Table V-1

Liver Tumors in F344 Rats Fed Diets Containing DEHP for 2 years[a]

	Dietary Concentration		
	0	0.6%	1.2%
Male			
Neoplastic nodule	2/50[b]	5/49	7/49
Hepatocellular carcinoma	1/50	1/49	5/49
Neoplastic nodule or hepatocellular carcinoma	3/50 (6%)	6/49 (12%)	12/49 (24%)
Female			
Neoplastic nodule	0/50	4/49	5/50
Hepatocellular carcinoma	0/50	2/49	8/50
Neoplastic nodule or hepatocellular carcinoma	0/50 (0%)	6/49 (12%)	13/50 (26%)

[a]From NTP Technical Report Series No. 217.

[b]Number of animals with lesions/number of animals whose livers were examined microscopically.

Table V-2

Liver Tumors in B6C3F1 Mice Fed Diets Containing DEHP for 2 years[a]

	Dietary Concentration		
	0	0.3%	0.6%
Male			
Hepatocellular adenoma	6/50[b]	11/48	10/50
Hepatocellular carcinoma	9/50	14/48	19/50
Hepatocelluar adenoma or carcinoma	14/50 (28%)	25/48 (52%)	29/50 (58%)
Female			
Hepatocellular adenoma	1/50	5/50	1/50
Hepatocellular carcinoma	0/50	7/50	17/50
Neoplastic nodule or hepatocellular carcinoma	1/50 (2%)	12/50 (24%)	18/50 (36%)

[a]NTP Technical Report Series No. 217.

[b]Number of animals with lesions/number of animals whose livers were examined microscopically.

Noteworthy non-neoplastic lesions were testicular degeneration in high dose male rats (90%) and mice (14%). Hepatomegaly was not reported in either species.

The Panel concluded that these studies, although conducted in only one laboratory, provide definitive evidence for the carcinogenicity of DEHP when administered in the diet to rodents. The weight of the evidence is strong because an increased incidence of malignant tumors was found in the same organ in both sexes of two species of animals in a dose-related way.

1. Discussion

In a critique of a draft report of the NTP bioassay, Northrup et al. (1982) raise essentially four concerns: (a) the maximum tolerated dose was exceeded in the majority of the test groups, (b) the tumor incidences in laboratory and historic control groups were variable, (c) there was potential for cross contamination from other test substances in the same room, and (d) the data show both causation of and protection from cancer.

In a response to the critique, Kluwe et al (1983) discuss the principles of dose setting for bioassays and suggest that Northrup et al (1982) may have confused the use of one element of dose selection, body weight gain, as a predictor of the maximum tolerated dose with the actual measurement of all the effects during the bioassay. The Panel has reviewed the arguments presented in both papers as well as the relevant data and conclude that the dose levels selected were not so high as to preclude interpretation.

Regarding the question of variations in tumor incidences among various control groups, Kluwe et al present summary data showing that there was relatively little variation in liver tumor incidences among contemporary NTP historic controls, and that the concurrent controls in the NTP DEHP bioassay were within the expected range. It is not surprising therefore that the statistical significance of the findings did not change when historic control groups were substituted for the concurrent controls for analysis.

The potential for cross-contamination from other test substances within a facility always exists. Chronic feeding studies in progress in the same facility at the time of the DEHP bioassay were gum agar, butyl benzyl phthalate, and di(2-ethylhexyl) adipate (DEHA). The liver tumor incidences in control and dosed rats and mice in all these studies were in the expected range for control animals except for mice exposed to DEHA, which developed increased numbers of liver tumors. Although the apparent response to DEHA could represent cross-contamination by DEHP, the close structural similarity of the two compounds and the lack of tumor response in other animal groups make it highly likely that DEHA is also hepatocarcinogenic to mice and that cross-contamination has not occurred.

The last point, that tumor incidences were reduced at some sites while increased for the liver in the DEHP bioassays, is difficult to explain. It is tempting to speculate, as have Kluwe et al., that the decreased testicular tumors were causally related to testicular toxicity and secondary pituitary hypertrophy. Reductions from expected incidences in testicular, thyroid, and pituitary tumors in rats may be related to decreased body weight in dosed animals. While these kinds of

associations are frequently observed in bioassays, few data exist upon which to draw solid scientific conclusions and the relationship remains uncertain (Haseman 1983). It is noteworthy, however, that decreased tumor incidences were not found at other sites in dosed mice that developed cancers of the liver.

2. Conclusion.

The Panel concludes that the evidence is sufficient to establish the carcinogenicity of DEHP for rats and mice. That conclusion is consistent with the separate evaluation by the IARC (1982).

B. Animal Bioassays for Tumor Initiation or Promotion

As DEHP has been demonstrated to have properties of a complete carcinogen, it would not be surprising if DEHP were shown to be effective both in initiating the carcinogenic process and in promoting the growth and development of neoplastic cells after they have formed. There are two reasons, however, to inquire about DEHP's initiating and promoting capacities. First, it is sometimes argued that the mouse liver is already initiated naturally in some way, or that it is an unusually sensitive organ to induction of cancer and, therefore, that tumor production in the mouse liver may represent principally tumor promotion rather than complete carcinogenesis. Second, the capacity of DEHP to promote cancers induced by other agents may have important public health implications.

1. Initiation.

Ward et al. (1983, and Ward 1985) administered DEHP by gavage in single doses of either 50 or 25 g/Kg to 8 groups of ten 4-week-old male B6C3F1 mice followed 2 weeks later by administration of 500 ppm phenobarbital (PB) in the drinking water to half of them until the mice were sacrificed 6 and 18 months later. After 6 months, proliferative focal lesions were found in the livers of mice given the higher DEHP dose,

but not at the lower DEHP dose or in mice also given PB. After 18 months, focal proliferative hepatic lesions were found in all 4 groups receiving DEHP. The two groups of mice receiving DEHP and PB had more hepatic foci than those receiving only DEHP, but the incidences were similar to those of mice receiving only PB. Although the authors concluded that there was no evidence of liver tumor initiation in this experiment, the panel believes that the PB effect was so great (76% of mice exposed only to PB had hepatic foci) as to possibly have obscured an increase in initiated foci detectable by PB promotion. The experiment also suggests the carcinogenicity of DEHP alone for the liver of male mice after a single administration of 25 g/kg (four mice with hepatic foci and with hepatocellular carcinoma in the group of ten mice receiving 25 g/kg DEHP as compared to no foci or tumors in the untreated group).

In another experiment, Ward and his associates (Diwan et al 1983; Diwan et al 1985) applied DEHP (98.1)g in acetone) to the skin of CD-1 mice followed by twice weekly applications of either DEHP or TPA (10 ug in acetone) for 40 weeks. No skin tumors formed after the DEHP treatments and the few papillomas produced by DEHP and TPA were comparable to those produced by TPA alone. The positive control of DMBA (dose not given) followed by TPA produced an average of 14.5 papillomas per mouse in 26 weeks.

The Panel concluded that the tumor initiating activity of DEHP has not yet been explored adequately.

2. Promotion

Studies designed to demonstrate the capacity of a substance to promote the development and growth of tumors after one or more cells have

been transformed into neoplastic cells typically employ standardized model systems in which tumor promotion can be reproducibly measured with known initiating and promoting agents. Foremost among the model systems is application to the skin of mice where developing tumors can be observed and counted. DEHP has been investigated for promoting activity in mouse skin in two studies using different strains of mice. Another commonly used model system that is directed toward the rat liver is particularly relevant for DEHP, as the liver appears to be the only target site for tumor development in rodents after exposure to DEHP. Two such studies with DEHP using different strains of rats have been reported. In addition, the methods used to study promotion in rat liver were applied to an investigation of DEHP's effects in mouse liver.

Diwan et al (1983, 1985 and Ward 1985,) conducted classical skin painting studies in CD-1 mice using single topical applications of 50 ug 7,12-dimethylbenz[a]anthracene (DMBA) as the initiator, followed by DEHP (98.1 ug in acetone) twice weekly for 40 weeks to test for promotional activity. The known tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) (10 ug in acetone twice weekly) was used as a positive control. Under the conditions of the experiment, DMBA followed by DEHP produced no skin tumors (no promotion), while with DMBA followed by TPA 97% of mice developed skin tumors. In a second set of skin painting experiments, female SENCAR mice were painted with DMBA once (20 ug), then TPA (2 ug, twice a week for 2 weeks), followed by DEHP (100 ug, twice weekly) or by TPA, mezerein, or acetone as controls. With this protocol, DEHP showed some late promotional activity for mouse skin but was not as strong a second-stage promoter as mezerein (DMBA + TPA + DEHP gave 6.4 papillomas

per mouse, DMBA + TPA + mezerein produced 23 papillomas per mouse, DMBA + TPA + TPA gave 26.4 papillomas per mouse, DMBA + DEHP - DEHP gave 0.9 papillomas per mouse, and DMBA alone produced no tumors).

