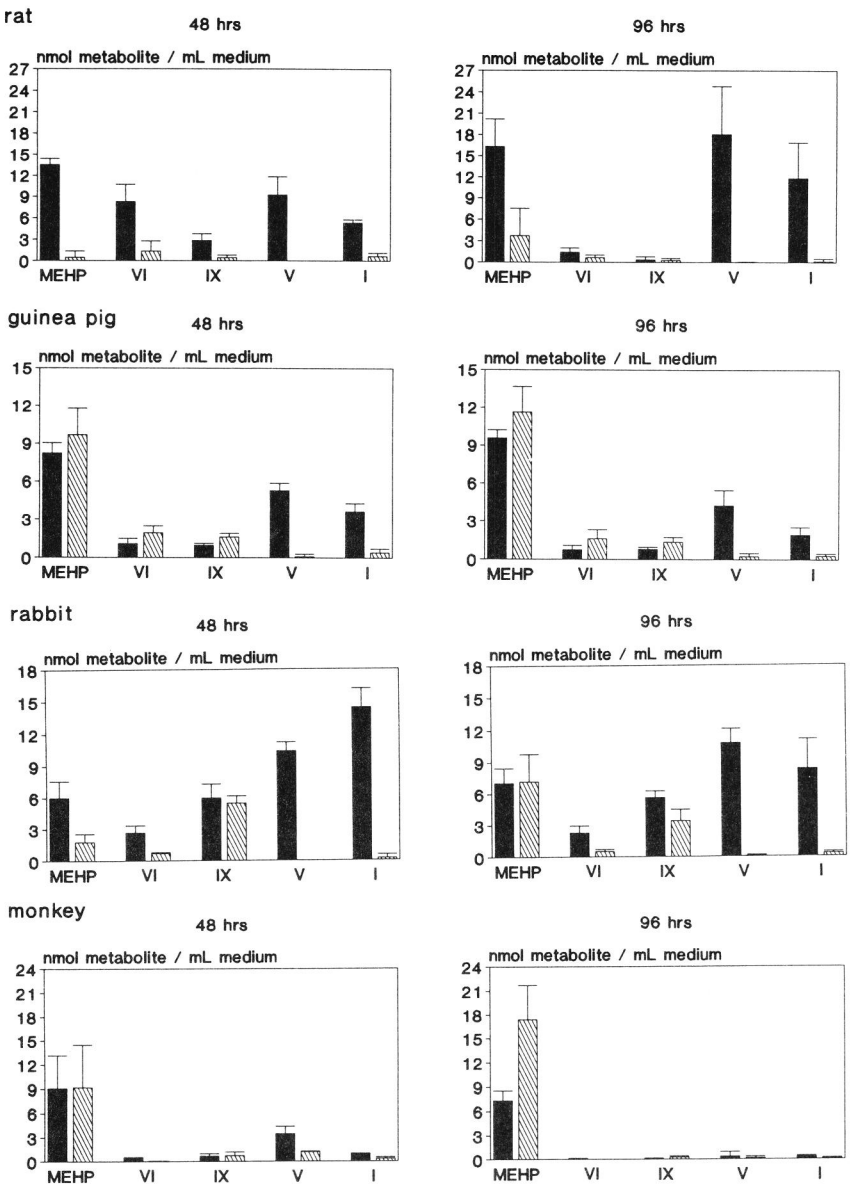

Figure 6 - Concentrations of MEHP and its metabolites in media from primary hepatocyte cultures of rat, guinea pig, rabbit and (male) monkey treated in vitro with 100 μM MEHP.
Results after 48 h (24-48h) and 96 h (72-96 h) in culture are shown.
Hepatocytes were preincubated for 24 hr, followed by 72 hr of treatment. Each metabolite was tested in 3 experiments, in each experiment hepatocytes of different animals were used. Mean values ± SD of these 3-5 experiments are presented.
Key: ▨ = glucuronidated metabolites, ■ = free metabolites

were conjugated. After treatment with metabolite VI no other metabolites were detected. A small proportion of metabolite VI was conjugated (results not shown).

Guinea pig, rabbit and monkey: in media of hepatocytes of these three species treated in vitro with MEHP, the same 4 metabolites were identified as with the rat hepatocytes. In media from guinea pig and rabbit hepatocytes the concentration of oxidized metabolites in 24-48 h and 72-96h periods was comparable to the findings in the rat (figure 6). In media of primary cultures of monkey hepatocytes, lower concentrations of the oxidized metabolites were detected compared to rabbit and guinea pig. Furthermore, the concentrations of these metabolites in media from monkey hepatocytes declined in time. In guinea pig, rabbit and monkey, a major part of the parent compound MEHP and a part of the metabolites VI and IX, was conjugated. This conjugation activity was not lost during culture.

Summarizing, all species showed metabolic capacity towards MEHP with a low oxidizing activity in monkey and a low conjugating capacity in rat.

Discussion

A major question in the assessment of carcinogenicity of DEHP and other peroxisome proliferators, is what mechanisms underly species differences in sensitivity for peroxisome proliferation. In addition, to assess the risk of these compounds for man it is necessary to quantify these species differences.

In rat hepatocytes peroxisomal palmitoyl-CoA oxidase activity is already significantly increased (2.9-fold) at concentrations of 60 μM MEHP. In rabbit, guinea pig and monkey hepatocytes, a significant induction of this activity (1.8 fold) is observed only after treatment with 600 μM MEHP. These results indicate that MEHP can induce peroxisome proliferation in monkey, guinea pig and rabbit, but that these latter species are less sensitive for peroxisome proliferation than rat. Using linear regression analysis, we have calculated at which MEHP concentration the palmitoyl-CoA oxidase activity is 50% increased in the four species tested (table 1). This value is at least 30-fold higher for rabbit, guinea pig and monkey, than for rat.

Our results with primary cultures of monkey and guinea pig hepatocytes, are in agreement with observations that these species are less sensitive for peroxisome proliferation than rat and mouse after in vitro and in vivo treatment with peroxisome proliferating compounds [4-11, 18]. Gibson [8] considered rabbits and hamsters to be intermediate sensitive for peroxisome proliferation. This conclusion was made after in vivo treatment with the peroxisome proliferating compound ciprofibrate. Our results on in vitro treatment of rabbit hepatocytes with MEHP, indicate that rabbits are not very sensitive for peroxisome proliferation.

After treatment of rat hepatocytes with MEHP, we have found a close association between induction of lauric acid ω-hydroxylation activity and peroxisomal palmitoyl-CoA oxidase activity, in accordance with earlier

Table 1 - Estimation of the concentration of the DEHP metabolites causing a 50% increase in enzyme activity after in vitro treatment of hepatocytes of different species.

		Palmitoyl-CoA oxidase activity	Lauric acid ω-hydroxylation activity
Rat	MEHP	14.8 μM	4.5 μM
	Metabolite VI	12.1 μM	6.9 μM
	Metabolite V	262 μM	78.6 μM
Rabbit	MEHP	440 μM	ND[1]
Guinea pig	MEHP	485 μM	ND[1]
Monkey	MEHP	408 μM	ND[1]

[1] It was not possible to calculate these values since activities decreased with increasing MEHP concentration.

published studies [3, 4, 19]. Using linear regression analysis, we have calculated that a 50% increase in lauric acid hydroxylation activities in rat hepatocytes is found at lower MEHP concentrations than a 50% increase in peroxisomal palmitoyl-CoA oxidase activities (table 1), again demonstrating that induction of lauric acid ω-hydroxylation activities is the more sensitive marker for peroxisome proliferation. The increase in activity of these enzymes is accompanied by increases in mRNA level for these enzymes. This is partly, if not entirely, due to an increase in the transcription of the respective genes [32, 33]. However, the observed increase in palmitoyl-CoA oxidase activities in guinea pig, monkey and rabbit hepatocytes after treatment with MEHP, is not accompanied by an increase in lauric acid ω-hydroxylation activity. Similar findings have been reported after in vivo treatment of marmoset monkeys with ciprofibrate [10]. The proposed mechanistic inter-relationship between induction of peroxisomal enzymes and induction of lauric acid ω-hydroxylation activities as proposed by Sharma et al. [3], might therefore not be valid in rabbits, guinea pigs and monkeys.

When we studied the triglyceride concentration in the MEHP treated hepatocytes, we observed different trends in the species tested. In guinea pig hepatocytes, hepatic triglyceride levels were increased by the MEHP treatment. In rabbit these levels were much less altered, while in monkey a decrease in hepatic triglyceride levels was found. Watanabe *et al.* [7] reported that after in vivo treatment of guinea pigs with the peroxisome proliferator bezafibrate, an increase in hepatic triglyceride level was observed, in rhesus monkeys a decrease was found, while in rat and rabbit no alteration of liver triglyceride levels was observed. The effects observed in our in vitro studies

with rabbit, monkey and guinea pig, show striking similarities with those observed after in vivo exposure. Further studies on species differences in fatty acid metabolism are necessary to explain these results. Kocarek and Feller [34] found a slight increase in triglyceride biosynthesis in rat hepatocytes treated in vitro with three peroxisome proliferating compounds.

Mitchell *et al.* [14] have proposed that metabolite VI is the active peroxisome proliferator of DEHP in rats. We doubt if this is true since our studies indicate that both MEHP and metabolite VI have the same potency to induce palmitoyl-CoA oxidase activities and lauric acid ω-hydroxylation.
In addition our biotransformation studies indicate that the concentration of metabolite VI formed from MEHP in rats is low according to the effects found.

Our metabolism studies indicate that in all species the pattern of oxidized metabolites does not differ much between the species. Metabolite VI is formed in all four species. In monkeys the formation of oxidized metabolites decreased in time, indicating a loss of cytochrome P450 activity, since the hydroxylation of MEHP is P450 dependent [35]). In guinea pig and rabbit the amounts and pattern of oxidized metabolites found between 24-48 h and between 72-96 h were comparable. In rats higher concentrations of metabolite V and metabolite I were formed between 72-96 h compared to 24-48 h. This indicates that ω-hydroxylation activity towards MEHP is induced after 48 h. Lhuguenot *et al.* [36] studied the metabolism of MEHP in vivo and in vitro in the rat and observed that after repeated administration the concentrations of ω-oxidation metabolites of MEHP were increased. It is possible that the ω-hydroxylation of MEHP is mediated by P450 enzymes associated with the ω-hydroxylation of fatty acids [35]. Induction of these enzymes by MEHP results not only in an increased ω-hydroxylation of fatty acids, but might also result in an increased ω-hydroxylation of MEHP.

Metabolite I is supposed to be formed by (peroxisomal) ß-oxidation of metabolite V, and Turnbull and Rodricks [37] have proposed that monitoring the formation of metabolite I might be useful for quantifying the target-dose. Since guinea pig and rabbit, although less sensitive for peroxisome proliferation, do form high concentrations of metabolite I, we think that formation of metabolite I is not per se an indicator of increased peroxisomal ß-oxidation activities.

In media of hepatocytes from rabbit, guinea pig and monkey, glucuronide conjugates of MEHP, metabolite VI and metabolite IX were found. Metabolites V and I were hardly conjugated. The rat did hardly form any conjugated products at all. This finding is in accordance with the observations that rats do not excrete conjugated metabolites of DEHP in urine [38]. A lower conjugating activity towards DEHP metabolites in the rat might mean an increased bioavailability of these metabolites in the hepatocytes. However, we doubt whether there is a causal relationship between induction of peroxisome proliferation and conjugating activity, since mice are very sensitive for peroxisome proliferation and do form conjugated DEHP metabolites [38].

Our studies show that the observed species differences in peroxisome proliferation, are not the result of species differences in the formation of active metabolites. We propose that species differences in peroxisome

proliferation might be due to differences in fatty acid metabolism, in particular species differences in the partitioning of fatty acids between ß-oxidation and esterification. With the recent description of a Peroxisome Proliferator Activated Receptor (PPAR) as a transcription factor for peroxisomal ß-oxidation genes (review: Green [12]), it is also possible that species differences in peroxisome proliferation might be caused by variation in expression of PPAR, or in the regulation of the genes that are under control of this receptor. To assess the toxicity of peroxisome proliferating compounds to humans, further studies on species differences in peroxisome proliferation should focus on these two aspects.

Acknowledgments
The authors wish to thank dr AAJ van Iersel and A Timmerman (National Institute of Public Health and Environmental Protection (RIVM, Bilthoven) for assistance in obtaining the monkey livers. We wish to thank dr. Baadehuysen from the Clinical-Chemical Laboratory of the Radboud Hospital (University of Nijmegen) for performing the triglycerides and cholesterol determinations. We wish to thank dr. GG Gibson (University of Surrey, UK) for his hospitality and advice in determining RNA levels in the hepatocytes. HD received financial support from the European Science Foundation to work at the laboratory of Dr. Gibson.

