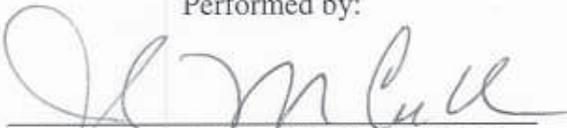


Expert Review of Revised Technical Review of Diisononyl Phthalate

Performed for the American Chemistry Council

8/31/2005

Performed by:



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I have reviewed the Revised Technical Review of Diisononyl Phthalate (DINP) issued by the Office of Environmental Information, Environmental Analysis Division, Analytical Support Branch, dated March 4th, 2005 as well as related documents from studies conducted in nonhuman primates (Webley, 1998), rats (Moore, 1998; Lington et al., 1997), a PWG report from studies in rats conducted by the Experimental Pathology Laboratories, Inc. and a number of ancillary references with the purpose of investigating the conclusions of the Revised Technical Review of DINP with an emphasis on the liver-related findings. My background includes more than 20 years as a veterinary pathologist with a specific interest in liver disease on the faculty of North Carolina State University College of Veterinary Medicine. I also have an adjunct appointment in the Toxicology Department at NCSU. I have a PhD in Comparative Pathology in addition to my veterinary degree. I am a board certified pathologist, being certified in 1982. Over the last 20 years I have consulted for a variety of governmental agencies, including the National Toxicology Program, serving a term on the Board of Scientific Councilors. I have attached my C.V. for reference. My analysis follows and is divided into a background section on spongiosis hepatitis and separate discussions of spongiosis hepatitis, increased liver weight/ peroxisomal proliferation issues and hepatic liver enzyme changes.

Background on Spongiosis Hepatitis

Spongiosis hepatitis, or as it is also known, cystic degeneration, has an uncertain pathogenesis and has a history of controversy regarding its significance. It is a spontaneous lesion in the liver of ageing rats, appearing most often in the second year of life, with a strong predilection for male animals. The incidence of spongiosis hepatitis can reach 34% in male Fischer rats as a spontaneous lesion and it can be increased and the age of onset reduced by exposure to a diverse number of chemicals. Some of these chemicals are genotoxic, but also a number of nongenotoxic agents as well, including Di(2-ethylhexyl)phthalate (DEHP), a known peroxisome proliferator, have been shown to produce spongiosis hepatitis (Karbe and Kerlin, 2002; David et al. 2000).

The lesion is composed of altered sinusoidal lining cells interpreted as hepatic stellate cells (Ito cells or fat-storing cells). The characteristic histologic appearance, as described by Bannasch and co-authors in rats treated with carcinogens, is that of a uni- or multilocular cyst-like formation filled with finely granular or flocculent acidophilic material (Bannasch, 1981). The lesion tends to replace lost hepatic parenchyma or interdigitate between hepatocytes, rather than compress adjacent parenchyma. The cystic spaces are lined, not by endothelium or epithelium, but by extracellular matrix components typical of those produced by hepatic stellate cells among others. These elements include collagen III and IV, notably not collagen I as would be typical for activated hepatic stellate cells. This extracellular matrix abuts flattened fibroblastic-like cells that contain small lipid vacuoles typical of hepatic stellate cells (see below for discussion of this family of cells). Cell markers typical of hepatic stellate cells include desmin and occasionally smooth muscle actin. The contents of the cyst-like structures are suggestive of proteoglycans, consistent with products of stellate cell metabolism.

Ultrastructurally, cells forming the walls of the lesions resemble hepatic stellate cells both in conformation (interdigitations between hepatocytes and a subendothelial location in early lesions) and on the basis of cytoplasmic lipid droplets. Spontaneous spongiosis hepatitis is less likely to be multilocular and is less often associated with stellate cell aggregates. Spongiosis hepatitis lesions can, in carcinogen-treated rats, be associated with or found within aggregates of hepatic stellate cells. Spongiosis hepatitis is often found within other hepatic lesions such as altered foci and hepatocellular neoplasms. One group of authors associated with the Bannasch laboratory regard spongiosis hepatitis to be a preneoplastic or neoplastic lesion (Bannasch and Zerban, 1986; Bannasch and Zerban, 1997) based primarily on its association with exposure to hepatic carcinogens, the increased rate of cell proliferation and the persistence of cell proliferation following withdrawal of the initial chemical treatment. Karbe and Kerlin (Karbe and Kerlin, 2002) have argued that there is insufficient evidence to warrant this designation, since these characteristics are not necessarily indicative of a neoplastic process and that spongiosis hepatitis is more likely a degenerative response.

The broad consensus of pathologists appears to support the view that spongiosis hepatitis is a degenerative change. In the definitive text written largely by pathologists from the National Toxicology Program, Pathology of the Fischer Rat and published in 1990, spongiosis hepatitis is described as a degenerative lesion (Boorman et al., 1990). (This remains the leading text in the field.) It is clear that the response is multifocal and represents altered metabolism and proliferation of a population or populations of subendothelial lining cells of the sinusoids.

The cell of origin of spongiosis hepatitis, regarded as the hepatic stellate cell, is probably less clear than assumed. In recent years, several fibroblastic cell types in addition to hepatic stellate cells have been described in the liver sinusoids and connective tissue of the portal tract and perivenular region of the central veins. These include, in addition to the hepatic stellate cell, transitional cells and myofibroblasts. All are reported to be present in the perisinusoidal spaces (Space of Disse). In addition, other lineages with distinct immunohistochemical signatures, septal myofibroblasts and interface myofibroblasts have been recently described in rats (Cassiman and Roskams, 2002). Thus, there are a number of cells, some of which may be species-specific (i.e., rat only) or have species-specific responses, within the group that has previously been aggregated into a single category of hepatic stellate cell.