In rats, Popp et al (1985a) gave single intraperitoneal injections of 150 mg/kg diethylnitrosamine (DEN) to groups of 10 female F344 rats. After a two-week recovery period, one group received 1.2% DEHP in the diet and other groups received basal diets or diets containing 0.05% phenobarbital (PB) as negative and positive controls, respectively. Five rats in each group were killed after 3 months, the remaining five after 6 months, and the liver tissues examined for foci of cellular alteration using six histological stains and histochemical reactions. DEHP did not increase the number or the size of the foci induced by DEN. No tumors were reported in any of the groups. The Panel considered this a well designed and conducted study without evidence for tumor promotion at a dietary dose level that was carcinogenic in the two-year NTP bioassay. It should be emphasized, however, that the end-point measured was altered cellular foci rather than tumor formation. It is possible that the hepatocellular tumors produced by DEHP arise in normal appearing tissue rather than from progression from altered foci to tumors.

In another study in rats, DeAngelo et al (1983, 1984) administered single intraperitoneal injections of 30 mg/kg DEN to male Sprague-Dawley rats 18 hours after partial hepatectomy. Ten days after DEN initiation, groups of 4 or 5 rats were placed on diets that were either choline deficient or choline sufficient and supplemented with either 2% DEHP or 0.06% PB. The rats were killed after 10 weeks on the experimental diets and the liver tissues examined for gamma-glutamyltranspeptidase positive foci. In control rats not receiving DEN none of the dietary regimens

induced the appearance of foci. In positive control groups, rats receiving DEN followed by diets that were either choline deficient or supplemented with PB or both, the number of hepatic foci were increased as expected. When DEHP was added to choline deficient or PB supplemented diets, the number of foci was decreased indicating inhibition of promotion. Adjustments for differences in body weights or liver weights did not affect the conclusions. The Panel considered the partial hepatectomies and choline deficient diets to be useful test variables but noted that the limited histochemical reactions used may have caused some types of cellular foci to be missed.

Ward et al. (1983) used an approach in mice similar to that used by Popp et al (1985b) in rats. They gave single intraperitoneal injections of DEN (80 mg/kg) to 4-week-old male B6C3F1 mice followed, after a two-week interval, by DEHP in the diet at 12,000, 6,000 or 3,000 ppm. Groups of 10 mice were killed 2, 4 or 6 months after DEN injection. A few proliferative hepatic foci were found after DEN or DEHP alone, while numerous foci and neoplasms were seen in mice given DEHP after DEN (promotion). With the combined treatments, the numbers of foci did not increase between 4 and 6 months but the foci increased in size. With higher doses of DEHP, foci and tumors appeared earlier. DEN-induced lung tumors were not affected by DEHP administration. Non-neoplastic lesions in mice receiving the high dose level of DEHP included oval cell hyperplasia and pigmented macrophages in the liver and tubular degeneration, hyperplasia, and cysts in the kidneys. Other mice in the same study were continued on dietary DEHP after DEN exposure for 18 months (Ward et al 1985). At the low dose of 3,000 ppm DEHP, 5 of 10 control mice receiving DEHP alone had hepatic foci and 1 of 10 had

hepatocellular carcinoma as compared to 10/10 mice with hepatic foci and carcinoma in the mice receiving DEN plus DEHP. With a similar DEN/DEHP protocol, female F344 rats were said not to develop proliferative foci within the 14-week experimental period even though peroxisome induction was demonstrated. In a separate report (Ward et al 1984), tumor promotion by DEHP in the liver of mice was demonstrated as early as 28 days.

3. Discussion.

The demonstration of tumor promotion by DEHP in the livers of mice but not rats may be surprising to some. It may be, however, that the few experiments performed thus far have not adequately explored the possibilities for tumor promotion in view of the evidence that DEHP is carcinogenic for both species. Alternatively, the preneoplastic lesions induced by DEN may not respond to promotional stimuli in the same way as those lesions that arise spontaneously. Another possibility is that the assumption that the altered foci are the precursors of neoplasms may be partly or entirely untrue. The experiments reported thus far tend to concentrate on young animals and it may be that the promotional activity of DEHP would be more effective after one year of age when spontaneous altered foci and hepatic neoplasms develop in both sexes of both species (Ogawa et al 1981; Ward 1983; Popp et al 1985b). It is tempting, too, to speculate that mice might be predisposed to hepatic carcinogenesis because type C retroviruses have been found in some hepatic tumors (Becker, 1984) as have cellular oncogenes that transform NIH 3T3 cells (Fox and Watanabe 1985). Lack of comparable data for the rat and man, however, preclude transspecies comparisons.

4. Conclusion

The Panel concludes that there is some experimental evidence for tumor promotion by DEHP in mouse liver and mouse skin. The evidence for tumor initiating activity is inadequate for conclusions to be drawn. Because oral administration of DEHP to rats and mice produces hepatocellular tumors by some mechanism, DEHP or its metabolites must be presumed to have both initiating and promoting activities.

C. Carcinogenicity of Structurally Related Compounds

The results of dietary animal bioassays by NCI/NTP on four compounds related to DEHP have been summarized recently (Griesemer 1982). Phthalamide at 3% in the diet (NCI TR-161) produced toxic effects in the livers and urinary tracts of rats and mice but no tumors. Phthalic anhydride (NCI TR-159) at 5% in the diet produced no clear evidence of carcinogenicity in rats and mice but raised the possibility of a leukemogenic effect in female rats. Di(2-ethylhexyl)adipate (DEHA) (NTP TR-212) produced liver tumors in mice but not in rats. Butylbenzylphthalate (BBP) (NTP TR-213) provided only suggestive evidence for leukemias in female rats. BBP produced both thymic and testicular atrophy. Subsequently Huff and Kluwe (1984) have reviewed the bioassay data for these compounds again, along with that for diallylphthalate (DAP) (NTP TR-242 and 284) which was administered by gavage. DAP administration was hepatotoxic for rats and associated with leukemia in females. In mice DAP caused hyperplasia and papilloma formation in the forestomach in both sexes and equivocal production of leukemia in males. It can be concluded that several phthalic ester compounds possess some carcinogenic activity but that the target sites are dissimilar.

Kluwe (1985) has examined structure activity relationships for three esters of phthalic acid (DEHP, butyl benzyl phthalate, diallyphthalate), phthalic anhydride, and three non-phthalate chemicals containing a 2-ethylhexyl moiety: di(2-ethylhexyl)adipate; tris(2- ethylhexyl)phosphate (NTP TR-274); and 2-ethylhexyl sulfate (NTP-TR-256).

In contrast with the dissimilar target sites for carcinogenicity of the phthalic acid esters, Kluwe (1985) reported that all the 2-ethylhexyl containing compounds possessed some hepatocarcinogenic activity in mice and particularly in female mice. The 2-ethylhexyl compound that produced the greatest response in mice (DEHP) was also hepatocarcinogenic in rats.

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VI. PEROXISOME PROLIFERATION AND ITS ROLE IN CARCINOGENESIS.

A. Introduction.

A study of the mechanism of the carcinogenic action of DEHP is bound to examine the possibility that the carcinogenicity of DEHP is associated with its role as an inducer of peroxisome proliferation. The term "peroxisome proliferation" refers to the increase in the number of subcellular organelles called peroxisomes in response to administration of a series of compounds collectively called "peroxisome proliferators". The phenomenon of peroxisome proliferation has so far been described - mainly for the liver (Tolbert and Essner 1981; Reddy and Lalwani 1983), kidney and intestine (Lalwani et al 1981; Reddy et al 1975; Thorp 1970). The compounds referred to as peroxisome proliferators have diverse and unrelated chemical formulas. Despite the lack of common chemical structure these compounds have similar effects. Their functional common denominators are:

a. Induction of increase in the number of peroxisomes of the liver in several species.
b. Induction of variable degrees of hepatocyte hyperplasia.
c. Lowering of the concentrations of plasma lipids.
d. Induction of liver carcinogenesis.
e. Weak or absent mutagenicity in in vitro mutagenesis bioassays. For a excellent review of the role of peroxisome proliferation and carcinogenesis and for more detailed analysis of the issues covered in this section of the report the reader is referred to the recent review by Reddy and Lalwani (1983).

In this part of the report we will attempt to provide answers to the following questions:

1. Is peroxisome proliferation a cellular state that by itself can lead to carcinogenesis?
2. How is peroxisome proliferation induced?
3. Is DEHP and/or any of its metabolites a peroxisome proliferator?
4. Is DEHP acting solely as a peroxisome proliferator or are there additional mechanisms that should be considered in order to explain it carcinogenic effect in rodent liver?

B. Peroxisomes: Occurrence, Structure, Biochemistry and Function

In this section only a summary presentation of the large literature available on peroxisomes will be attempted. More than 650 references directly relating to peroxisomes have appeared in the international literature since the original description of these organelles by DeDuve and Baudhuin in 1965 (and DeDuve 1966). For a recent review on peroxisomes the reader is referred to the reviews by Tolbert (Tolbert 1981, and Tolbert and Essner 1981).

Peroxisomes are also variously referred in the scientific literature under the terms "microbodies" and "glyoxysomes". The latter term is mostly applied to the peroxisomes of the plants and yeasts in which the enzymes related to the glyoxylate metabolic pathway are frequently found. The term "microbodies" is currently used synonymously with the term "peroxisomes". The term "peroxisome" as used in this report is the most frequently used term for the mammalian organelles and is meant to encompass the terms "microbodies" and "glyoxysomes" under which the peroxisomes are still occasionally referred.