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Chapter 7

Determination of four metabolites of the plasticizer di(2-ethylhexyl)phthalate in human urine samples.

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Int Arch Occ Env Hea, in press

Abstract

A method for biological monitoring of exposure to the plasticizer di(2-ethylhexyl)phthalate (DEHP) is described. In this method the four main metabolites of DEHP (i.e. mono(2-ethylhexyl)phthalate (MEHP), mono(5-carboxy-2-ethyl)pentylphthalate, mono(2-ethyl-5-oxohexyl)phthalate and mono(2-ethyl-5-hydroxyhexyl)phthalate), are determined in urine samples. The procedure includes enzymatic hydrolysis, ether extraction and derivatization with triethyloxonium tetrafluoroborate, respectively. Analysis is performed by gas chromatography-electron impact mass spectrometry. The detection limit for all four metabolites is less than 25 μg/L urine. The coefficient of variation based on duplicate determinations of urine samples of workers occupationally exposed to DEHP, was 16% for MEHP (mean concentration 0.157 mg/L) and 6 - 9% for the other three metabolites (mean concentrations 0.130-0.175 mg/L).

The method described here was used to study DEHP metabolism in man. Most persons excrete mono(2-ethyl-5-oxohexyl)phthalate and mono(2-ethyl-5-hydroxyhexyl)phthalate as a (glucuronide) conjugate. Mono(5-carboxy-2-ethyl)pentylphthalate is mainly excreted in free form, while for MEHP a large interindividual variation in conjugation status was observed.

Of the four metabolites quantified, 52% are products of a (ω-1)-hydroxylation reaction of MEHP (i.e. mono(2-ethyl-5-oxohexyl)phthalate and mono(2-ethyl-5-hydroxyhexyl)phthalate), 22% is the product of a ω-hydroxylation reaction of MEHP (i.e mono(5-carboxy-2-ethyl)pentylphthalate), and 26% is not oxidized further (i.e. MEHP). A good correlation is obtained when the amount of MEHP ω-hydroxylation products is compared with the amount of MEHP (ω-1)-hydroxylation products in urine samples.

When the internal dose of DEHP has to be established, we recommend to study the levels of all four metabolites of DEHP in urine samples.

Introduction

Di(2-ethylhexyl)phthalate (DEHP) is a plasticizer commonly used in polyvinylchloride (PVC) plastics. In 1982 a study by the National Toxicology Program of the USA was published in which DEHP was shown to induce liver tumors in rats and mice (NTP, 1982). The International Agency on Research of Cancer has classified DEHP as a possible human carcinogen (group 2b) (IARC, 1987). Since DEHP is not genotoxic, mechanisms leading to carcinogenicity are not very well understood (for review: Conway *et al.*, 1989).

Our understanding of the metabolism of DEHP is largely due to the work of Albro and co-workers (for review: Albro *et al.*, 1982 and Albro, 1986). In most species, including man, DEHP is rapidly hydrolyzed to mono(2-ethylhexyl)phthalate (MEHP) and 2-ethyl-1-hexanol (see figure 1). In the liver, MEHP can be oxidized by cytochrome P450 dependent reactions (Albro *et al.*, 1984). Between species there are large differences in the excretion pattern of urinary DEHP metabolites. Rats for example, excrete more of the further oxidized urinary metabolites than man (Albro *et al.*, 1982, Albro, 1986). Rats do hardly excrete (glucuronide) conjugated metabolites, in contrast to monkeys and man (Albro *et al.*, 1982).

Schmid and Schlatter (1985) described that after oral dosing of 2 volunteers with DEHP, 11-15% of the dose was excreted in the urine, mainly in the form of conjugated metabolites. The halflife was estimated to be 12h. Schmid and Schlatter (1985) described seven metabolites, and the most important were MEHP, mono-(2-ethyl-5-hydroxyhexyl)pthalate (metabolite IX), mono(2-ethyl-5-oxohexyl)phthalate (metabolite VI) and mono(5-carboxy-2-ethyl-pentyl)phthalate (metabolite V). Numbering of the metabolites is according to Albro (1986).

Exposure to DEHP has been studied in patients receiving a hemodialysis treatment, a blood product transfusion, undergoing cardiopulmonary bypass surgery and infants receiving an exchange transfusion (Sjöberg *et al.*, 1985; Nässberger *et al.*, 1987; Flamino *et al.*, 1988; Rock *et al.*, 1988; Barry et al., 1989). Little is published on occupational exposure to DEHP.

In order to study the uptake of DEHP in man, we have developed a biological monitoring method. In this paper we describe a GC-MS method based on the simultaneous determination of four metabolites of DEHP (i.e. MEHP, metabolite IX, VI and V) in urine of workers occupationally exposed to DEHP.

Methods

Chemicals

MEHP (> 99% pure) was synthesized as described elsewhere (Dirven *et al.*, 1992). The internal standard mono(pentyl)phthalate (MPP, >99% pure) was synthesized according to the same procedures using 1-pentanol in stead

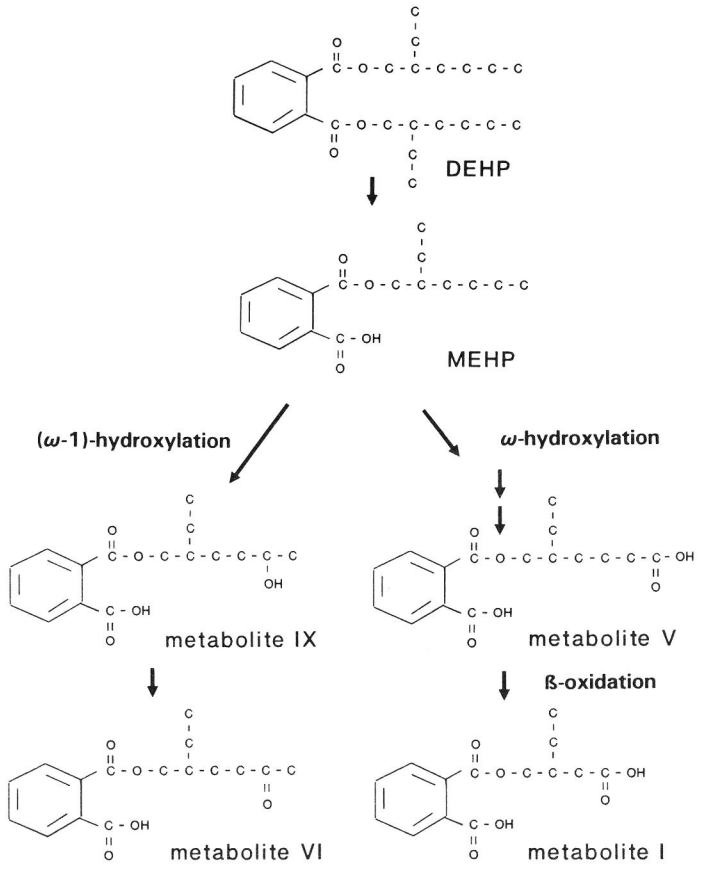

Figure 1 - Biotransformation of DEHP. DEHP is hydrolyzed by nonspecific hydrolases to MEHP. Hydroxylation by cytochrome P450 enzymes may occur at the ω- or at the (ω-1)-position. Further metabolism by alcohol dehydrogenase enzymes and aldehyde dehydrogenase enzymes yield keto-metabolites or dicarboxylic acids. Dicarboxylic acids can undergo a ß-oxidation reaction (for review see Albro, 1986).

of 2-ethyl-1-hexanol.

Metabolite VI (99% pure) and metabolite IX (91% pure) were synthesized by Drs. Nefkens and van Zeist (Department of Organic Chemistry, University of Nijmegen, The Netherlands). Metabolite V (96% pure) was a gift of Dr. Sjöberg (Department of Drugs, National Board of Health and Welfare, Uppsala, Sweden). The identity of all four metabolites was confirmed with NMR and MS.

ß-Glucuronidase/aryl sulphatase was obtained from Boehringer (Mannheim, Germany). Triethyloxonium tetrafluoroborate was bought in diethylether from Lancaster Synthesis ltd (Morecamb, UK). When stored as a salt

at -20 °C, no loss of ethylating activity was observed. A working solution was obtained after pouring off the diethylether and dissolving the salt in dichloromethane (final concentration 0.5 M). The solution was filtered using a glass fiber filter. This working solution could be stored at -20 °C for up to 1 month.

All other chemicals were of the highest purity obtainable.

Calibration samples

The metabolites were dissolved in methanol. Pooled urine samples of non-exposed persons were spiked with MEHP, metabolite V, VI and IX, yielding concentrations of 0, 0.1, 0.25, 0.5 and 1 mg of each metabolite/L urine (final concentration of methanol in urine was less than 1% (v/v). These calibration samples were aliquoted and stored at -20 °C for maximal 5 months.

Treatment of urine samples

Enzymatic hydrolysis To an aliquot of urine (5 ml), 10 ml 0.1 M acetate buffer (pH 4.9) was added and the pH was adjusted to pH 4.85-4.9 with 1 M HCl. This mixture was incubated in duplicate overnight (16 h) with 12.5 µl ß-glucuronidase/aryl sulphatase at 37 °C in a rotary shaking bath (200 rpm).

Extraction. To all samples 100 µl of a 200 µM solution of monopentyl phthalate was added as an internal standard. The pH of the mixture was adjusted to 1 with 3 M HCl and 2 x 20 ml of diethylether was added. Samples were shaken for 15 min at high speed and the ether layer was removed. The combined ether extracts were evaporated at 30 °C under a gentle stream of nitrogen.

Derivatisation: The metabolites were ethylated using a triethyloxonium tetrafluoroborate solution. To the dried ether extracts 25 µl triethylamine was added followed by 0.5 ml of a 0.5 M triethyloxonium tetrafluoroborate solution in dichloromethane. The formation of hydrogen fluoride was used for monitoring the derivatization reaction. The same volume of triethyloxonium tetrafluoroborate solution was added to all samples within a serie. Excess dichloromethane was evaporated at 40 °C under a gentle stream of nitrogen. To the dried extract 1 ml of water was added followed by extraction with 2 x 3 ml n-hexane. The combined extracts were evaporated to dryness under nitrogen at 45°C. The extract was dissolved in 0.5 ml toluene.

Analysis by GC-MS

GC-MS analysis was performed on a Varian Saturn GC-MS system controlled by a Compaq 386-20 personal computer using software version A (Varian, Houten, The Netherlands). A 30 m DB-5 column (J & W Scientific Folsom, California, USA) was used with 0.32 mm i.d. and 0.25 µm film thickness.

The 'on column injection' mode was used (SPI: Septum equipped temperature Programmable Injector). An aliquot (1 µl) of toluene extracts was

injected in a deactivated high-performance liner (injection rate 5 µl/min). The initial injector temperature was 110 °C. After 1 minute the temperature rose to 280 °C (80 °C/min). After 4 minutes at 280 °C the temperature was lowered to the initial temperature by cooling with liquid carbon dioxide. The initial oven temperature was 110 °C. After 1 min the temperature rose to 280 °C (10 °C/min) where it remained constant for 6 min. Helium was used as carrier gas (column flow was 0.8 ml/min). The interface temperature was 280 °C and electron impact was used as ionization mode. Data acquisition was performed in the auto ion mode in the mass range 100-249, scan speed was 1 scan/second.

Identification was carried out by the combination of full scan spectra (100-249 atomic mass units), selected ion fragments (m/z 149 and m/z 177) and retention times of pure standards.

Quantification was performed on mass fragment m/z 149 for the internal standard MPP, metabolite VI and metabolite V; mass fragment m/z 177 for MEHP and on mass fragments m/z 149 and m/z 177 for metabolite IX. Retention times were: 585 sec for MPP; 710 sec for MEHP; 810 sec for metabolite VI, 820 sec for metabolite IX and 910 sec for metabolite V.

The metabolite/MPP areas ratio was calculated for each sample and quantified by comparing to a calibration curve, which was processed along with the series of samples. The mean of the duplicate determinations was used.

The intercept of the calibration curves for metabolite VI, IX and V was not statistically significant different from zero. However, the intercept of the MEHP calibration curve was significant different from zero. This probably indicates that in the pooled urine sample used for preparation of the calibration curves a small background of MEHP was present. During quantification no correction was made for this background concentration.