Peroxisomes are intracellular organelles that are ubiquitously present in most eucaryotic cells at all levels of the phylogenetic scale. They have been described in algae, fungi, protozoa, leaves, germinating seeds, fishes, birds and mammals, including rodents and primates. (Tolbert 1981, Tolbert 1971, Gerhardt 1978, Beevers 1969, Beevers 1979, Tolbert 1972, Maxwell et al 1977, Muller 1975, Muller and Moller 1969). In these tissues peroxisomes are seen by electron microscopy as particles ranging in diameter from 0.1 to 1.5 um, with an average diameter of 0.5 um. They are limited by a single-layer tripartite membrane and in their interior there is a finely granular matrix. Crystalloid structures, often associated with uricate oxidase, are occasionally also seen in the interior of these organelles (Hruban and Swift 1964). The enzymic contents of the peroxisomes vary in the different tissues and species and probably reflect differing functions. In all instances however peroxisomes contain catalase and at least one flavin oxidase.

The term "microperoxisome" has been used by Novikoff et al (1973) to denote small peroxisome like organelles that are seen in association with the endoplasmic reticulum. They lack the nucleoid core often seen in peroxisomes. Their membranes often appear in direct continuity with the membranes of the endoplasmic reticulum. This has led to the hypothesis that the microperoxisomes are intermediate stages in the biogenesis of these organelles (Novikoff et al 1973, Kindle 1982). This hypothesis is further supported by the finding that in tissues (e.g. liver, kidney) in which peroxisome proliferation is induced the predominant type of peroxisome seen are the microperoxisomes (Tolbert

1981). Microperoxisomes have been seen in all cells of all mammalian tissues other than red blood cells (Novikoff et al 1973). Aside from their difference in mean size diameter and the fact that their membrane frequently appears continuous with the smooth endoplasmic reticulum, no other functional differences have been found between microperoxisomes and peroxisomes. In view of the association between the microperoxisomes and the state of peroxisome proliferation, the term "peroxisomes", as used in this report, is meant to encompass also the microperoxisomes.

The abundant peroxidase activity of these organelles is also used for their cytochemical identification, using a diaminobenzidine procedure as described by Novikoff and Goldfisher (1969).

This section will focus on the biochemical functions and enzymes associated with the mammalian hepatic peroxisomes and the implications of these functions for the association between peroxisome proliferation and carcinogenesis in mammalian liver.

In general, the peroxisomes contain a variety of enzymes associated predominantly with lipid metabolism. Several oxidation reactions can be catalyzed by the enzymes residing in the peroxisomes. These reactions employ as their terminal oxidase an H_2O_2 generating flavin oxidase. The H_2O_2 produced is converted to H_2O by catalase. Catalase accomplishes this either by directly converting H_2O_2 to $H_2O+1/2$ O_2 or by utilizing two hydrogen atoms from suitable molecular donors to convert H_2O_2 to two molecules of H_2O plus an oxidized substrate.

These reactions are shown in a, b, and c below. The catalase reactions b and c are also called "catalatic" and "peroxidatic" reactions respectively.

$$RH_2 + O_2 \longrightarrow H_2O_2 + R \quad (a)$$

$$H_2O_2 \longrightarrow H_2O + 1/2\ O_2 \quad (b)$$

$$H_2O_2 + RH_2 \longrightarrow 2H_2O + R' \quad (c)$$

In mammalian hepatic peroxisomes the following enzymes have been described (taken after Reddy and Lalwani 1983) with slight modifications):

TABLE VI-1.
Enzymes in Mammalian Hepatic Peroxisomes

Enzymes	Substrates
Catalase	Catalatic: H_2O_2 Peroxidatic: ethanol, methanol, formate, nitrite etc.
Oxidases (H_2O_2 generating enzymes)	
Glycolate	Alpha-hydroxy fatty acids.
Urate	Uric acid
D-amino acid	D-amino acids
Fatty acyl-CoA	(C10, C22,) Long chain fatty acylCoA
Polyamine	Spermine and spermidine.
Acyl transferases	
Carnitine acetylCoA	AcetylCoA
Carnitine octanoylCoA	-C18 Acyl CoA
AcylCoA:Dihydroxyacetone-P	Dihydroxyacetone phosphate; palmitoyl CoA
Dehydrogenases	
NAD:glycerol-P	Glycerol-P
NAD:isocitrate	Isocitrate
NAD:L-3 hydroxy fatty-acyl-CoA	C_4-C_8 Fatty acyl-CoAs
Other	
Enoyl-CoA hydratase	Crotonyl-CoA; enoyl-CoA (C_6-C_{16})
Thiolase	C_6-C_{12} fatty acids
Fatty acyl-CoA synthetase	Long-chain fatty acids
Membrane Oxidase	Glycerol Phosphate, Xanthine, Aldehydes

As shown in the above Table VI-1, most of the enzymes identified in peroxisomes are involved in lipid metabolism. The consumption of oxygen by the peroxisomes during the formation of H_2O_2 has been estimated to amount to 20% of the oxygen consumption by the liver (DeDuve and Baudhuin 1966). Release of the oxygen of the H_2O_2 by catalase is associated with production of heat. Catalase is present in peroxisomes in amounts larger than any other single enzyme. Catalase has been estimated to be approximately 33% of the hepatic peroxisomal protein (DeDuve and Baudhwin 1966).

C. Hepatic changes associated with administration of peroxisome proliferators.

An excellent detailed description of the changes associated with feeding peroxisome proliferators is given in the review by Reddy and Lalwani (1983). A summary of the responses described in the literature is presented here, with emphasis on the aspects that are relevant to the carcinogenicity of DEHP and the other peroxisome proliferators.

It should be emphasized again at this point that the term "peroxisome proliferators" refers to a group of compounds with diverse chemical structure, whose only common aspects are the fact that they are capable of inducing increasing numbers of peroxisomes in (primarily) liver and kidney and of inducing hypolipidemia. There is no apparent common aspect of chemical structure that characterizes these compounds. A large number of them are derived from clofibrate and are considered its structural analogs. Several other compounds structurally unrelated to clofibrate (tibric acid; Wy-14,643; BR-931; Tiadenol; RPM-14,514; DG-5685; DH-6463; LK-903; chlorocyclizine; acetylsalicylic acid (aspirin))

also result in peroxisome proliferation if given in sufficient doses. In addition to the above compounds several plasticizers can also induce peroxisome proliferation. Such compounds (with known peroxisome proliferator activity) are di(2-ethylhexyl)phthalate (DEHP), di(2-ethylhexyl)adipate (DEHA), di(2-ethylhexyl)sebacetate (DEHS), etc. (Moody and Reddy 1978; Reddy et al 1976; Lake et al 1975). The plasticizers also have a hypolipidemic effect, in common with the other peroxisome proliferators. Nutritional conditions such as high fat diet or deficiency in vitamin E can also induce peroxisome proliferation, but on a much lesser scale as compared to the administration of the peroxisome proliferators.

1. Peroxisome Proliferation

a. General aspects of peroxisome proliferation.

In general there is no observed difference in the biochemical functions of the peroxisomes as determined by the type of the peroxisome proliferator used. The magnitude of the response depends on the dose of the particular peroxisome proliferator. Effects related to peroxisome proliferation are seen after sufficient concentrations are given. The relationship between dose and response varies with the index used. A typical example is shown in the following table (after Reddy et al 1985):

Table VI-2 - Peroxisome Proliferation

DEHP % in diet	Catalase (units/mg protein)	Palmitoyl-CoA oxidation (umoles/min/g liver)
0.25	48 ± 3	4.4 ± 0.1
0.50	55 ± 2	6.1 ± 0.3
1.0	58 ± 3	5.9 ± 0.9
2.0	70 ± 3	10.7 ± 1.0

In other studies much higher increases in some enzymatic functions have been reported. Palmitoyl CoA oxidation increased more than 15 fold as a result of feeding 2% DEHP (Lake et al 1984).

Peroxisomal proliferation at high doses starts within 24 hours of the first dose of the peroxisome proliferator and reaches a plateau at 14 days. It is maintained at the plateau level for as long as the peroxisome proliferator is administered. There is a rapid decline immediately after the removal of the peroxisome proliferator (Moody and Reddy 1976) and within 10-15 days the number of peroxisomes is down to normal. Thus administration of peroxisome proliferators is not associated with any persistent changes in peroxisome numbers that would remain after the drug is withdrawn. The only persistent change in normal hepatocytes seen after prolonged administration of peroxisome proliferators is the presence of increased amounts of lipofuchsin, interpreted as indicative of prolonged peroxidation of membrane lipids (Reddy et al 1982d).