Determination of the creatinine concentration

The urinary creatinine was determined with an automated analyzer technique based on the reaction of creatinine to alkaline picrate.

Calculation of the coefficient of variation

The coefficient of variation was calculated by using the formula: coefficient of variation = $\Sigma\%D / n\sqrt{2}$, in which n = the total number of duplicate observations. %D was calculated using the formula $\%D = (| x_1 - x_2 | / 0.5(x_1 + x_2)) \times 100\%$, in which x_1 and x_2 are the values of a duplicate observation.

Results

Chromatograms

In figure 2A and 2B a mass chromatogram of a blank urine sample and a spiked urine sample is shown. A total ion current (or reconstructed ion

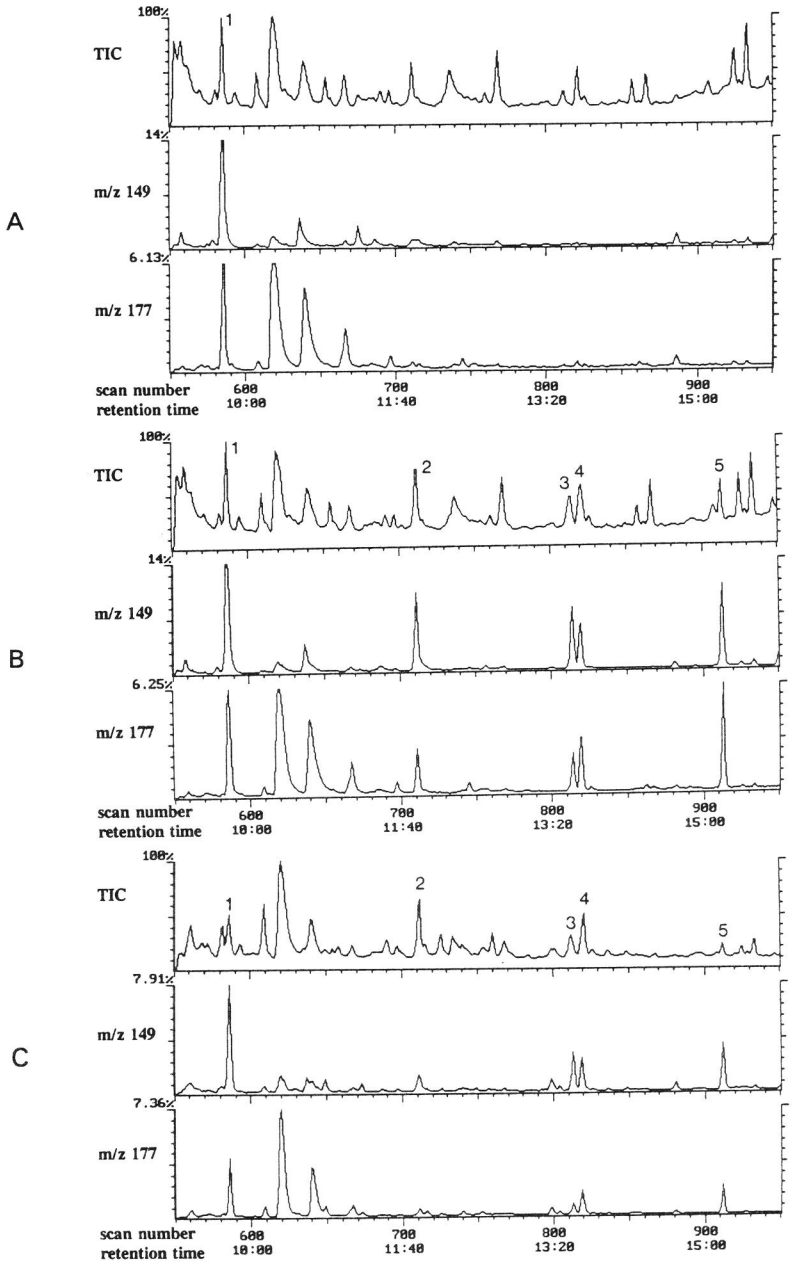

Figure 2 - CG-MS total ion chromatogram (TIC) and single-ion chromatograms at m/z 149 and m/z 177 of A) a blank urine sample and B) a spiked urine sample and C) an urine sample of a worker occupationally exposed to DEHP.
peak 1 = MPP (internal standard), peak 2 = MEHP, peak 3 = metabolite VI, peak 4 = metabolite IX and peak 5 = metabolite V

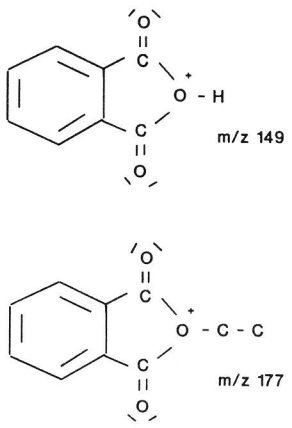

Figure 3 - Fragments of interest when DEHP metabolites are analyzed by mass spectrometry in the electron-impact mode. Mass fragment m/z 149 is formed from all ortho phthalic esters, mass fragment m/z 177 is formed when mono-esters of ortho-phthalic acid are ethylated.

current) chromatogram is shown as well as single-ion chromatograms at m/z 149 and m/z 177. Mass fragment m/z 149 is characteristic for ortho-phthalate compounds (both di- as mono-esters), while mass fragment m/z 177 is characteristic for ethylated mono-esters (see figure 3).

The mass spectra of the metabolites are not very characteristic due to the lack of fragments of the ethylhexyl moiety with sufficient abundance. The main fragments found were m/z 149 and m/z 177. The spectra of metabolite V showed a characteristic fragment of m/z 171[1], in the spectra of metabolite IX a characteristic fragment of m/z 157[2] was found and mass spectra of metabolite VI showed a characteristic fragment of m/z 127[3].

In figure 2C a chromatogram of an urine sample of a worker exposed to DEHP is shown. Although the concentrations of MEHP and metabolites V, VI and IX were relatively high, no major other peaks are detected with mass fragments m/z 149 or m/z 177, which means that the four metabolites determined were the main DEHP-metabolites.

When studying the metabolism of MEHP in primary hepatocyte cultures of rat, rabbit, guinea pig and monkey, we have identified metabolite I. The retention time of this metabolite is 790 sec. In chromatograms of urine samples of workers exposed to DEHP, minor peaks were detected with this retention time.

[1] $[\cdot CH_2\text{-}CH(C_2H_5)\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}COO\text{-}CH_2CH_3]^+$
[2] $[\cdot CH_2\text{-}CH(C_2H_5)\text{-}CH_2\text{-}CH_2\text{-}CHO(C_2H_5)\text{-}CH_3]^+$
[3] $[\cdot CH_2\text{-}CH(C_2H_5)\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}CH_3]^+$

Table 1 - Quantitative distribution of free- and conjugated metabolites in 5 human urine samples. Values are expressed as nmol metabolite/mmol creatinine.
Values in parenthesis is the percentage of the free metabolites in relation to the total amount of metabolite.

	MEHP		VI		IX		V	
	free	con-jugated	free	con-jugated	free	con-jugated	free	con-jugated
worker 1	15 (20%)	61	8 (8%)	96	11 (8%)	123	77 (68%)	36
worker 2	27 (38%0	45	8 (9%)	86	ND[1] -	152	95 (60%)	63
worker 3	27 (77%)	8	4 (12%)	29	4 (9%)	42	28 (67%)	14
worker 4	4.2 (100%)	ND[1]	3 (20%)	12	2 (12%)	15	6 (55%)	5
worker 5	12 (30%)	32	2 (5%)	35	4 (7%)	52	18 (58%)	13

[1] = not detectable, concentration below the detection limit.

Conjugated metabolites

In urine samples of 5 men occupationally exposed to DEHP, we have determined the concentrations of MEHP and the metabolites V, VI and IX, with and without enzymatic hydrolysis. The results are presented in table 1. Metabolites IX and VI were almost completely conjugated in all 5 persons, while metabolite V was for 32-45% present in conjugated form. For MEHP a large interindividual variation was found. In urine samples of worker 3 and 4, MEHP was for 77-100% present in free form, while in the urine samples of 3 other men, only 20-38% of the MEHP was not conjugated.

Sensitivity and reproducibility of the analysis

The detection limits (signal/noise > 3) were 25 μg/L urine for MEHP, 25 μg/L urine for metabolite IX, 13 μg/L urine for metabolite VI and 16 μg/L urine for metabolite V.

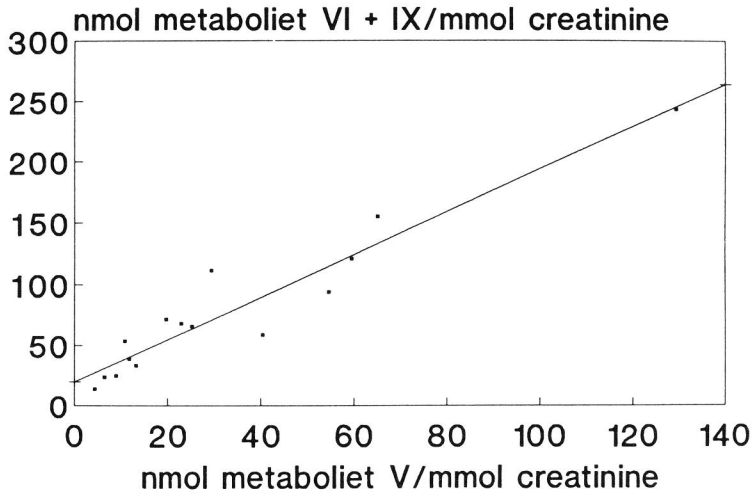

Figure 4 - The amount of metabolite VI + IX versus the amount of metabolite V in urine samples from 15 men occupationally exposed to DEHP. Metabolite V is formed after ω-hydroxylation of MEHP, while metabolite VI and IX are both products of (ω-1)-hydroxylations.
$y = 20 + 1.77x$, $r = 0.958$, $p < 0.001$

A pooled urine sample from workers exposed to DEHP was aliquoted and 5 aliquots were analyzed in the course of 3 months. The following values (± SD) were obtained from these 5 determinations: for MEHP 86 ± 17 μg/L (range 71-106 μg); for metabolite IX 127 ± 14 μg/L (range 109-146 μg); for metabolite VI 70 ± 10 μg/L (range 52-76 μg) and for metabolite V 91 ± 8 μg/L urine (range 79-101 μg). The day-to-day reproducibility is in the range of 9 to 20 percent.

69 Urine samples from workers exposed to DEHP were analyzed in duplicate. When the concentrations determined were above the detection limits a coefficient of variation based on duplicate measurements was calculated. This value is 16% for MEHP (n = 55), mean concentration 0.157 mg/L; 6% for metabolite VI (n = 52), mean concentration 0.169 mg/L; 9% for metabolite IX (n = 60), mean concentration 0.175 mg/L and 9% for metabolite V (n = 60), mean concentration 0.130 mg/L.

These data show that the reproducibility of the method at the μg/L range is acceptable.

Interindividual variation in metabolism of DEHP

In order to select one metabolite as marker for DEHP-exposure, we were interested whether there are any interindividual variations in the metabolism of DEHP. Variation can occur in the rate of ω-hydroxylation or (ω-1)-hydroxylation activity of MEHP (see figure 1). In figure 4, the amount of

the product of the ω-hydroxylation reaction (metabolite V) is compared with the amount of the product of the (ω-1)-hydroxylation reaction (metabolite VI + metabolite IX) in post-shift urine samples of 15 men. A high correlation ($r = 0.95$) was obtained and no substantial interindividual differences in the concentrations of the products of ω- or (ω-1)-hydroxylation were found. The absolute concentration of the metabolites VI+IX was 1.7 times as high as the concentration of metabolite V, which indicates that in human metabolism the (ω-1)-hydroxylation reaction is favoured over the ω-hydroxylation reaction.

Discussion

The use of the described GC-MS method in this study allowed the detection of concentrations of the four DEHP metabolites as low as 13-25 μg/L urine. Since the four DEHP metabolites were detected in urine samples of workers occupational exposed to DEHP (Dirven *et al.*; 1993, see accompanying paper), this method is sensitive enough for the determination of occupational and medical exposure to DEHP.

The determination of the four metabolites of DEHP is specific for exposure to DEHP, in contrast to earlier studies in which the total phthalic acid concentration in urine samples was used to assess exposure to DEHP (Liss *et al.*, 1985 and Nielsen *et al.*, 1985). The method described here, is reproducible and not very time consuming. The use of triethyloxonium tetrafluoroborate as derivatization agent is an improvement for the determination of monoesters of phthalic acid. This very powerful ethylating agent can be stored for a longer period, and the reaction is very fast and can be monitored by measuring the pH of the reaction mixture.