The number of peroxisomes in a hepatocyte from a normal rat liver is approximately 1,000. This number increases 8-10 fold at the plateau

phase of the response seen at the highest effective doses of peroxisome proliferators. Several investigators have described differences between the peroxisomes seen in the normal liver and the peroxisomes that appear increased in numbers in livers of rodents after feeding of peroxisome proliferators. (For reviews see Tolbert 1981, Reddy and Lalwani 1983). As mentioned above, the peroxisomes are smaller than usual and they frequently appear to connect directly with the membranes of the endoplasmic reticulum. The term "microperoxisomes" more aptly describes the type of peroxisomes seen in these conditions (Novikoff et al 1973). The enzymes that are normally present within peroxisomes also increase in overall concentration in hepatic homogenates that contain fragmented peroxisomes, indicating an increase of the overall enzyme levels per hepatocyte in parallel to the increase of peroxisomes, the organelles within which these enzymes are contained. Not all the enzymes contained within the peroxisomes increase to the same extent. The increase in catalase is proportionately less than the increase in the number of peroxisomes, whereas the increase in enzymes involved in beta oxidation is proportional to the increase in number of peroxisomes. Increase in carnitine-acetyl transferase parallels the increase in number of peroxisomes whereas the increase in uric acid oxidase seems to be less than the increase in peroxisome number. (Moody et al 1976, Reddy and Kumar 1979, Kolde et al 1976; Leighton et al 1975, Reddy et al 1970, Lalwani et al 1983). Other enzymes have not been as extensively studied. This disproportion in enzyme induction results in peroxisomes that are relatively deficient in catalase compared to the peroxisomes of the normal rodent liver. It was mentioned above that catalase is the most abundant enzyme in peroxisomes, estimated to comprise up to 33% of

the peroxisomal protein. It has been argued on theoretical grounds that the disproportion between catalase and the enzymes of beta-oxidation could result in increased production of H_2O_2. On the other hand, given the excess in catalase concentration in the normal peroxisomes compared to other peroxisome components, it could be possible that the levels of catalase are capable of converting all the H_2O_2 into H_2O despite the increased production of H_2O_2.

A direct attempt to resolve this issue was made by Fahl et al (1984). Peroxisomes were isolated from livers of normal rats and of - rats treated with peroxisome proliferators. The authors employed purified peroxisomal preparations and found that the peroxisomes from livers treated with peroxisome proliferators produced 30-70 times more H_2O_2 compared to control peroxisomes. The results obtained by the use of purified preparations do not necessarily reflect the concentrations that may be available to the intranuclear compartment in view of the fact that in vivo the cytoplasmic catalase and glutathione peroxidase would limit the probability that reactive oxygen species would reach the nucleus. Furthermore, in these studies, cyanide was used as a mitochondrial inhibitor. Since cyanide is also a catalase inhibitor, it interferes with the interpretation of the role of catalase in view of the fact that catalase would be nonfunctional under the conditions of the assay. The findings of the authors of this study, however, are impressive in that they showed for the first time an increased production of reactive oxygen species by peroxisomes during peroxisome proliferation.

b. Differences Between Species.

The phenomenon of peroxisome proliferation was considered in the past to be relevant only for the rodent liver and kidney. Several recent studies have shown, however, that the phenomenon can be observed in the livers of a variety of species. By using transplanted hepatocytes of several species in nude mice (Reddy et al 1984) and by direct studies in livers of animals given peroxisome proliferators it has now been shown that a variety of experimental animals, including rodents, birds and primates, respond to the administration of peroxisome proliferators in a manner similar to rodents (Reddy et al 1982b). In a recent study by Lalwani et al (1980), it was shown that, at doses that exceed the ones required for the hypolipidemic effect, the full spectrum of the peroxisome proliferation could be demonstrated in the liver of rhesus monkeys.

There have been relatively few direct studies of the effects of peroxisome proliferators in human liver. A study by Hanefeld et al (1980) has shown that after prolonged treatment with CPIB (p-chlorophenoxy isobutyric acid) there was an increase in endoplasmic reticulum and peroxisomes in livers of patients. The increase was smaller compared to that seen at maximal doses in rodent liver but was measurable and was reported to persist for months after discontinuation of the treatment. The latter finding is at variance with the above mentioned findings with rodents where the discontinuation of the drug results in rapid decline in peroxisomal numbers. Other investigators (De la Iglesia et al 1981) found no increase in numbers of peroxisomes in livers of patients after long term gemfibrozil therapy. In a recent report (Ganning et al 1984) an increased number of peroxisomes was noted in the liver of patients

that had been on prolonged renal dialysis. Though there may be several factors that can account for this effect, one of the factors may be the documented increased exposure of humans on renal dialysis to high levels of DEHP.

In general the studies with human liver are sparse and not as conclusive compared to the studies with rodents. This has been ascribed (Reddy and Lalwani 1983) to several factors. Human peroxisomes lack the characteristic nucleoid core seen in the rodent peroxisomes. This makes them more difficult to identify. There is more variation in size with human as compared to rodents peroxisomes. In addition the histochemical (diaminobenzidine) reaction most commonly used for peroxisome identification (Novikoff et al 1969) proceeds optimally at 40°C in human liver samples as opposed to 37° C for rodent liver. There is a need for more defined studies for a better quantitation and comparison of the effects of peroxisome proliferators in human liver. The clearly demonstrated effects of peroxisome proliferators in the livers of so many other species, including, as mentioned above, birds, rodents, and primates, likely indicate that peroxisome proliferation can also be expected to be induced in the human liver under the appropriate conditions, as in the livers of these other species. These conditions, however, need to be defined in view of the fact that the available studies show considerable variation among species in the peroxisome proliferation response. In a recent study dramatic differences were shown in the response of different species to gemfibrozil (Gray and De la Iglesia 1984). For that chemical the response was in the descending order of rat > hamster > monkey > dog. DEHP also induces a stronger response in the rat as compared to the hamster (Lake et al 1984). The

following description and analysis of the effects of peroxisome proliferators is derived from published studies in the livers of rodents.

c. Differences in Potency Between Inducers.

Substantial variation in the potency of peroxisome proliferators has been found in experimental studies. Aspirin is a very weak peroxisome proliferator, whereas Wy-14,643 is one of the strongest. On an equivalent weight basis they can be roughly ranked (Lalwani et al - 1983c) by declining potency in the sequence: methyl clofenapate > ciprofibrate > Wy-14,643 > nafenopin > BR-931 > Tiadenol > fenofibrate > tibric acid > clofibrate > bezafibrate > gemfibrozil > DEHP > aspirin.

Though the plasticizers, exemplified by DEHP, are the weakest peroxisome proliferators on an equal weight basis, it should be emphasized that, given adequate dose (for DEHP 1-2% in the diet), the magnitude of peroxisome proliferation achieved is comparable to that achieved by some of the strongest peroxisome proliferators, albeit at smaller doses.

d. Mechanisms of the Induction of Peroxisome Proliferation.

The general mechanisms of action of the compounds that induce peroxisome proliferation is not clear. As mentioned above the chemical structures of the compounds that fall into this category do not have any apparent common structural denominator. The phenomenon of peroxisome proliferation has been induced in hepatocytes in primary culture (Mitchell et al 1984). In culture conditions hydrocortisone appears to

play a permissive role and carnitine maximizes the inducing effect. The demonstration of the effect with primary cultures of hepatocytes proves that peroxisome proliferation is due to a direct effect of the proliferators on hepatocytes and not to an indirect effect mediated on the hepatocytes through another cell type. Previous studies (Svoboda et al 1967) had raised the issue that peroxisome proliferation is seen only in male rats and not in females. More recent studies have shown that this is not the case (Reddy et al 1979, Reddy and Kumar 1979, Svoboda et al 1969) and that the phenomenon can be seen in both sexes.

Other studies have shown that the proliferation of peroxisomes is associated with new RNA and protein synthesis (Warren et al 1982). The precise way that the signal is transmitted from the entry of the proliferator compound in the hepatocyte to the mechanisms for gene regulation in the nucleus is not clear. Presumably a suitable message should reach intranuclear sites of gene regulation so that the new protein required for the production of the parts used in the peroximal assembly by the endoplasmic reticulum is synthesized. The nature of this putative signal has resulted in much speculation, centered around three main hypotheses. These are as follows:

i. The receptor theory.

According to this theory the peroxisome proliferators interact with a receptor molecule that is present in the cytoplasm or the plasma membrane of the hepatocytes. The formation of a receptor-peroxisome proliferator complex is the beginning of a chain of events that lead to the generation of a message that is transmitted to the nucleus and initiates the process for the production of peroxisomal components. The precise sequence of events for the signal transduction from the

receptor-ligand interaction to the final response can be considered as analogous to existing models of similar such interactions that are well documented in the literature. Such models are: the estrogen receptor model, the corticosteroid receptor model, the adrenergic receptor models, as well as the models with peptide hormones. All of these models (not to be reviewed here) are well characterized in the literature and share as a common property that the interaction between the ligand and the receptor results in measurable effect only after considerable numbers of receptors are occupied by the ligand. There are definite thresholds that characterize these interactions and these thresholds (in terms of receptor occupancy or ligand concentration) need to be exceeded before a response is seen in the target cell.

The main exponents of this theory are Drs. Reddy and Lalwani (Lalwani et al 1983a,c). In a recently published study they showed that "[3H]nafenopin, a known inducer of liver peroxisomal enzymes, was shown to bind to a specific, saturable pool of binding sites in cytosols from rat liver and kidney cortex. Tissue levels of this binding protein (liver greater than kidney cortex; not detectable in myocardium, skeletal muscle) were seen to correlate with the ability of nafenopin to induce peroxisomal enzymes in these organs. Clofibrate and ciprofibrate, which are structurally similar to nafenopin, competitively blocked the specific binding of [3H]nafenopin. Phenobarbital, a non-inducer of peroxisomes, and [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid and 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio-(N-beta-hydroxyethyl)acetamide, which are structurally unrelated peroxisome proliferators, did not compete for the specific [3H]nafenopin binding sites. The [3H]nafenopin binding protein is proposed as a

mediator of the drug-induced increase in peroxisomes and associated peroxisomal enzymes". This is tne only study published so far in which direct evidence for the presence of a receptor is presented. This binding protein has not so far been purified and nothing else is known in terms of its molecular composition. Purity of this protein would be essential in studies aimed to precisely quantify the levels of the peroxisome proliferators required, so that a sufficient receptor ligand interaction is generated that can result in measurable peroxisome proliferation.

ii. The substrate overload theory.

According to this theory the effect of the peroxisome proliferators is on peripheral targets rather than in the liver itself. The administration of these compounds results in an increased influx of substrates for the peroxisomal enzymes such as long chain fatty acids, etc. This in turn results in an increase in the number of peroxisomes, presumably due to the induction and increased synthesis of the enzymes and other components that constitute these organelles. In support of this theory is the finding that feeding rats in a diet high in fats results in a slight increase in the number of peroxisomes. The increase, however, is very slight compared to the increase in peroxisome numbers induced by the peroxisome proliferators. Furthermore, there is no direct evidence that the peroxisome proliferators exercise a direct lipolytic effect on peripheral tissues. Further evidence against this hypothesis is also furnished by a recent paper (Lake et al 1983) in which the effects of several phthalate esters are compared. Whereas both the phthalate esters DEHP and MNOP had hypolipidemic effects, peroxisome proliferation was seen only with DEHP and its metabolites.

Similar findings that dissociate the hypolipidemic effect of peroxisome proliferators were shown in relation to bezafibrate by Lazarow and DeDuve (1976).

iii. Peroxisome proliferators as substrates for peroxisomal enzymes.

According to this theory the peroxisome proliferators themselves are substrates for the peroxisomal enzymes and especially for the enzymes involved in beta-oxidation. Thus, though the overall influx of fatty acids even in high fat diets is insufficient to produce more than marginal peroxisome proliferation, the peroxisome proliferators themselves also have to be counted as substrates, and the combined effect of peripheral fatty acid influx as well as the influx of the molecules of the peroxisome proliferators is a sufficient signal to result in the degree of observed peroxisomal proliferation. In support of this theory is the fact that some components of the molecular structure of the peroxisome proliferators appear as suitable candidates for metabolism by the peroxisomal enzymes. Phthalate esters themselves appear to undergo multiple rounds of beta oxidation and some of this could be taking place in the peroxisomes. Recent evidence, however, (Albro PW, personal communication) indicates that the peroxisome proliferators are not metabolized to any substantial degree by peroxisomes. Incubation of several peroxisome proliferators with peroxisomes did not result in demonstrable metabolites that could be ascribed to the peroxisomes. Metabolite VI of DEHP was the most efficient inducer of peroxisome proliferation in hepatocyte cultures and yet it did not appear to be produced by peroxisomes either.

Studies by Lhuguenot et al (1985), however, propose that MEHP at high doses is partly metabolized by peroxisomes. A direct proof to

substantiate this proposal (incubation of peroxisomes with MEHP and isolation of metabolites) is lacking. In another report by the same team (Mitchell et al 1985) it is postulated that the peroxisome proliferation is associated with the concomitant increase of a cytochrome P450 species that is associated with omega oxidation of lauric acid and other long chain fatty acids. It is proposed that the two phenomena (peroxisome proliferation and cytochrome P450 induction) are attempts of the rat hepatocyte to cope with a disturbed lipid metabolism. Due to the inability of the rat to conjugate these compounds, their presence creates a substrate load which is alleviated by the creation of these alternate pathways.

In summary, there is no definitive evidence to totally prove or disprove any of the above theories and all three appear as plausible. Regardless of which of these three theories is the correct one, it should be stressed that all of them view the mode of action of the peroxisome proliferators as one that would only be manifest after a certain concentration of effector molecules were to be reached at the sites responsible for regulating the event of peroxisome proliferation.

2. **Hepatic Hyperplasia and Hypertrophy**

Enlargement of the liver in response to administration of peroxisome proliferators is always seen to accompany the proliferation of the peroxisomes. The phenomenon is well characterized. It has been studied mainly in rodents. The enlargement of the liver starts shortly after the initiation of the feeding of the proliferator compound. There is a gradual increase in liver size for the first two to three weeks. The final increased size is maintained for as long as the drug is administered, with the liver returning to normal weight within days

after the peroxisome proliferator is discontinued. In rats the liver weight can reach 9-10 grams and in mice it can reach up to 20% of the body weight. The thymidine nuclear labelling index and the mitotic index increases during the increase in liver weight, indicating a genuine hyperplastic response associated with new DNA synthesis. (Reddy et al 1979). This hyperplastic response is not the same as the regenerative response seen in liver after damage by toxic agents. The final liver weight in liver regeneration due to toxic effects does not exceed the original weight. The mechanism that is responsible for the observed hyperplastic response is not at all understood. It is not clear whether the mechanisms responsible for the peroxisome proliferation and the hyperplastic hepatomegaly are the same or unrelated.

3. Other Effects of Peroxisome Proliferators on the Liver

There have been several reports concerning effects of peroxisome proliferators on other subcellular organelles. Regarding DEHP, a report (Ganning et al 1983) has claimed that feeding DEHP resulted in an increased number not only of peroxisomes but also of mitochondria. The oxidative phosphorylation pathways appeared intact, indicating lack of toxicity. The authors pointed out that the induction of mitochondria and peroxisomes in the absence of induction of endoplasmic reticulum membranes constitutes a response that is unique for DEHP. These findings are corroborated by work of other investigators that show an increase in synthesis of mitochondrial enzymes after feeding DEHP (Ozasa et al 1984).

Morphometric analysis of the numbers of mitochondria comparable to that of Ganning et al (Ganning et al 1983) has not been performed by

other groups. An effect of DEHP on the profiles of the cytochrome P450 isozymes and a moderate increase in overall cytochrome P450 (Ganning et al 1983) has also been reported. Several studies have shown (Lake et al 1983, Gibson et al 1982, Orton and Parker 1982) that peroxisome proliferators are associated with the induction of a cytochrome P450 species that is associated with high specificity towards omega and omega-1 oxidation and low specificity towards typical cytochrome P450 substrates. More studies are necessary for a better understanding of the role of peroxisome proliferators in induction of specific components of the microsomal enzyme system.

D. Peroxisome Proliferation in Other Tissues

There are few detailed studies that examine the tissue range of the peroxisome proliferation response. In mammalian tissues, recent studies (Reddy et al 1985) have shown that the response is seen mainly in the liver and, to a lesser extent, in the kidney. Previous studies had also reported a small increase in peroxisome-associated enzymes in intestinal tissue (Small et al 1983, Svoboda et al 1967). In the study by Reddy and his associates, the peroxisome protein PPA-80 was used as a marker in two dimensional protein electrophoresis of proteins from small intestine, heart, skeletal muscle, testis, adrenal, brain, and lung of rats fed ciprofibrate. No induction of PPA-80 was seen in those tissues though this protein is easily identified in similar analysis of liver proteins after peroxisome proliferator feeding (Watanabe et al 1985).

Induction of peroxisome proliferation was also seen in hepatocytes transplanted into the anterior chamber of the eye of rats. In those studies the other attached eye tissues had no evidence of peroxisome

proliferation (Rao et al 1984b). Similarly, when peroxisome proliferation was demonstrated in transdifferentiated pancreatic hepatocytes, there was no peroxisome proliferation seen in the attached pancreatic acinar or ductal tissues. These studies address mainly mammalian epithelial tissues and muscle. Other tissues (e.g. connective tissue, nonparenchymal hepatic cells, etc) were not directly addressed in these studies. There have not been any studies with morphological or biochemical markers to address the issue of peroxisome proliferation in cell cultures used for in vitro genotoxicity bioassays.

E. Peroxisome Proliferation and Carcinogenesis

1. Possible Mechanisms

This section will examine the possible mechanisms that are responsible for the carcinogenic effect of peroxisome proliferators. Since liver is the target organ for the carcinogenic effect of these compounds, pertinent findings from other defined models of liver carcinogenesis should provide the conceptual framework for the understanding of this phenomenon. Several well defined models of experimental liver carcinogenesis are now available (Pitot et al 1978, Solt et al 1977, Shinozuka et al 1979, Peraino et al 1984). From these models it appears that two prerequisites need to be fulfilled before liver carcinogenesis develops: genotoxic damage to target cells and hepatocyte proliferation.

a. Genotoxic Damage to Target Cells.