After exposure of man to DEHP by inhalation, MEHP, metabolite V, metabolite VI and metabolite IX, are the major metabolites found. Our results indicate that man excretes DEHP metabolites in conjugated form, which is a major contrast to the rat, which hardly excretes conjugated metabolites of DEHP (Albro *et al.*, 1982).

In table 2 the urinary metabolite profile as found in the 15 men in our study is compared with the profile described in 2 other studies (Albro *et al.*, 1982; Schmid and Schlatter, 1985). The differences in these profiles reflect probably large interindividual differences in metabolism and/or routes of administration.

In selecting metabolites of interest for biological monitoring of DEHP 3 questions have to be studied:

1) do interindividal differences in the metabolism of DEHP exist?;
2) are there any metabolites which formation is specific for a toxic effect?;
3) are there any practical (analytical-chemical) arguments which favours the choice for a specific metabolite?

ad 1) Our results indicate that there are some but no large interindividual differences in the formation of the four major metabolites of DEHP in man.

Table 2 - Quantitative distribution of metabolites of DEHP in urine of humans.

metabolite	this study	Schmid and Schlatter, 1985	Albro *et al.*, 1982
MEHP	26.2%	10.5%	25.4%
VI	18.2%	24.2%	16.8%
IX	33.8%	32.5%	50.3%
V	21.8%	33.0%	7.4%
total	100%[1]	100.2%[2]	99.9%[3]

[1] = metabolites were determined in urine samples of 15 man after a 8 hr exposition to DEHP. Intake of DEHP was probably a combination of inhalation during work and ingestion (background).
[2] = these 4 metabolites accounted for 91% of total metabolites quantified. Metabolites were determined in urine samples of 2 man after oral dosing with 30 mg DEHP.
[3] = these 4 metabolites accounted for 71.9% of total metabolites quantified. Metabolites were determined in pooled urine samples of 2 leukemia patients after intravenous administration of DEHP.

The rate of formation of ω-hydroxy metabolites is linear with the rate of formation of (ω-1)-hydroxy metabolites.

ad 2) Studies about toxic effects linked to specific metabolites are limited. None of the four metabolites were mutagenic in the Ames test using several typhimurium strains and different metabolic activation systems (Dirven *et al.*, 1991). Metabolites associated with a toxicological effect include metabolite I (see figure 1), since this metabolite is supposed to be formed in (peroxisomal or mitochondrial) ß-oxidation (Albro, 1986; Turnbull and Rodricks, 1985). It is suggested that the quantity of metabolite I formed, might serve as an indicator of peroxisomal activity, or as a surrogate of the target dose (Turnbull and Rodricks, 1985). However, we have found that metabolite I is not a major metabolite in human metabolism of DEHP. Metabolite VI was mentioned as the proximate peroxisome proliferator in rats (Elcombe and Mitchell, 1986).

Another metabolite of interest is metabolite V. It has been hypothesized that the induction of fatty acid ω-hydroxylation activity by peroxisome proliferating compounds, is an early, and possible obligatory, step in the induction of peroxisome proliferation (Sharma *et al.*, 1988; Dirven *et al.*, 1992). Since there is evidence that the ω-hydroxylation of both MEHP and

fatty acids is catalyzed by the same P450 enzymes (Albro *et al.*, 1984), the formation of metabolite V might be indicative for induction of these fatty acid ω-hydroxylating enzymes. This view is supported by the findings that, in rats treated in vivo or in vitro with DEHP, the metabolism is shifted to metabolites formed by ω-hydroxylation reactions (Lhuguenot *et al.*, 1985). However, when tested in primary cultures of rat hepatocytes, no induction of peroxisome proliferation was found after treatment with metabolite V (Elcombe and Mitchell, 1986).

ad 3) Quantification of metabolite V in urine samples is somewhat favoured, since this metabolite appears in the latter part of the chromatogram free of interfering compounds, resulting in a baseline separation and therefore in a low detection limit and a low coefficient of variation.

On the basis of the arguments mentioned above, we recommend to study all four metabolites of DEHP when the internal dose to DEHP has to be established.

Acknowledgments
We thank Dr. A.J. van Zeist, Dr. H.H.L. Nefkens and Prof. Dr. B. Zwanenburg of the Department of Organic Chemistry of the University of Nijmegen for synthesizing the metabolites. We thank Rob Anzion and Paul Sessink for practical advice using the GC-MS equipment. We thank Prof. Dr. P.Th. Henderson for critical reading of the manuscript.

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Chapter 8

Metabolites of the plasticizer di(2-ethylhexyl)phthalate in urine samples of workers in polyvinylchloride processing industries.

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Int Arch Occ Env Hea, in press

Abstract

Little is known about occupational exposure to the plasticizer di(2-ethylhexyl)phthalate (CAS number 117-81-7), a compound widely used in polyvinylchloride plastics. We have studied the uptake of DEHP in workers by determining the concentration of four metabolites of DEHP in urine samples, i.e. mono(2-ethylhexyl)phthalate (MEHP), mono(5-carboxy-2-ethyl)pentylphthalate, mono(2-ethyl-5-oxohexyl)phthalate and mono(2-ethyl-5-hydroxyhexyl)phthalate. In addition DEHP concentration in the air was determined by personal air sampling. Nine workers in a PVC boot factory exposed to maximal 1.2 mg/m^3 DEHP showed an increase in urinary concentration of all four metabolites over the workshift. These results were found on the first day of the workweek as well as at the last day of the workweek. With the exception of MEHP the increase in concentration of the metabolites during a workday was statistically significant. Six workers from a PVC cable factory exposed to maximal 1.2 mg/m^3 DEHP showed a 1-4 fold increase in the concentration of the four metabolites over the workshift, but these increases were not statistically significant.

These results indicate that measurement of DEHP metabolites in urine samples may have utility for monitoring the occupational exposure to DEHP.

Introduction

Di(2-ethylhexyl)phthalate (DEHP; CAS number 117-81-7) is a odourless oily liquid. The compound is used in large quantities to make PVC plastics more flexible. Some synonyms of DEHP are dioctylphthalate, bis(2-ethyl-

hexyl)phthalate, hexaplas DOP, bisoflex DOP and flexol DOP (Burg, 1988). In 1985 approximately 24000 tonnes plasticizer were used in the Netherlands and 49% of this amount was DEHP. PVC plasticized with DEHP was mainly used in the production of wrapping materials, tubes, shoe soles and floor covering (de Groot, 1987).

The toxicological effects of DEHP have been reviewed recently (Turnbull and Rodricks, 1985; Burg, 1988; Albro, 1986; Stott, 1988). The most pronounced toxic effects of DEHP are the hepatocarcinogenic properties of this chemical in rats and mice. The International Agency on Research of Cancer has classified DEHP as a possible human carcinogen (IARC, 1987). Since DEHP is not genotoxic, mechanisms leading to carcinogenicity are not very well understood. DEHP might have promotional activity on tumor formation and/or might act as an epigenetic carcinogen (for review: Conway et al., 1989). This latter activity is probably associated with a biochemical disturbance in the liver called peroxisome proliferation (Rao and Reddy, 1987). Identification of the hazard of DEHP to man is complicated by the fact that man and primates are less sensitive for peroxisome proliferation than rats and mice (for review: Rodricks and Turnbull, 1987; Stott, 1988). Mechanisms behind these species differences in sensitivity for xenobiotic induced peroxisome proliferation are not known. However, in a recent workshop on the health risks by DEHP the participants concluded that DEHP possibly induce peroxisome proliferation in man and therefore is possibly carcinogenic to man (Schulz, 1989).

The American Conference of Governmental Industrial Hygienists has classified DEHP as a substance of low toxicity by all routes of exposure (ACGIH, 1986). Many countries have set a threshold exposure level for DEHP at the workplace at 5 mg/m^3 (ILO, 1989). In establishing this threshold exposure level no reference was made to carcinogenic effects of DEHP.

Absorption of DEHP during occupational exposure is most likely to occur by inhalation since the dermal absorption of DEHP in rats is low (Elsisi et al., 1989). DEHP has a low vapor pressure at ambient temperatures and a moderate vapor pressure at enhanced temperatures (0.04 mbar at 120 °C; 4 mbar at 200 °C; data obtained from product information on hexaplas DOP from Imperial Chemical Industries PLC, Cleveland, England). These data suggest that exposure to DEHP by inhalation can be expected at high process temperatures

Little is known about occupational exposure to DEHP. In a recent study in 9 PVC processing plants DEHP concentrations in the air were determined ranging from 0.02 to 0.5 mg DEHP/m^3 (Vainiotalo and Pfäffli, 1990).

No increase in the number of chromosome abberations were found in 10 workers exposed for 10-30 years to DEHP (Thiess and Fleig, 1978). In two studies the excretion of phthalic acid by workers in PVC industries was determined as marker for exposure (Liss et al., 1986, Nielsen et al., 1985). Determination of the phthalic acid concentration in urine samples is however not an agent-specific method for biological monitoring of DEHP. It still is questionable whether biological monitoring of DEHP provides additional

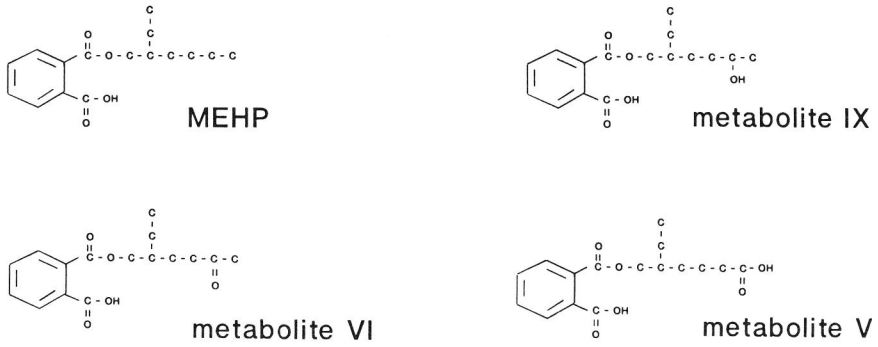

Figure 1 - Structures of the metabolites of DEHP determined in this study. Numbering of the metabolites is according to Albro *et al.*, 1986.

information about the exposure of workers. We have recently developed a method for the determination of four metabolites of DEHP (figure 1) in urine samples (Dirven et al., 1993; see accompanying paper). This report is intended to investigate the utility of measuring the concentrations of four metabolites of DEHP in urine samples as an index of occupational exposure to DEHP.

Methods

Chemicals

DEHP (99% pure) and carbon disulfide were obtained from Merck, Darmstadt, Germany. Tricosane was obtained from Aldrich Chem. Co. Milwaukee, WI, USA. All other chemicals were of the highest purity obtainable.

Field measurements

Studies were performed in 2 plants: a PVC boot factory and a cable factory. Personal data of the workers involved are shown in table 1.

Boot factory. In this factory hexaplas DOP was added to recycled PVC together with coloring materials, dibutylphthalate and other additives. Every week approximately 30 tonnes of DEHP were used. The mixture was warmed and mixed in a semi-open 'papenheimer' at 160 °C. After 30 minutes the liquid PVC paste was transported to an extruder (working temperature 170 °C). Granules were cooled in water and stored for further processing. Production occurred in a batch wise manner.

In each week three workers in a shift were monitored (working hours 6.00 a.m.-2.00 p.m.). Measurements were performed for 3 consecutive weeks so nine workers were involved in this study. When the men started at monday 6.00 a.m. no (occupational) DEHP exposition had occurred in

Table 1 - Personal data of the male workers involved in the biological monitoring study.

Subject	function	age (a)	body wt (kg)	smoking[1]	alcohol consumption[2]
Boot factory					
1	operator	41	100	-	5
2	operator	59	78	-	4
3	operator	24	66	-	50
4	operator	55	105	-	10
5	operator	33	110	-	6
6	operator	31	92	10-20	14
7	operator	59	84	-	2
8	operator	30	78	<10	8
9	operator	62	96	-	6
Cable factory					
10	extruder	28	64	10-20	12
11	granulating	23	104	10-20	4
12	extruder	57	74	-	0
13	granulating	39	83	10-20	10
14	extruder	?	65	20	15
15	extruder	43	82	-	0

[1] number of cigarettes/day.
[2] number glasses (beer,wine,spirit)/week.

the last 48 h. Urine samples were collected at the first and the last day of the 5-day workweek, before work and after work. Workers 1-3 were studied in the first week, workers 4-6 were studied in the second week and workers 7-9 in the third week. At the first and last day of the workweek personal air sampling was employed on all three workers in a shift (two times 2 h for each worker on the first day and once 2 h for each worker on the last day).

Cable factory. In this factory a PVC paste was produced from DEHP, PVC granules and other additives in a closed, batchwise process (temperature 200 °C). Workers employed in the mixing and granulating processes stayed most of the time in a noise-closed cabin. In a semi-open process the produced PVC granules were used for the coating of electricity cables (process temperature 200 °C). The production and use of DEHP containing

PVC was alternated with non-DEHP containing polyethylene. When we started our studies 12500 kg DEHP/week had been used for at least 6 weeks.

Five men worked in each shift. When the men started at the first day in the workweek no (occupational) DEHP exposure had occurred in the last 48 h. Six workers in two shifts (6.00 am-2.00 pm, 2.00 pm-10.00 pm) collected urine samples at the first and fourth day of the workweek, before work and after work. At the first day of the workweek personal air sampling was employed on all workers in a shift (two times 2 h for each worker).

Air sampling and analysis

DEHP concentrations in the ambient air were determined by personal sampling as a 2h Time Weighted Average (TWA) concentration, according to method No. S40 of the U.S. National Institute for Occupational Safety and Health (NIOSH, 1977). Flow rate was 1 L/min and was checked before and after each air sample. The DEHP on the filters (mixed cellulose ester membrane filters, 37 mm, 0.8 μM, Schleicher and Schuell GmbH, Dassel, Germany) was extracted in carbon disulfide after the addition of the internal standard tricosane. 1 μl of the extract was injected on a Carlo Erba instrumentiazone, FTV series 4300 gas chromatograph with a flame-ionization detector. Separation was achieved on a 25 m CP-SIL-5 column (Chrompack, Middelburg, The Netherlands) with 0.22 mm i.d. and 0.20 μm film thickness. Helium was used as carrier gas. The 'on column' mode was used. Injector and detector temperature was 300 °C. Initial oven temperature was 75 °C. After 1 min the temperature rose to 250 °C (15 °C/min) and was hold for 7 min. Retention time for tricosane was 12.05 min and for DEHP 13.7 min. Peak areas were compared with a calibration curve of DEHP in carbon disulfide. The detection limit was 1 μg/filter (= 8.3 μg/m^3).

In control experiments, filters were spiked with 30 μg DEHP in carbon disulfide. The filters were dried at room temperature and used as described above to test the stripping from filters during sampling. No loss of DEHP was found. With the analysis of each series of filters, a filter spiked with DEHP and a blank filter were analysed to test for interferences. On the blank filters no DEHP was detected. The recovery of DEHP from spiked filters was > 97%.

Urine sampling and analysis

All urine samples were stored in glass containers. Urine samples were frozen at -20 °C immediately after the collection.

The concentration of four DEHP metabolites was determined with GC-MS as described in detail in an accompanying paper (Dirven *et al.*, 1993). Detection limits for the four metabolites were 25 μg/L for MEHP, 25 μg/L for metabolite IX, 13 μg/L for metabolite VI and 16 μg/L for metabolite V, and the coefficient of variation ranged from 6-16 %. When concentrations of the metabolites were below the detection limit, a value of 2/3 of the detection limit was used for further calculations.

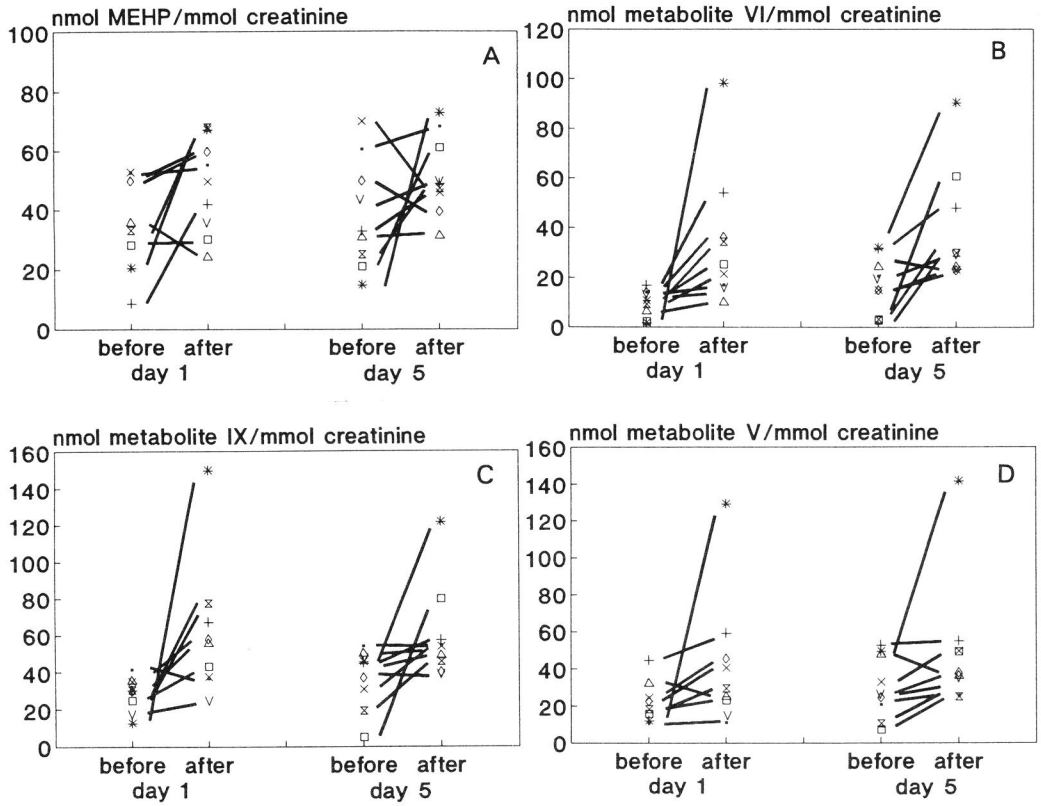

Figure 2 - Concentration of four metabolites of DEHP (A = MEHP, B = metabolite VI, C = Metabolite IX and D = metabolite V) in urine samples of 9 workers in a boot factory.

Urinary creatinine was determined by an automated technique based on the reaction of creatinine to alkaline picrate.

Results

Urinary excretion of DEHP metabolites.

Since the halflife for DEHP is approximately 12 h (Schmid and Schlatter, 1985) we assumed that DEHP absorption during a 8 h workday could be demonstrated by comparing the concentration of the DEHP metabolites in urine samples collected at the end of a 8 h work period with the concentration of the DEHP metabolites in urine samples collected before work started. In addition, we expected that due to the short halflife of DEHP, urine samples collected after a period of more than 48 h without occupational exposure to DEHP could be considered as blank samples.

Table 2 - Median values of the concentrations of four DEHP metabolites in urine samples collected from 9 workers in a boot factory. Within parenthesis the range of the values is expressed.
All values are expressed as nmol/mmol creatinine.

	day 1		day 5	
	before work	after work	before work	after work
MEHP	35.6 (8.6-52.6)	49.5 (24.1-67.9) $p=0.29^1$	32.7. (14.5-69.8) $p=0.29^2$	48.9 (31.2-72.8) $p=0.20^1$
Met VI	10.5 (1.5-17.4)	24.9 (9.9-94.1) $p=0.01^1$	17.1 (2.3-31.8) $p=0.07^2$	24.2 (20.4-90.3) $p=0.01^1$
Met IX	30.2 (12.4-41.2)	57.9 (24.7-149.6) $p=0.02^1$	44.5 (4.7-53.9) $p=0.21^2$	54.0 (39.8-121.3) $p=0.05^1$
Met V	24.4 (10.5-44.6)	29.3 (10.7-129.2) $p=0.05^1$	24.6 (6.94-52.7) $p=0.08^2$	37.9 (25.0-141.4) $p=0.03^1$

[1] = the metabolite concentration in the after work sample was compared with the metabolite concentration in the urine sample collected before work by means of the Wilcoxon test for paired samples, two tailed.
[2] = the metabolite concentration in the urine samples collected at day 5 before work was compared with the metabolite concentration in the urine samples collected at day 1 before work by means of the Wilcoxon test for paired samples, two tailed.

Boot factory. The concentrations of the DEHP metabolites determined in urine samples of 9 workers are represented in figure 2 and table 2. On day 1 as well as on day 5, the concentration of metabolite VI, IX and V was 1.2-2.3 fold increased in urine samples collected at the end of the workday compared to urine samples collected at the start of the workday. The increase was statistically significant. Median concentration of all four metabolites in the prework samples on day 1 and day 5 were equal.

Cable factory. Urine samples from 6 different men in the cable factory were collected at day 1 and day 4 of the workweek at the start and at the end of the workday. In all urine samples collected prework at the start of the workweek all four DEHP metabolites were detected (table 3). Median

Table 3 - Median values of the concentrations of four DEHP metabolites in urine samples collected from 6 workers in a cable factory. Within parenthesis the range of the values is expressed.
All values are expressed as nmol/mmol creatinine.

	day 1		day 4	
	before work	after work	before work	after work
MEHP	15.1 (4.9-25.5)	17.9 (3.6-61.4) p=0.43[1]	16.2 (4.0-26.1) p=0.56[2]	34.5 (4.9-75.9) p=0.15[1]
Met VI	4.0 (3.0-5.3)	11.0 (6.3-45.9) p=0.03[1]	3.7 (1.8-20.5) p=0.78[2]	16.6 (2.2-37.5) p=0.14[1]
Met IX	11.3 (7.3-16.9)	17.8 (7.3-108.6) p=0.14[1]	13.9 (3.7-29.7) p=0.68[2]	25.9 (17.9-75.5) p=0.06[1]
Met V	5.2 (3.1-10.8)	11.0 (4.3-64.9) p=0.16[1]	6.1 (2.2-25.8) p=0.31[2]	15.1 (7.1-37.9) p=0.16[1]

[1] = the metabolite concentration in the after work sample was compared with the metabolite concentration in the urine sample collected before work by means of the Wilcoxon test for paired samples, two tailed.
[2] = the metabolite concentration in the urine samples collected at day 4 before work was compared with the metabolite concentration in the urine samples collected at day 1 before work by means of the Wilcoxon test for paired samples, two tailed.

concentration of the metabolites in the urine samples collected after work was 1.2-4.5 fold increased compared to median values in the urine samples collected before work, but these increases were not statistically significant. The median concentration of the four metabolites in prework samples during the 2 days in the workweek was comparable.

Personal air sampling

In table 4 the mean concentrations of DEHP in the ambient air in the 2 plants as determined by 2h TWA personal air samples, are shown. Within each plant exposure levels were determined by men working at the mixing as well as by men working at the extruding processes. Within each plant a

Table 4 - Mean concentrations of DEHP in the ambient air as determined by personal air sampling. All values are expressed in $\mu g/m^3$.
n = the numbers of samples analysed and the values within parenthesis is the range.

plant	mixing	extruder
boot	261 n = 16 (100-1214)	120 n = 11 (48-278)
cable	180 n = 8 (9-809)	239 n = 13 (10-1266)

wide variation in exposure levels was found even during a day, probably due to the batch wise production processes. Between the plants the average DEHP concentrations in the ambient air were in the same range. The highest DEHP concentration found was 1.26 mg/m^3.

Discussion

An uptake of DEHP during work was demonstrated in a PVC processing plant. For nine workers in a boot factory median values for three DEHP metabolites, i.e. metabolite VI, IX and V were higher in urine samples collected at the end of the workday compared to urine samples collected at the start of the workday. However, the increased postshift urinary DEHP metabolite concentrations of workers in a cable factory were not statistically significant.

The concentration of the metabolites in urine samples of workers collected prework at the start of the workweek were higher than in a pooled urine sample of non-occupationally exposed persons used for constructing calibration curves (Dirven *et al.*, 1993), although no occupational exposure to DEHP had occurred for at least 48 h (4 times the halflife). This might indicate that the halflife of DEHP is much larger than 12h (Schmid and Schlatter, 1985).

The concentration of the DEHP metabolites in the urine samples at the start of the workweek were higher for workers in the boot factory than for the workers in the cable factory. The difference in excretion of DEHP metabolites between the workers in the boot- and cable factory might be caused by the fact that in the boot factory the weekly amount of DEHP plasticized PVC produced is constant, while in the cable factory the

production is more variable since the production of PVC is interchanged with the production of polyethylene. Continuous exposure to DEHP might result in higher levels of DEHP (metabolites) in slow releasing compartments, like adipose tissue. The concentration of the DEHP metabolites in urine samples collected prework at the start and at the end of the workweek however, were in the same order of magnitude. This indicates that during a workweek no observable accumulation of DEHP did occur.