In most of the protocols currently applied, an initiating carcinogen is administered, followed, in some models, by a regenerative

stimulus such as partial hepatectomy. In other models (Shinozuka et al 1979) partial hepatectomy may or may not be performed, but the initiating carcinogen is followed by dietary regimens that induce a continuous wave of liver cell proliferation due to low grade continuous hepatic necrosis associated with hepatocyte damage. The latter triggers a low grade regenerative response. The most popular of these dietary regimens is a choline-deficient diet. In a recent modification prolonged feeding of a choline and methionine deficient diet has been shown to be associated with development of hepatic neoplasia in the absence of initiating carcinogen (Ghoshal and Farber 1984). In the latter protocol, however, it is very likely that the initiating stimulus is provided from genomic alterations associated with or resulting from hypomethylated DNA. The latter alterations would be playing the role of the initiating carcinogens used in the other regimens.

It should also be emphasized that there are numerous studies that have shown that the effect of the initiating carcinogen alone is not sufficient for the development of the hepatic neoplasia. In several studies the administration of well known carcinogens (e.g. benzo(a)-pyrene) is followed by the appearance of stable DNA adducts. Yet carcinogenesis is not seen unless the carcinogen is followed by a step involving hepatocyte proliferation (Solt et al 1977).

Current evidence on the mode of action of peroxisome proliferators shows that they may be able to provide an initiating genotoxic damage due to the generation of reactive oxygen species. They are definitely capable of providing the background of liver cell proliferation required (but not sufficient) for the induction of liver carcinogenesis.

b. Hepatocyte Proliferation

The hepatic hyperplasia due to hepatocyte proliferation was described above. It is seen in rodent liver with all the peroxisome proliferators, including DEHP (Reddy and Lalwani 1983) if given at sufficient dose. As mentioned above, the evidence from all the studies with the existing liver carcinogenesis models shows that the induction of hepatocyte hyperplasia alone is not sufficient to induce hepatic neoplasia, except in situations where severe hypomethylation of the DNA is expected. Thus, it is unlikely that the hyperplasia associated with peroxisome proliferators should by itself, and in the absence of any associated genotoxic damage, be sufficient to cause carcinogenesis. It is very likely that the hyperplastic response potentiates the carcinogenic potential of the genotoxic damage. It is of interest in this regard, in view of the lack of carcinogenic effects of these compounds for the kidney, that there has been no evidence in the literature of kidney hyperplasia associated with peroxisome proliferation, though peroxisome proliferation is also induced in the proximal tubules.

2. Carcinogenicity Studies - Theories

Studies on the carcinogenicity of these compounds have focused on the following theories:

a. Unbalanced Production of Reactive Oxygen Radicals

There is strong direct evidence that peroxisomes obtained after treatment with peroxisome proliferators are capable of producing more hydrogen peroxide than the peroxisomes obtained from livers of untreated animals. The direct evidence was furnished by the work of Fahl et al

(1984) who showed that the peroxisomes from treated animals were producing 30 to 70 times more H_2O_2 compared to peroxisomes from normal livers. They also showed that both peroxisome types, but especially the ones from the animals fed peroxisome proliferators, induced DNA breaks in circular SV40 DNA. When the authors added excess catalase to these preparations to prevent the single strand DNA breaks they found that a large percentage of these breaks was not preventable by the addition of catalase. This finding, however, is difficult to interpret in view of the presence of the cyanide during the assay.

Previous studies by several investigators, referred above, had also shown that during peroxisome proliferation the induction of the peroxisomal constituent enzymes is disproportionate, with the catalase (the enzyme that hydrolyzes H_2O_2) being induced to a lesser degree than the enzymes associated with beta-oxidation (the enzymes mainly responsible for interacting with the terminal flavin oxidase and resulting in H_2O_2 production). As pointed out above, the production of excess H_2O_2 may not be the only reason for the generation of reactive oxygen species, in view of the fact that excess catalase did not prevent all the peroxisome induced DNA damage in in vitro studies. The hypothesis assumes that the reactive oxygen species can reach the cellular DNA without being inactivated by other cellular components. This can easily be visualized for the mitochondrial DNA but less so for the nuclear DNA. The same limitation should hold for all the reactive electrophiles generated from chemical carcinogens in liver cytosol.

Numerous studies have shown that in the latter occasion the activation of the carcinogenic chemical is associated with the formation of DNA adducts. The same possibility can be entertained for the excess

production of H_2O_2 from peroxisomes of peroxisome proliferator treated rats. The feasibility of long range effects of the reactive oxygen species generated in these situations may be further enhanced if the findings of Ciriolo et al (1984) are true with other peroxisome proliferators. These investigators found that when fenofibrate was given to rats, in addition to the hypolipidemic effects and peroxisomal proliferation, there was also a marked suppression of the superoxide dismutase and glutathione peroxidase, enzymes that would be involved in scavenging reactive oxygen species produced by the peroxisomes. Evidence for the increased production of H_2O_2 during peroxisome proliferation was furnished by a recent study (Tomaszewski et al 1985). These investigators estimated that as a result of peroxisome proliferation induced by DEHP there should be an increased steady state level of H_2O_2 in liver homogenates. Results of direct measurements were also reported by the same team (Tomaszewski et al, 1985) in mice and rats of both sexes, after feeding nafenopin, DEHA, and DEHP. The levels of the H_2O_2 produced at steady state by liver homogenates after addition of palmitoyl-CoA varied amongst the three compounds and between the species and sexes. These levels, however, correlated in all instances with the relative carcinogenicity of these compounds in the different species and sexes.

The production of reactive oxygen species may also cause DNA damage due to peroxidation of membrane lipids. This has been demonstrated in several publications unrelated to the study of peroxisome proliferators. Peroxidation of lipids of the cellular membranes, especially of the nuclear membrane, has been associated with the potential of causing DNA damage in several studies (Summerfield et al 1984a, Summerfield et al

1984b). Attempts to directly measure lipid peroxidation after treatment with peroxisome proliferators have met with mixed success. Reddy et al have repeatedly shown that the hepatocarcinogenesis associated with peroxisome proliferators is accompanied by dramatic accumulation of the pigment lipofuchsin, generally regarded as due to accumulation of lipochromes resulting from lipid peroxidation. On the other hand, it was recently shown (Kornbrust et al 1984) that there is no measurable lipid peroxidation during administration of DEHP in regimens associated with peroxisome proliferation.

It is possible that the use of peroxisome proliferators has to be very prolonged (of the order of months as seen in carcinogenesis protocols) in order for lipid peroxidation to be demonstrable. The notion, that peroxisome proliferation can cause DNA damage due to release of reactive oxygen species, appears to clash with some reports in which unscheduled DNA synthesis (UDS) in primary cultures of rat hepatocytes could not be demonstrated after feeding of peroxisome proliferators (Kornbrust et al 1984, Butterworth et al 1984). In another report (Glauert et al 1984) some of the peroxisome proliferators did induce UDS in hepatocyte cultures, albeit a weak response. The argument can be made that if genotoxic damage were to be produced it should be demonstrable by measurable DNA repair synthesis. It should be pointed out, however, that the assay of UDS in hepatocyte culture is more efficient for large DNA adducts, due to the fact that they tend to be associated with repair of large DNA patches. The damage due to the reactive oxygen species is known to be associated with short patch repair and hence more difficult to detect. This was shown to be true for the DNA damage induced by x-rays (Regan and Setlow 1974). X-rays

are also considered by some to cause DNA damage partly due to the formation of active oxygen species resulting from interactions between x-rays and the molecules of water in the vicinity of macromolecules. In addition, the UDS assay in hepatocytes can only assess UDS for incubations no longer than 24 to 48 hours and it is likely that more prolonged release of reactive oxygen species is required for DNA damage to be measurable.

The assays of DNA damage and repair in hepatocytes have been rather inadequate in studies aimed to demonstrate the damaging effect of x-rays. Use of alkaline elution to detect DNA damage in liver from x-rays could not demonstrate any DNA damage until at least 1,000 rads were used (Meyn and Jenkins 1983). This shows that detection of hepatic DNA damage due to reactive oxygen species may be very difficult to detect even with the most sensitive of the current techniques. This, however, may not be true in other cell systems. In the chapter on mutagenesis of this report studies are discussed that demonstrate that the techniques of unscheduled DNA synthesis and alkaline elution are capable of detecting similar types of damage in other cell types. Further evidence that the carcinogenic effect of peroxisome proliferators is associated with reactive oxygen species is also furnished by the studies in which it was shown that the antioxidants ethoxyquin and 2(3)-tert-butyl 4-hydroxyanisole inhibited the carcinogenesis induced by ciprofibrate, whereas they did not inhibit the peroxisome proliferation associated with this agent (Lalwani et al 1983b, Rao et al 1984a). The interpretation of these experiments is complicated, however, by the fact that these antioxidants inhibit the

carcinogenic effect of other carcinogens (Kahl 1982)that do not have any demonstrable effect in the generation of reactive oxygen species.

b. The Peroxisome Proliferators Themselves are Directly Genotoxic.