The personal air sampling data of the 2 plants show that the occupational exposure to DEHP is in the $\mu g/m^3$-range. Our data are in the same order of magnitude as previous published exposure levels (Vainiotalo and Pfäffli, 1990; Thiess and Fleig, 1978). No apparent relation was found between the concentration of DEHP in the air and the metabolite concentration in the urine. However, since we have only monitored workers for 2-4 h during a 8 h workperiod and the intra-day variation of DEHP concentrations was large, our dataset on the DEHP concentration in air is too limited to find such a correlation. In a forthcoming study it is necessary to monitor the air concentrations for 8 h.

The lack of a relation between DEHP air levels and the levels of DEHP metabolites in urine samples might also be caused by the time schedule we have used for collection of urine samples. We have collected urine samples after a 8 h workperiod. It remains to be elucidated whether the concentrations of the metabolites are higher in urine samples collected at a later point in time.

With our limited dataset we tried to estimate whether the increase in excretion of urinary DEHP metabolites during a workday was in the same order of magnitude as the DEHP uptake by inhalation. A worse-case approach was followed. For this calculation we used the data collected at the first day of the workweek of all 15 workers in both the boot- and the cable plant. The absorbed dose was calculated with equation 1 and the excreted dose was estimated with equation 2.

Equation 1:
absorbed dose = C_l * BV * t * r, in which:
C_l is the DEHP concentration in the air. In our calculations we have used the median value of all air samples collected on the first day of the workweek in the boot- and cable factory (= 137 $\mu g/m^3$);
BV is the breathing volume, and was assumed to be 28.6 L/min (moderate workload, based on literature data from Diem and Lenther, 1976);
t is the exposure time, and was 8 h (= 480 min);
r is the retention of DEHP in the lungs, and was assumed to be 100%.

Equation 2:
The excreted amount of DEHP metabolites in urine = (C_{mehp} + $C_{met\ IX}$ + $C_{met\ VI}$ + $C_{met\ V}$) * daily creatinine excretion, in which:
C_{mehp}, $C_{met\ IX}$, $C_{met\ VI}$ and $C_{met\ V}$ is the median value of the mentioned metabolite after work minus the median value of the mentioned metabolite before work;

daily creatinine = the creatinine excretion in 24 h. According to the literature this excretion is 16 mmol/24 h (Diem and Lenther, 1976).

According to equation 1 the maximum daily uptake of DEHP is 1.9 mg. Equation 2 shows that the total excretion of DEHP metabolites in urine is 0.49 mg. These values are in the same order of magnitude which indicate the value of biological monitoring studies to assess exposure to DEHP. The remaining DEHP might be excreted via bile and faeces. In studies with humans and with Cynomolgus monkeys (Schmid and Schlatter (1986); Astill, 1989) it was found that of an oral dose of DEHP 10-50 % of the dose was recovered in urine. According to this calculation the daily uptake of DEHP during a 8 h workday is estimated to be 1.9 mg (= 27 μg/kg per day). Lacking a clear understanding of the mechanism of carcinogenicity of DEHP, human risk of cancer is based on rodent data. Application of various low dose extrapolation models lead to the prediction that the daily dose resulting in a lifetime risk on hepatocellular tumors of no more than 1 in 1 million would be between 1.5 and 791 μg/kg per day, with the most likely figure being 116 μg/kg per day (Turnbull and Rodricks, 1985). In this risk assessment humans and rats/mice were assumed to be at the same risk at the same daily dose level of DEHP and it was assumed that DEHP's mechanism of carcinogenicity in rodents most likely involves its ability to induce peroxisome proliferation. There is however a substantial doubt if these assumptions are right. Our own studies have shown that primates are at least 30-fold less sensitive for peroxisome proliferation than rats (Dirven *et al.*, unpublished results).

We therefore conclude that occupational exposure to DEHP at concentrations as found in our study is not a major risk factor for developing hepatocellular tumors.

Acknowledgments

We gratefully acknowledge the fruitful co-operation with Mark Fleuren, Ruud Duine and Bart Roberti in performing the studies in the plants.

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Chapter 9

General discussion and summary

Few compounds have been so thoroughly tested with respect to their potential toxicity as some of the peroxisome proliferators, but still the hazard of these compounds for man is hard to assess. As described in chapter 1 the carcinogenicity of DEHP and other peroxisome proliferating compounds might be due to a combination of initiation of DNA damage by oxidative stress and propagation of this damage by proliferative responses. Biological mechanisms leading to these responses, as well as the biological basis for possible species differences in these mechanisms are unknown. The incidence of primary liver tumors is low in the USA and West-Europe (Simonetti *et al.*, 1991) so it is difficult to perform epidemiological studies aiming at identifying human risks of these compounds. A reliable risk assessment of DEHP and other peroxisome proliferating compounds will be fascilitated by the identification of the biological mechanisms of action.

9.1 - On the mechanism of peroxisome proliferation

Peroxisome proliferating compounds can cause a variety of effects in rats/mice. In chapter 2 experiments are described in which effects of DEHP on the liver were compared with effects found in serum. The relationship between the observed changes in serum parameters with the effects found in the liver was not clear. Therefore we have focussed our further studies on effects of DEHP in the liver.

A number of hepatic enzyme activities have been reported to be disturbed by peroxisome proliferating compounds. In 1988 Sharma *et al.* proposed a mechanistic inter-relationship between induction of peroxisomal palmitoyl-CoA oxidase activity and cytochrome P450 4A1 related lauric acid ω-hydroxylation activity. In order to determine lauric acid ω-hydroxylation activity, we developed a new assay based on the derivatization of lauric acid and its two metabolites with a fluorescent label. After derivatization metabolites were separated using reversed-phase HPLC (chapter 3).

Applying this assay in a number of experiments (described in chapter 3,4, 5 and 6) we have shown that the described method is sensitive and useful for determining lauric acid ω-hydroxylation activity in hepatic microsomal fractions and whole liver homogenates of different species. In the experiments described in Chapter 4 we have explored the proposed mechanistic inter-rela-

tionship between cytochrome P450 4A1 induction and peroxisome proliferation. Rats were treated in vivo with different doses of DEHP and a dose-dependent induction of both lauric acid ω-hydroxylation and peroxisomal palmitoyl-CoA oxidase activities was found. These results were confirmed after administration of metabolites of DEHP like MEHP, 2-ethylhexanol and 2-ethylhexanoic acid and other inducers of peroxisomal ß-oxidation activity.

In addition a number of other studies demonstrated:

1. co-induction of peroxisomal ß-oxidation enzyme and microsomal lauric acid ω-hydroxylation activities in liver of rats (Lake *et al.*, 1984; Bacher *et al.*, 1988; Makowska *et al.*, 1990; Chinje *et al.*, 1991; Bell *et al.*, 1991a and 1991b; Kozaku *et al.*, 1991; Bell *et al.*, 1992; Gibson, 1992),
2. induction of both enzymes in the same part of rat liver (Bell *et al.*, 1991a),
3. appearance of mRNA encoding cytochrome P450 4A1 earlier than RNA encoding peroxisomal ß-oxidation enzymes after treatment of rats with peroxisome proliferating compounds (Milton *et al.*, 1990; Bieri *et al.*, 1991: Bell *et al.*, 1991a and 1991b),
4. prevention of peroxisome proliferation by inhibition of lauric acid ω-hydroxylation activity (Ortiz de Mantellano *et al.*, 1992).

These results and the results obtained in our own experiments provide further evidence for a close linkage between xenobiotic induction of cytochrome P450 4A1 and peroxisome proliferation in rats. Since a Peroxisome Proliferator Activated Receptor (PPAR) has been described to be involved in the transcription of acyl-CoA oxidase genes (Tugwood *et al.*, 1992), it is tempting to speculate about the role of PPAR in the induction of cytochrome P450 4A1. We suppose that PPAR is also involved in the transcription of cytochrome P450 4 genes.

9.2 - Species differences in peroxisome proliferation

Since a mechanistic inter-relationship between induction of cytochrome P450 4A1 and peroxisome proliferation may exist we wondered whether species differences in peroxisome proliferation are reflected by species differences in cytochrome P450 4A1 induction. As a first step in this approach we studied whether cytochrome P450 4A1 enzymes are present in human liver (Chapter 5). In microsomal liver fractions of 12 kidney transplant donors we detected pronounced lauric acid ω-hydroxylation and (ω-1)-hydroxylation activities. In contrast to rats, lauric acid ω-hydroxylation activity was two-fold higher than (ω-1)-hydroxylation activity. Using an antibody raised against rat hepatic cytochrome P450 4A1, we were able to identify proteins in the microsomal fractions of human liver which were immunochemically related to rat cytochrome P450 4A1. However, no correlation was observed between the amount of protein present and lauric acid ω-hydroxylation activity. We concluded that cytochrome P450 4A proteins are present in human liver but that they have different physicochemical properties when

compared to rat cytochrome P450 4A1. Final identification of human cytochrome P450 4A1 must await the sequencing of human cytochrome P450 4 genes.

Species differences in induction of cytochrome P450 4A1 and peroxisomal palmitoyl-CoA oxidase activities have been studied after treatment of primary hepatocyte cultures of rat, rabbit, guinea pig and male and female monkey with a metabolite of DEHP, i.e. mono-(2-ethylhexyl)phthalate (MEHP) (Chapter 6). In hepatocytes of rat a 50% increase in peroxisomal palmitoyl-CoA oxidase activity was found at a MEHP concentration of 15 μM. In hepatocytes of rabbit, guinea pig and monkey a 50% increase in palmitoyl-CoA oxidase activity was observed at a concentration of 410-480 μM MEHP. These results indicate that the latter three species are at least 30-fold less sensitive to induction of peroxisome proliferation than rats. The induction of peroxisomal palmitoyl-CoA oxidase activity in rats is accompanied by an induction of lauric acid ω-hydroxylation activity. This induction was not observed in guinea pig, rabbit and monkey. Similar findings have been reported by Makowska *et al.* (1991) after treatment of marmoset monkeys with a peroxisome proliferating compound. It seems that the mechanistic inter-relationship between induction of peroxisomal palmitoyl-CoA oxidase activity and microsomal lauric acid ω-hydroxylation activity as found in rat hepatocytes is not observed in the other three species tested.

Treatment of primary hepatocyte cultures with MEHP caused a decrease in hepatic triglyceride levels in monkey hepatocytes and an increase in hepatic triglyceride levels in hepatocytes of rat and guinea pig. Similar results have been found after in vivo treatment of these species with peroxisome proliferating compounds (Watanabe *et al.*, 1989), which demonstrates the value of primary hepatocyte cultures in studying the effects of peroxisome proliferating compounds. The different trends in triglyceride levels observed among the species after treatment with peroxisome proliferating compounds, suggest that more basic research on species differences in hepatic fatty acid metabolism and the partitioning of fatty acids between ß-oxidation and esterification, is necessary to explain species differences in peroxisome proliferation.

Species differences in peroxisome proliferation were not the result of species differences in the formation of specific metabolites, since the pattern of oxidized MEHP metabolites in media of hepatocytes from all species was comparable (chapter 6).

9.3 - Human exposure to DEHP

In view of the assessment and management of health risks of DEHP in humans it is necessary not only to study the toxicity of DEHP but also to make a reliable estimate of the internal exposure to DEHP. In Chapter 7 a method is described for biological monitoring of DEHP based on the determination of four metabolites of DEHP in urine samples. Included in the method is

an enzymatic hydrolysis, an ether extraction, a derivatization-reaction and analysis by GC-MS. Exposure of DEHP in 15 workers in DEHP using industries was studied by environmental- and biological monitoring (Chapter 8). By means of biological monitoring an uptake of DEHP during a 8 h workday was demonstrated. Total uptake of DEHP during a 8 h workday was estimated to be 1.9 mg/day (= 27 μg DEHP/kg.day).

9.4 - Towards a risk-assessment of DEHP

Only a few attempts were made to assess the human carcinogenic risk of DEHP, although there is a growing need for these assessments by regulatory authorities. It is beyond the scope of this thesis to provide a risk-assessment on DEHP, although the results of our studies can be useful for such an assessment.