According to this hypothesis, the peroxisome proliferators themselves are capable of directly interacting with DNA, causing DNA damage in a manner analogous to other studies with chemical carcinogens such as aflatoxin, etc. This hypothesis is not mutually exclusive with the previous hypothesis and it is theoretically possible that both phenomena may occur. The proof that any compound is acting as a carcinogen by a direct genotoxic mechanism rests on the eventual demonstration of the formation of covalent DNA-carcinogen adducts composed of DNA bases and molecular moieties derived from the compound. This has been amply demonstrated for a variety of carcinogenic compounds. The peroxisome proliferators, including DEHP, have been shown in repeated studies not to be associated with DNA adduct formation.

The whole issue of the direct genotoxicity of DEHP is reviewed in another part of this report. In this section the peroxisome proliferators are viewed as a class of compounds. It should be mentioned in this section that even when the most sensitive technique for detection of DNA-adducts, the ^{32}P-post labelling of DNA hydrolyzates, was applied to livers of animals or hepatocytes exposed to many peroxisome proliferators including DEHP, no DNA-peroxisome proliferator adducts could be identified (Gupta et al 1985). This technique is capable of detecting adducts in the frequency of 1 in 10^{10} deoxyribonucleotides (Randerath et al 1982). As a class of compounds

the peroxisome proliferators have also been registered as negative in most of the in vitro genotoxicity bioassays, in contrast to genotoxic carcinogenic compounds. In general, the possibility that the peroxisome proliferators are directly genotoxic is rather weak and against the preponderance of the available evidence.

c. Peroxisome Proliferators Have Promoting Activity.

It is well established that in most tissues carcinogenesis proceeds through the steps of initiation and promotion. In this context, tumor promoters are considered to be substances that themselves do not possess carcinogenic activity but enhance the yield of tumors induced by an initiating carcinogen. In view of the fact that peroxisome proliferators induce tumors when they are fed by themselves they cannot be considered true promoters by the very definition of the term. It has been shown, however, in several studies that when small doses of peroxisome proliferators follow initiators they enhance the carcinogenic yield (Ward et al 1984). It was not clear from these studies whether the initiation of the tumors should be ascribed to the peroxisome proliferators or the other carcinogen. In view of the fact that the other carcinogen used for these studies (diethylnitrosamine) has never been shown to enhance the carcinogenic process in this manner, it was thought that the peroxisome proliferators acted in a "promoting manner" and enhanced the carcinogenic yield. Opposite results, showing no promoting effect for nafenopin after AAF administration, were reported recently in another report (Numoto et al 1985). When other promoters were examined for similar carcinogenic effects in their target tissues, including liver, very weak or absent carcinogenic activity could be demonstrated

(Pitot et al 1980). When peroxisome proliferators are fed at the proper doses 100% of the animals have hepatic neoplasms.

The issue of possible promoter effects is a recurrent theme in the literature on peroxisome proliferators in view of the fact that they, like phorbol esters (the well known mouse skin tumor promoters), are associated with formation of reactive oxygen species. Hydrogen peroxide itself has been shown to be a weak promoter for the mouse skin. For a review of the role of reactive oxygen species in tumor promotion see Cerruti (1985). Cerruti recently attempted to define a "pro-oxidant state" for cells by summarizing evidence from the scientific literature. According to Cerruti, the pro-oxidant state is associated with long term production of reactive oxygen species. During this state there is an orchestrated induction of cellular enzymes that result in cell proliferation and/or tumor promotion.

Despite the evidence that tumor promotion in the mouse skin is associated with increased production of oxygen radicals, there is no strong evidence that this phenomenon is associated with promotion in other tissues. In the case of peroxisome proliferators and the liver, the argument was made that somehow the formation of oxygen radicals leads to hepatic hyperplasia. There is no direct evidence that this association is a causal one. Hepatic hyperplasia associated with peroxisome proliferators can act in a "promoting" manner for tumor development, as discussed above. Hepatic cell proliferation alone (as, e.g., in chronic feeding with a choline deficient diet) does not result in hepatic neoplasia (Shinozuka et al 1979).

In summary, though the hepatic hyperplasia associated with peroxisome proliferators can act as a tumor "promoter", such a

phenomenon should not by itself result in hepatic neoplasia at the rates seen with peroxisome proliferators unless there is an accompanying source of genotoxic damage.

F. Peculiar Aspects of Hepatocarcinogenesis Associated with Peroxisome Proliferators.

In all rat hepatocarcinogenesis models used in experimental studies, at least 50% of the frank hepatocellular carcinomas and more than 80% of the early neoplastic clonal lesions are positive for the enzyme gamma glutamyl transpeptidase (GGT) (Pitot et al 1980). This enzyme is used for the demonstration of these tumors by histochemical means. In most instances of hepatocarcinogenesis associated with peroxisome proliferators the neoplasms are negative for GGT (Rao et al 1982). In addition, administration of DEHP and some other peroxisome proliferators (Perera et al 1984, De Angelo et al 1984) has been associated with rapid reversal of the GGT+ early clonal neoplastic lesions. Due to the apparent decrease in the number of the GGT+ foci, DEHP and other peroxisome proliferators were considered to be "anti-promoters" for liver carcinogenesis. This notion was in obvious contradiction with the hyperplastic state induced by peroxisome proliferators and the associated "promoting" effects of peroxisome proliferators discussed above.

The reason for this peculiarity was recently explained in a publication by Numoto (Numoto et al 1984). In that study, GGT+ lesions were preinduced by feeding the carcinogen fluorenylacetamide (N-Acetylaminofluorene, AAF). After the GGT+ lesions appeared, the animals were given peroxisome proliferators (clofibrate and nafenopin).

It was shown that the peroxisome proliferators quickly suppressed the GGT activity of the already GGT+ foci. The GGT activity was also suppressed in normal livers, by histochemistry or biochemistry. Thus, the peculiarities of the histochemistry of the liver neoplasms associated with peroxisome proliferators is due to a direct effect of these compounds on the expression of some plasma membrane enzymes. The mechanisms underlying this phenomenon and its significance are not clear.

G. Peroxisomes, Carcinogenesis and the Neonatal Liver.

Very few studies have been done on the phenomenon of peroxisome proliferation in the neonatal liver. The effect of nafenopin given to rats from 5 to 9 days post partum was examined by Staubli et al (1977). The study addressed only in a peripheral manner the issue of peroxisome proliferation as related to the neonatal liver. The data showed, however, that the phenomenon occurs in the neonate animal as intensely as it occurs in the adult animal, though a direct comparison was not made. A study by Seccombe et al (1980) addressed the development of peroxisomes by measuring the levels of hepatic catalase at different times after birth. It was shown that the catalase slowly increased till 22 days after birth. At that point there was a dramatic increase to adult levels, monitored till day 30 in that study. Similar results had been reported by Krahling et al (1979) for all the other enzymes of the peroxisomes. In a more recent study (Fahl et al 1983) it was shown that when nafenopin or Wy-14,643 were given to lactating rats, metabolites of these compounds were found in the milk and they resulted in induction of peroxisome proliferation similar to that seen in adult animals. There

have not been any studies directly aimed at comparing in detail the enzymatic profiles and the production of H_2O_2 between the adult and the neonatal liver after feeding of peroxisome proliferators. In addition, no studies have been performed to compare the sensitivity of the neonate liver to low doses of peroxisome proliferators.

Assuming that the phenomenon of peroxisome proliferation occurs in the neonatal liver as it does in the adult, an additional issue to be addressed is whether the neonate liver is intrinsically more sensitive for the development of neoplasia as compared to the adult for the same degree of genotoxic damage. A pertinent model for basing such an analysis is provided by Peraino et al (1984). In these studies administration of carcinogens to neonate animals (one day after birth) at doses that are not effective in adult rats resulted in a high incidence of neoplasia. The carcinogen benzo(a)pyrene given to adult rats does not result in hepatocarcinogenesis unless specific initiation - promotion protocols with a strong hyperplastic stimulus are used. This carcinogen is effective and results in measurable hepatocarcinogenesis when given to neonate rats (Toth 1968). The reasons for the increased sensitivity are probably related to a wave of development, including new DNA synthesis, that is seen in rat liver shortly after birth. The developmental wave is well described and is associated with the appearance of new isozymes and a change in cell ploidy. (Evans 1976, Sell et al 1974). Similar phenomena have been demonstrated for the human liver (Snell 1984, Greengard 1977). The interpretation of the data with the neonatal rat liver by Peraino et al is that when genotoxic carcinogens are administered to the neonate rat, the classic requirements for rat hepatocarcinogenesis (DNA-adduct formation and hepatic

cell hyperplasia) are met because of the hyperplastic state that is seen soon after birth with rat liver. In view of the similarity in the overall patterns of maturity between rat and human liver, similar sensitivities and responses should be expected though direct data are lacking.

H. DEHP and Peroxisome Proliferation.

In this section we will examine DEHP as a peroxisome proliferator and try to focus on aspects of that function that are unique to DEHP and may relate to its effects as a peroxisome proliferator and as a carcinogen.