Current models designated as non-threshold models used in risk assessment of genotoxic carcinogens assume that these compounds have mutagenic potency and that one or two mutations in a single cell are sufficient to initiate malignancy (Day and Brown, 1980). The multistage model is the model most widely used by regulatory agencies. The multistage model assumes additivity of background, which leads to low dose linearity. The Mantel-Bryan probit model assumes that the mechanism of the carcinogen is different from that of whatever causes the background incidence of tumors. Hence, risk may not be linear at low dose levels (Mantel *et al.*, 1975).

Turnbull and Rodricks (1985) have assessed possible carcinogenic risk of DEHP to humans, using four non-threshold models (i.e. multistage model using applied doses of DEHP, multistage model using surrogate data for target-site doses, Mantel-Bryan probit model using applied doses of DEHP and Mantel-Bryan model using surrogate data for target-site doses). Results are reviewed in table 1. In these assessments, data from the NTP bioassay were used and humans and rats/mice were assumed to be at the same risk at the same daily dose level of DEHP. It was stated that the mechanism of carcinogenicity by DEHP most likely involves peroxisome proliferation. Application of the various extrapolation models lead to the prediction that the daily dose resulting in a lifetime risk of no more than 1 in 1 million would be between 1.5 and 791 μg/kg per day (see table 1) . The authors concluded that the multistage model with the surrogate target site dose adjustment was the most appropriate model and that the most likely figure was 116 μg/kg per day. Surrogate target-site doses were calculated from the production of metabolite I (see chapter 7) since the authors assumed that production of this metabolite is an indicator for peroxisomal activity. Since our studies demonstrate that rabbits and hamsters, i.e. species not very sensitive for peroxisome proliferation do produce this metabolite at the same rate as rats (chapter 6), we assume that this concept needs reconsideration.

For genotoxic compounds, the presumption is made that there is no threshold-effect. For non-genotoxic chemicals it seems likely that a no-effect

Table 1 - Estimates of safe levels of exposure to DEHP (from: Turnbull and Rodricks, 1985)

Extrapolation model	Virtually safe dose (risk < 10^{-6}) (μg/kg per day)	
	Maximum likelihood estimate	Lower 95th percentile estimate
Multistage with applied dose	2.2	1.5
Multistage with surrogate dose	116	86.3
Mantel-Bryan with applied dose		11.9
Mantel-Bryan with surrogate dose		791

threshold does exist. The threshold model (application of a safety or uncertainty factor to a no-observed-effect level (NOEL)) is normally used to predict safe levels of exposure for noncarcinogenic effects (National Academy of Sciences, 1977). Generally, this method has also been accepted to predict exposure levels for non-genotoxic carcinogens. Turnbull and Rodricks (1985) have also assessed possible carcinogenic risks of DEHP to humans applying a threshold model. In the case of DEHP such a threshold might be considered as the concentration of DEHP at which no induction of peroxisome proliferation is found. Turnbull and Rodricks (1985) identified a NOEL in rats for increases in peroxisomal ß-oxidation activity of 70-106 mg/kg per day. These levels are in the same range as we have found in our studies (chapter 4). In their assessment however they used a NOEL for all effects and applying a safety factor of 1000 they calculated a chronic acceptable daily intake of between 70 to less than 3,5 μg/kg per day, with the most likely value being 11 μg/kg per day. This value is lower than the estimated total uptake of DEHP during a 8 h workday (= 27 μg DEHP/kg per day, chapter 8).

A no-effect level for peroxisome proliferation is strongly species dependent as indicated by our data on species sensitivity for peroxisome proliferation (chapter 6). However, with the current knowledge that proliferative responses induced by peroxisome proliferating compounds might be important in the carcinogenic process as well (chapter 1.4.2), there is a urgent need for more information on these responses in primates/man.

An adequate risk-assessment for DEHP or other peroxisome proliferating compounds seems hard to achieve. To our opinion the threshold model is at this moment the best model to predict safe exposure levels to DEHP. If

peroxisome proliferation is indeed an intermediate step in the development of cancer by DEHP, the finding that primates are at least 30-fold less sensitive for peroxisome proliferation than rats, should be used in establishing a threshold. Present occupational exposure to DEHP seems to be low and it is unlikely that at this level of exposure a high risk for developing livertumors will be found.

The best option for an adequate risk assessment of DEHP is a risk assessment based on understanding the biological mechanisms leading to tumor formation and knowledge about the biological basis of species differences in these mechanisms. The discovery of PPAR might provide an unique insight in the mechanism leading to peroxisome proliferation. Green (1992a) proposed that PPAR might also mediate changes in key genes for growth and differentiation like oncogenes and/or growth factors and their receptors. This might offer opportunities for receptor-mediated models for risk assessment (Green, 1990; Cohen and Ellwein, 1991; Green, 1992a and 1992b). A threshold might be included in this model since it is likely that it is possible to determine concentrations below which no receptor activation will occur. A further implication of this model is that the receptor acts as a trigger for the biological effect of the chemical and therefore the abundance of the receptor in species and/or tissues is an important factor.

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Samenvatting

Mensen komen binnen en buiten hun werk in contact met chemische stoffen. Een aantal van deze stoffen kan kanker veroorzaken. Deze kankerverwekkende stoffen kunnen opgespoord worden door middel van testen, waarin bestudeerd wordt of chemicaliën het erfelijk materiaal van cellen beschadigen. Er zijn echter verbindingen die niet het DNA van cellen beschadigen, maar die toch kanker kunnen veroorzaken. In dit proefschrift is één zo'n stof bestudeerd, namelijk de weekmaker di-(2-ethylhexyl)ftalaat oftewel DEHP. Deze stof wordt aan polyvinylchloride plastics toegevoegd om het plastic flexibel te maken. PVC plastics met daarin DEHP verwerkt worden o.a. gebruikt in regenpakken, vinyl vloerbedekking, laarzen, elektriciteitskabels, medische apparatuur en verpakkingsmaterialen voor voedsel. Het lijkt aannemelijk om te stellen dat iedereen elke dag wel in contact komt met een produkt waarin DEHP is verwerkt.

Ratten en muizen die iedere dag met grote hoeveelheden DEHP worden behandeld krijgen leverkanker. Fabrikanten, werknemers en de overheid willen daarom graag weten of DEHP ook bij mensen leverkanker kan veroorzaken. Om deze vraag te kunnen beantwoorden is het noodzakelijk om eerst te bestuderen hoe DEHP kanker veroorzaakt. Op dit moment is dat nog niet bekend. Er zijn twee theorieën over hoe DEHP leverkanker kan veroorzaken. Theorie 1 gaat er van uit dat dit mogelijk iets te maken heeft met het vermogen van DEHP om in levercellen een toename van het aantal peroxisomen te veroorzaken. Peroxisomen zijn net als mitochondriën en andere celonderdelen in iedere cel aanwezig. Met behulp van microscopen zijn peroxisomen zichtbaar te maken in weefsel (zie chapter 1, figure 1). Peroxisomen spelen een belangrijke rol bij het vetzuur metabolisme. Bij de afbraak van vetzuren in de peroxisomen ontstaat waterstofperoxyde, een stof die het DNA kan beschadigen. Doordat DEHP het aantal peroxisomen in levercellen doet toenemen, zou zoveel waterstofperoxyde binnen een levercel ontstaan dat het DNA beschadigd wordt.

Theorie 2 verklaart het kankerverwekkend zijn van DEHP als een gevolg van een toename in het aantal celdelingen in de lever. Welk mechanisme deze toename veroorzaakt is op dit moment nog niet bekend.

In hoofdstuk 1 wordt beschreven dat het aannemelijk lijkt dat juist een combinatie van de effecten zoals beschreven in de twee theorieën leidt tot het optreden van tumoren.

Om de risico's van DEHP voor mensen te voorspellen, is het nodig om te weten of de toename in het aantal peroxisomen en het aantal celdelingen ook bij mensen wordt gevonden na blootstelling aan DEHP. In dit proefschrift is vooral bestudeerd hoe de toename van het aantal peroxisomen tot stand komt en of er soort verschillen bestaan in de gevoeligheid voor dit effect. Daarnaast is bestudeerd hoeveel DEHP mensen precies binnen krijgen als zij werken in fabrieken waar DEHP wordt gebruikt.

Uit de resultaten van de uitgevoerde experimenten blijkt dat de effecten

van DEHP in de lever veel sterker en specifieker zijn dan de effecten van DEHP op enzymen in het bloed (hoofdstuk 2). Het is dus niet mogelijk om via het bloed van mensen te bestuderen of er een toename van het aantal peroxisomen in de lever is.

De toename van het aantal peroxisomen wordt waarschijnlijk veroorzaakt door een verstoring van de vetzuurhuishouding in levercellen. Deze verstoring zou mogelijk een gevolg kunnen zijn van een toename in de activiteit van een enzym dat zorgt voor de hydroxylering van vetzuren. Dit enzym wordt cytochroom P450 4A1 genoemd.

In hoofdstuk 3 wordt een methode beschreven om de activiteit van dit enzym te meten. Nieuw aan deze methode is dat gebruik wordt gemaakt van een chemische reactie waarbij een fluorescerende groep aan de vetzuren wordt gekoppeld, zodat de produkten gevoelig kunnen worden gemeten.

In hoofdstuk 4 worden experimenten beschreven waarbij de relatie tussen een toename in cytochroom P450 4A1 en een toename in peroxisomen wordt bestudeerd. Ratten zijn behandeld met een aantal metabolieten van DEHP en met een aantal verbindingen die mogelijk een peroxisoom proliferatie kunnen veroorzaken. Er blijkt inderdaad een heel nauw verband te zijn tussen de mate waarin het cytochroom P450 4A1 is toegenomen en de toename in het aantal peroxisomen.

In hoofdstuk 5 worden experimenten beschreven waarin bestudeerd wordt of het cytochroom P450 4A1 enzym ook in levers van mensen aanwezig is. Uit de resultaten blijkt dat verwante enzymen inderdaad aanwezig zijn in levers van mensen, maar dat deze enzymen niet helemaal identiek zijn aan de cytochroom P450 4A enzymen, zoals die gevonden worden in de lever van de rat.

Soortverschillen in gevoeligheid voor DEHP zijn onderzocht met behulp van geïsoleerde levercellen die blootgesteld werden aan afbraak produkten van DEHP. Al bij een concentratie van 15 μM MEHP werd een toename in de activiteit van peroxisomale enzymen gevonden. Bij levercellen van konijnen, cavia's en apen waren 30x hogere concentraties MEHP nodig om dit effect te bereiken. Na behandeling met MEHP was de activiteit van het cytochroom P450 4A1 wèl toegenomen in de behandelde levercellen van de rat maar niet in levercellen van de drie andere soorten.

Als levercellen van apen een goed model zijn voor de levercellen van mensen, dan zou dit betekenen dat, in vergelijking met ratten ook bij mensen veel hogere doses DEHP nodig zijn om een toename van het aantal peroxisomen in de lever te krijgen. Als de toename in het aantal peroxisomen op een of andere manier zorgt voor het ontstaan van tumoren, dan betekent dit dat de mens pas bij veel hogere doseringen DEHP levertumoren krijgt dan de rat. Definitieve uitspraken over de risico's van DEHP voor mensen moeten wachten tot het moment dat precies bekend is welke mechanismen leiden tot de toename in het aantal peroxisomen. Met de recente ontdekking van een receptor die betrokken is bij de toename van het aantal peroxisomen lijkt het aannemelijk dat binnenkort meer inzicht in deze mechanismen zal ontstaan. Om de risico's van DEHP voor mensen echt goed te begrijpen, is het echter

ook noodzakelijk om de rol van de toename in celdelingen in het proces van tumorvorming te begrijpen. Nog onbekend is of de toename van celdelingen die door DEHP wordt veroorzaakt ook bij andere diersoorten dan ratten wordt gevonden.

Voor het vaststellen van gezondheidsrisico's van DEHP is het ook belangrijk de blootstelling aan DEHP bij werknemers te onderzoeken. Een nieuwe methode is ontwikkeld om vier afbraakprodukten van DEHP in urinemonsters aan te tonen. Deze methode is beschreven in hoofdstuk 7. In urinemonsters van 15 werknemers, die tijdens het werk met DEHP in contact komen, konden alle vier de afbraak produkten van DEHP aangetoond worden. In urinemonsters die aan het eind van de werkdag waren verzameld waren de hoeveelheden van de afbraakprodukten hoger, dan in urine monsters die aan het begin van de werkdag waren verzameld. Hiermee is aangetoond dat de werknemers lucht met daarin DEHP hebben ingeademd en dat dit DEHP is opgenomen in het lichaam (hoofdstuk 8). De opgenomen DEHP is echter erg laag, zeker als deze wordt vergeleken met de hoeveelheden DEHP die bij ratten en muizen levertumoren veroorzaakt. Als bovendien het optreden van levertumoren een gevolg is van peroxisoom proliferatie, dan moet rekening gehouden worden met het feit dat er zeer sterke soortverschillen zijn in gevoeligheid voor peroxisoom proliferatie.

Op basis van deze argumenten kan geconcludeerd worden dat de beroepsmatige blootstelling aan DEHP zo laag is dat de kans op het krijgen van leverkanker door DEHP waarschijnlijk verwaarloosbaar klein is.

Curriculum vitae

Hubert Dirven is op 11 januari 1962 geboren in Dordrecht. Na het behalen van het Havo diploma (Titus Brandsma College, Dordrecht), heeft hij in 1984 een tweedegraads onderwijsbevoegdheid in de biologie en de scheikunde behaald aan het Mollerinstituut in Tilburg. In datzelfde jaar is hij met de doctoraal studie Biologie aan de Katholieke Universiteit Nijmegen begonnen. Bijvakken in de Toxicologie (Prof. P.Th. Henderson) en Microbiologie (Prof. Dr. Ir. G.D. Vogels) zijn gevolgd. Tijdens het bijvak microbiologie is onderzoek verricht op het Instituut voor Bodemvruchtbaarheid in Haren (Groningen). Binnen het hoofdvak Dierfysiologie (Prof. S.E. Wendelaar Bonga) is o.a. onderzoek verricht bij de afdeling Genetische Toxicologie van MBL-TNO. In december 1987 heeft hij het doctoraalexamen Biologie afgelegd.
Van maart 1988 tot februari 1992 is hij als universitair docent in tijdelijke dienst werkzaam geweest binnen de vakgroep Toxicologie van de Katholieke Universiteit Nijmegen. Naast het uitvoeren van het in dit proefschrift beschreven onderzoek, heeft hij ook het biochemisch toxicologisch onderwijs aan derde- en vierdejaars studenten gezondheidswetenschappen verzorgd.
In het kader van het promotieonderzoek heeft hij een werkbezoek (september - december 1991) gebracht aan het laboratorium van Dr. G.G. Gibson, University of Surrey, Guildford, Engeland. Ook heeft hij een aantal modules van de post-doctorale opleiding toxicologie gevolgd (proefdierkunde, ecotoxicologie, pathobiologie en risico-evaluatie).
Op dit moment is hij werkzaam als onderzoeker binnen de sectie Biologische Toxicologie van het TNO Instituut voor Toxicologie en Voeding in Zeist.

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As co-author

F.J. Jongeneelen, H.A.A.M. Dirven, Ch.-M. Leijdekkers, R.M.E. Brouns, K. Halm, P.Th. Henderson. S-Phenyl-N-acetylcysteine in urine of rats and workers after exposure to benzene. *J Anal Toxicol* **11**: 100-104, 1987.

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F.A.G. Reubsaet, J.H. Veerkamp, H.A.A.M. Dirven, M.L.P. Bruckwilder, T. Hashimoto, J.M.F. Trijbels and L.A.H. Monnens. The effect of di-(2-ethylhexyl)phthalate on fatty acid oxidation and carnitine palmitoyltransferase in various rat tissues. *Biochim Biophys Acta* **1047**: 264-270, 1990.

Tot slot,

Vier jaar lang ben ik bezig geweest om 'geleerde in de weekgemaakte peroxisomen' te worden, zoals mijn collega's mij bij het afscheid hebben gediplomeerd. Vier jaar lang heb ik mogen werken in een vakgroep waar de vrijheid van een promovendus bij de invulling van het onderzoek hoog in het vaandel staat. Een sfeer die ik buitengewoon heb gewaardeerd.

In die vier jaar is er veel veranderd. Naast goed nieuws over de hereniging van West- en Oost-Europa en meer democratie in Latijns Amerika was er ook slecht nieuws over het milieu, over de AIDS epidemie en over tal van andere zaken. Deze en gelukkig ook minder mondiale problemen waren altijd uitgebreide gespreksstof tijdens de koffie- en theepauzes. Het relativeringsvermogen dat uit deze gesprekken sprak, heeft er zeker toe bijgedragen dat ik met veel plezier terug denk aan mijn vier jaar in Nijmegen. Bij deze wil ik dan ook graag iedereen van de vakgroep Toxicologie bedanken voor de prettige samenwerking in de afgelopen vier jaar.

Met het risico dat ik de bijdragen van andere onderbelicht, wil ik graag een aantal mensen in het bijzonder bedanken.

Frans Jongeneelen als eerste omdat wij samen aan de wieg hebben gestaan van het in dit proefschrift beschreven onderzoek. Frans, naast het feit dat je nu co-promotor bent, was jij ook de begeleider van mijn eerste doctoraal stage. Ik heb in die stage en ook daarna veel van je geleerd over het opzetten van onderzoek. Ik heb met heel veel plezier met je samen gewerkt.

Pie Henderson en Jan Noordhoek wil ik bedanken voor de begeleiding van het onderzoek in een wat latere fase. Ik hoop met jullie nog vaak van gedachten te wisselen over onze wederzijdse interesses.

Petra van den Broek en Janny Peters waren belangrijke steunpilaren in dit onderzoek. Jullie inbreng in het onderzoek was zeer waardevol. Het was een genoegen om met jullie te mogen samenwerken.

Abco van Zeist en Gerard Nefkens wil ik bedanken voor de synthese van de DEHP metabolieten. Toen jullie in drie uur klaar speelden waar wij al 3 weken mee bezig waren, heb ik mij voorgenomen om organische syntheses maar aan chemici over te laten. In addition I like to thank Dr. Sjöberg and Dr. Lindgren of the National Board of Health and Welfare, Sweden for their generous gift of metabolite V.

Geert Poelen, Hennie Broekman en Theo van den Ing van afdeling 2 van het Centrale Dieren Laboratorium wil ik graag bedanken voor hun hulp bij het uitvoeren van de dierexperimenten. Ik hoop dat jullie in staat gesteld worden om de hoge graad van dienstverlening, ook aan toekomstige generaties promovendi te kunnen geven.

Frans Reubsaet wil ik bedanken voor het feit dat hij mij wegwijs heeft gemaakt in de peroxisomale biochemie.

De studenten Diny van der Geest, Arno de Bruijn, Ton Arends, Marian Peeters, Erik Nordkamp, Arianne de Lepper en alle niet bij name genoemde MLO-stagiaires wil ik bedanken voor hun bijdrage bij de uitvoe-

ring van dit onderzoek. Misschien is het allerleukste van onderzoek doen wel om samen met anderen naar oplossingen voor problemen te zoeken.

I like to thank Dr. G. Gibson of the University of Surrey, UK for his help and advice in our studies and for the opportunity to work in his laboratory. In addition I like to thank Edwin Chinje for his help during my visit to Guildford.

Wim Mennes en Bas Blaauboer wil ik graag bedanken voor de plezierige samenwerking rond de hepatocyten experimenten. Wilbert Peters voor de samenwerking rond de humane levers.

Niet in de laatste plaats spreek ik mijn speciale waardering uit voor drie mensen, die ook erg belangrijk zijn geweest in de afgelopen jaren. Mijn ouders, omdat zij mij altijd gestimuleerd hebben om datgene te gaan doen wat ik graag wilde doen. Bij deze draag ik dit boekje dan ook op aan mijn ouders. Marianne, twee promotie-onderzoeken binnen één relatie zijn eigenlijk teveel van het goede. Teveel vrije tijd werd opgeofferd aan de 'wetenschap'. Lang niet alle bergwandelingen, die wij wilden gaan maken, zijn ook daadwerkelijk gemaakt. Hopelijk wordt het beter.

Hubert

Amsterdam, 11 -12 - 1992.

Stellingen

1. De bij ratten gevonden correlatie tussen het optreden van peroxisoom proliferatie en van inductie van cytochroom P450 4A enzymen geldt niet voor primaten, konijnen en cavia's.

   Makowska *et al.*, *Arch Toxicol* **65**: 106-113, 1991;
   Dit proefschrift.

2. De conclusie van Mitchell *et al.* dat mono(2-ethyl-5-oxohexyl)ftalaat de metaboliet van di(2-ethylhexyl)ftalaat is, die in hepatocyten van de rat in vitro peroxisoom proliferatie veroorzaakt, is onjuist.

   Mitchell *et al.*, *Toxicol Appl Pharmacol* **80**: 23-32, 1985;
   Dit proefschrift.

3. Hoewel al een grote diversiteit van verbindingen bekend is, die een interactie aangaan met de Peroxisome Proliferator Activated Receptor (PPAR), is het op grond van de ligand specificiteit van dergelijke nucleaire hormoon receptoren te verwachten dat een nog onbekende intermediair de eigenlijke ligand vormt.

   Green *et al.*, *Biochem Pharmacol* **43**: 393-401, 1992;
   Göttlicher *et al.*, *Proc Natl Acad Sci* **89**: 4653-4657, 1992.

4. De stelling dat de mens, na blootstelling aan peroxisoom prolifererende verbindingen een kleinere kans heeft dan ratten/muizen op het krijgen van levertumoren, omdat mensen minder gevoelig zijn voor het optreden van peroxisoom proliferatie, gaat uit van een niet bewezen oorzakelijke relatie tussen peroxisoom proliferatie en carcinogenese.

5. Het classificeren van peroxisoom prolifererende verbindingen als verbindingen met een promotorwerking op "spontaan geinitieerde cellen" moet vooralsnog als wetenschappelijk opgepoetste abacadabra worden aangemerkt.

   Schulte-Hermann *et al.*, *Cancer Res* **43**: 839-844, 1983;
   Kraupp-Grasl *et al.*, *Cancer Res* **51**: 666-671, 1991;
   Cattley *et al.*, *Carcinogenesis* **12**: 469-472, 1991.

6. Het beter gebruik van statistische methoden in de toxicologie kan leiden tot een aanmerkelijke vermindering van het gebruik van proefdieren.

   Zwart *et al.*, *Inhalation Toxicol* **2**: 105-117, 1990.

7. De idee dat de verschillende celtypen van een embryonaal weefsel geïmmortaliseerd kunnen worden tot ontwikkelingstadium specifieke cellijnen onderschat het belang van cel-cel interacties voor het handhaven van dat ontwikkelingstadium.

   Marianne Dingemanse, persoonlijke mededeling.

8. Onderzoek naar en ontwikkeling van geneesmiddelen tegen tropische ziekten mag niet stagneren omdat de farmaceutische wereld vreest dat de geïnvesteerde ontwikkelingskosten niet terugverdiend zullen worden.

9. Verzilting van de bodem door irrigatie is een onderschat milieuprobleem, dat bovendien zeer ernstige gevolgen voor de wereldvoedselvoorziening heeft.

10. Om de rol van glutathion en glutathion S-transferases (GST) bij het optreden van tumor resistentie tegen alkylerende cytostatica te begrijpen, moet bij de bestudering van de door GST gekatalyseerde reacties ook de relatief hoge intracellulaire concentratie van deze enzymen betrokken worden.
Ciaccio *et al.*, *Cancer Comm* **2**: 279-286, 1990;
Bolton *et al.*, *Cancer Res* **51**: 2410-2415, 1991.

11. Het gebruik van de term personeelszaken in (semi-) overheidsdiensten voor de afdeling, die in de industrie vaak human resources management wordt genoemd, wijst op een (fundamenteel?) verschil in inzicht over het standpunt of werknemers wel of niet tot het kapitaal van een organisatie behoren.

12. Het feit dat in het hele wetboek van strafrecht de term slachtoffer nergens vermeld wordt geeft aan dat het justitiële apparaat meer aandacht heeft voor de rechten van de pleger van het misdrijf dan voor de rechten van het slachtoffer.
Wigbold, Vrij Nederland, 5 december 1992.

13. Het feit dat zoveel politici de doctorandus titel voeren maakt dat in bepaalde kringen van de Nederlandse samenleving met argwaan wordt gekeken naar niet-politici die deze titel ook voeren.

14. Het veelvuldig gebruik van grotere lettertypes, bredere kantlijnen en grotere regelafstanden in academische proefschriften doet vrezen dat sinds de invoering van het AIO-stelsel het gezichtsvermogen van de schrijvers sterk is verslechterd.

Stellingen behorend bij het proefschrift 'Biological effects of and exposure to the peroxisome proliferating agent di(2-ethylhexyl)phthalate'.
Hubert Dirven, Nijmegen, 12 mei 1993.