Numerous studies have established the fact that DEHP is a peroxisome proliferator. Feeding of DEHP at sufficient doses (1% to 2% of the diet) results in the following hepatic changes:

a. Proliferation of peroxisomes.
b. Increase in mitochondria and endoplasmic reticulum membranes.
c. Induction of cytochrome P450 species associated with omega oxidation.
d. Hepatomegaly associated with hepatic hyperplasia.

(For an excellent review on phthalates and liver please see the Proceedings of the Conference on Phthalates, Environmental Health Perspectives vol.45, November 1982).

1. Structure Activity Relationships.

A systematic attempt to determine the part of the structure of the DEHP that is responsible for the peroxisome proliferation effect was

undertaken by Moody and Reddy (1978). Rats were fed diets containing 2% DEHP, di(2-ethylhexyl)sebacetate (DEHS), adipic acid, and diethylphtnalate (DEP) for two weeks. The typical peroxisome proliferation responses were seen with DEHP and DEHS but not with the other two compounds. In view of the finding that the di(2-ethylhexyl) moiety appeared to be the important part of the DEHP molecule, additional rats were fed diets with 2% 2-ethylhexanol, hexyl alcohol, 2-ethylhexanoic acid, hexanoic acid, 2-ethylhexyl aldehyde, hexyl aldehyde and 2-ethylhexylamine. Only the di(2-ethylhexyl) derivatives demonstrated activity.

As mentioned in other parts of this report, DEHP is rapidly hydrolyzed in most situations to 2-ethylhexanol and MEHP. Each one of these compounds is further metabolized extensively into other products. In recent studies conducted by Elcombe and his collaborators it was shown that MEHP, but not 2-ethylhexanol, induced peroxisome proliferation in hepatocyte cultures. In view of previous studies that have shown that several responses differ in magnitude between hepatocytes in culture and liver in vivo (Michalopoulos et al 1976), it is probable that the loss of responsiveness of the hepatocytes to 2-ethylhexanol only reflects adaptation to the in vitro environment.

In general, the studies performed to date have not established any single metabolite or metabolic pathway as essential for peroxisome proliferation. The maintenance of the response of hepatocytes in culture to MEHP, however, prompted Elcombe and his collaborators to conduct studies aimed at identifying the metabolites of MEHP that are responsible for the peroxisome proliferation. When several metabolites were tested Mitchell et al found that the omega oxidation products did

not induce peroxisome proliferation. The proliferation was induced, however, by MEHP and the omega-1 oxidation products (mono-(2-ethyl-5-oxohexyl)phthalate and mono(2-ethyl-5-hydroxyhexyl)phthalate (metabolites VI and IX of the Albro classification system). In another report using hepatocyte culture from the same team (Lhuguenot et al 1985), it was shown that metabolite IX is converted by dehydrogenation to metabolite VI. It appears thus as highly likely from these studies that the metabolite VI of MEHP is one of the proximate peroxisome proliferators. Though it has been shown from other studies that MEHP is rapidly converted to its metabolites, the data from these two reports do not allow one to rule out the possibility that MEHP itself is not also a peroxisome proliferator. This would be ruled out if it were possible to block the conversion of the MEHP to its products of metabolism. This does not apply to the metabolite VI because it is an end product and is not metabolized any further.

It should be noted, however, that the stoichiometry of the observed dose-response leaves some open questions about the conclusions drawn in that study. Mitchell et al (1984) showed that substantial peroxisome induction did not occur in the cultured hepatocytes until the concentration of metabolite IX or VI was 500 uM. Lhuguenot et al (1985), however, showed that when cultured rat hepatocytes were incubated with 500 uM MEHP for three days the maximum concentrations of metabolites IX and VI were 69 uM and 74 uM respectively. Thus, it is unlikely that the peroxisome induction observed when cultured rat hepatocytes were incubated with MEHP can be totally accounted for by the conversion of MEHP to metabolites IX and VI. It should also be mentioned that although metabolite I has been observed to be produced at

rates that are species- and dose-related, the role of metabolite I or any other metabolite as an index of peroxisome proliferation has not been established.

2. Relationship Between the Carcinogenic Effect of DEHP and its Peroxisome Proliferator Activity.

The whole issue of the relationship between peroxisome proliferation and carcinogenesis was discussed above. Only points that are directly relevant to DEHP will be addressed here.

Several studies have shown that whereas DEHP induces the full response of a peroxisome proliferator, other phthalates, e.g., dioctylphthalate, do not have these properties. It would be of interest to conduct carcinogenesis studies with such compounds that are chemical congeners to DEHP and have no peroxisome proliferator activity. If such studies were to be conducted and compared to the carcinogenic effects of DEHP they would help in determining in a more focused manner the relative contribution of the peroxisome proliferator properties of DEHP in the induction of hepatocarcinogenesis. At this point, as mentioned above, all compounds that are peroxisome proliferators and have been tested have been found to be carcinogenic for the rodent liver. The studies for the chemicals tested untill 1983 are summarized in an itemized form for every chemical in the 1983 review by Lalwani et al (1983c). In the case of DEHP and some other peroxisome proliferators the results by Gupta et al (1985) clearly show that in the target tissue (liver) the formation of DNA adducts, if it occurs, must be below the limit of detection of the most sensitive techniques currently employed.

balance of the available evidence with morphological and biochemical studies indicates that primates in general tend to be less sensitive to induction of peroxisome proliferation compared to rodent species (Gray et al 1984).

4. Relationship Between DEHP Dose and Peroxisome Proliferation.

There are no sufficient data to allow one to accurately determine a dose of DEHP below which peroxisome proliferation should not be expected in a long term study. In studies where the fed dose of DEHP was varied all the doses of DEHP tested induced peroxisome proliferation. In the study sponsored by the CMA the doses used were 1000, 6000 and 12000 ppm. Peroxisome proliferation was seen at all doses and it varied with the dose. The same variation was seen in the doses of 0, 50, 200 and 1000 mg/kg/day (approximately 1000, 4000, 20000 ppm) used in the study sponsored by CEFIC. A study by Morton (1979) employed the lower doses of 50, 100, 500, 1000, 2500 and 5000 ppm. Though serum triglycerides were significantly reduced at all levels tested, liver weight increased only after 1000 ppm and liver carnitine acetyltransferase and palmitoyl-CoA oxidation in the liver (the most sensitive available markers for peroxisome proliferation) increased in relation to the dose from 100 ppm and above. All three studies were of short duration (3, 4 and one week respectively). In another short term (30 day) study by Reddy et al (1985) detectable effects were found with 0.001% ciprofibrate. The same author considers the effects of 2% DEHP equivalent to 0.01% ciprofibrate.

There have been no long term studies with lower doses to allow a more precise definition of the relationship between DEHP dose and

peroxisome proliferation at long term exposures at low levels. As mentioned above, though the identification of peroxisomes in the rat is relatively easy, due to the presence of the crystalline core and a reliable histochemical reaction, this is not so in the primates. The carnitine acyltransferases of the liver should be a very sensitive and reliable assay to use for detecting peroxisome proliferation, because these enzymes are induced at high levels compared to control as opposed to other peroxisomal enzymes such as catalase, etc. These markers may be of value in studies that aim to detect the lowest possible levels of peroxisome proliferation in low dose experiments with DEHP.

I. Summary Appraisal

In the preceding sections of this chapter we presented the evidence that peroxisome proliferation and hepatic hyperplasia can result in hepatic carcinogenesis. The positive aspects of this evidence are as follows:

a: The peroxisome proliferators are compounds of diverse chemical structure. Yet most, if not all of them result in hepatic carcinogenesis. Their common functional characteristics are induction of peroxisome proliferation, hepatic hyperplasia and hypolipidemia.

b: In all the established initiation-promotion protocols for the liver, a genotoxic effect accompanied by hepatic hyperplasia (short or sustained) is required. In the absence of DNA damage the carcinogenity seen with the initiation-promotion protocols disappears. In the absence of hyperplasia, the incidence of carcinogenesis is substantially diminished.

c: It is generally acknowledged that DEHP and the other peroxisome proliferators induce hepatic hyperplasia. It has also been adequately demonstrated that these compounds lack sufficient direct genotoxic activity. Adducts formed between any of these compounds and DNA have not been identified.

d: It has been shown that during induction of peroxisome proliferation by DEHP there is a steady state increase in H_2O_2 in the hepatic cytoplasm.

e: H_2O_2 and other active oxygen species are known to be capable of inducing damage to DNA and mutagenesis.

There are also, however, some negative aspects of evidence concerning the validity of this theory. These are as follows:

a: A missing link to the proof of the hypothesis that peroxisome proliferation has the potential of resulting in hepatic DNA damage is the absence of the direct proof of formation of modified DNA bases or any other type of DNA damage during peroxisome proliferation. If this direct evidence were available, then one could make the statement that peroxisome proliferation definitely causes hepatic DNA damage. In the absence of this direct evidence, the hypothesis that peroxisome proliferation can cause DNA damage is unproven. It is, however, with all the available evidence, the most plausible of all the hypotheses that we have available, in order to explain the mode of action of DEHP and the other peroxisome proliferator compounds.

b: Despite the fact that peroxisome proliferation is also induced in the kidney, there are no reports for tumors associated in