Importantly, spongiosis hepatitis is a lesion that appears to be confined to rats, particularly male rats¹, and teleost fish. Careful review of rodents over the last twenty or more years by the National Toxicology Program has led to only a rare incidence of neoplasms arising from stellate cells in mice (13 cases from more than 90,000 mice), but these lesions differ morphologically from spongiosis hepatitis. There was no evidence of a lesion resembling spongiosis hepatitis in a review of 163 human livers conducted by members of the Bannasch laboratory (Su et al., 1997). Indeed, in the chapter on liver neoplasia from a definitive text on human liver disease, Pathology of the Liver edited by R.N.M. MacSween et al., the authors, including Dr. Bannasch, state: "To the best of our knowledge no human counterpart of the spongiotic pericytoma [*spongiosis hepatitis*] has ever been described." (Term in brackets added.) I was unable to find any reports of

¹ Although spongiosis hepatitis occurs spontaneously in female Fischer 344 rats, the incidence is lower than that found in the male rats, and was not increased by treatment with DINP.

lesions with similar characteristics in humans or non-human primates in the available literature. This lesion or lesions with similar appearances are not described in any of a number of standard texts on neoplasia or systemic pathology in domestic animals and there are no reports of this lesion in dogs in my literature review. Given the large number of laboratory dogs and primates that have been exposed to a broad variety of chemicals over a considerable number of years, the absence of descriptions of this lesion would support the view that spongiosis hepatitis is primarily confined to male rats and teleost fish.

Review of Revised Technical Review of Diisononyl Phthalate

Spongiosis Hepatis

My review of the data from the two rat studies and the marmoset study is essentially in accord with the findings of the Revised Technical Review. There is data to support the observation as stated in the integrated summary that: "The incidence of spongiosis hepatitis was dose-related and significantly elevated in rats chronically treated with DINP in two independent studies conducted by different laboratories." A review of the PWG report supports this conclusion. However, for reasons detailed below I do not believe that data presented concerning spongiosis hepatitis is evidence of *serious* liver injury in rats treated with DINP, nor does it support the conclusion that DINP can be anticipated to cause serious liver injury *in humans*.

With respect to the two chronic studies that formed the basis for this review (Lington et al., 1997; Moore, 1998) the deviation from the accepted practice of only diagnosing spongiosis hepatitis when it is found separate from altered foci or hepatocellular neoplasia, which was done in both studies, may have confounded interpretation of the incidence of spongiosis hepatitis. However, if spongiotic lesions were proportionately associated with preneoplastic or neoplastic hepatocellular lesions this altered evaluation system would have been unlikely to affect the final count of spongiotic lesions, since the aggregate number of preneoplastic and neoplastic hepatocellular lesions for each treatment group/sex was not dramatically different and, in my interpretation, not likely to significantly affect the total number of lesions of spongiosis hepatitis.

Clearly, more spongiotic lesions were identified in the Lington study (Lington, 1997) than the Moore study (Moore, 1998) given that there were more sections of liver examined. Since this method was applied to all groups in the Lington study (Lington, 1997) it should not affect the accurate comparison of groups.

The severity grade of spongiosis hepatitis in DINP-treated male rats that had diagnoses of spongiosis hepatitis from the Lington study (Lington, 1997) was determined during a re-evaluation using a subjective scale based on size and number of lesions in each of 4 liver lobes (Brown, 2000). A subjective scale with 1 as minimal, 2 as mild, 3 as moderate and 4 as severe was used. For rats found dead the average scores for all groups were: control, 1.0; 30 ppm, 1.17; 300 ppm, 1.5 and 600 ppm, 1.5. For rats from the terminal sacrifice the average scores were: control, 1.10; 30 ppm, 1.28; 300 ppm, 1.61 and 600 ppm, 1.57. Overall, when both groups are combined, the majority of the lesions were minimal to mild with some lesions graded as moderate. None of the lesions was considered to be severe. Approximately 94% of the grading in the top two doses was in

the minimal and mild categories. The utility of this type of subjective review has limitations. A morphometric analysis would be likely to be more meaningful. However, it is evident from this review that there is no dramatic dose-related change in the character of the spongiosis hepatitis in DINP-treated male rats and that these data do not provide evidence of a severe liver injury.

Conclusion Regarding Spongiosis Hepatis

In summary, the Revised Technical Review of Diisononyl Phthalate (EPA, 2005), related peer review documents and the findings of spongiosis hepatitis have been reviewed. The Revised Technical Review document (EPA, 2005) reflects the primary data in the original studies and the PWG review accurately. A dose-related increase in spongiosis hepatitis in male rats was evident in both studies (Moore, 1998; Lington et al., 1997), although the non-standard procedure of enumerating spongiotic lesions found in preneoplastic or neoplastic hepatocellular lesions may have affected the determinations of incidence of the lesions. However, the significance of spongiosis hepatitis is controversial. Clearly, spongiosis hepatitis is a spontaneous age-related lesion in rats and as such, the lesion cannot be interpreted as an indication of serious liver disease. Spongiosis hepatitis can be seen as an indicator of nonspecific liver injury. Specifically, the incidence of spongiosis hepatitis can be increased and the age of onset of the lesion can be lowered by treatment with carcinogens. The lesion is broadly regarded as a secondary change associated with exposure to carcinogens, but is not itself generally believed to pose a risk of malignant neoplasia or pose a significant health risk by itself (Karbe and Kerlin, 2002), although one group regards spongiosis hepatitis as a marker for hepatocarcinogenic effects (Bannasch, 2003).

The most significant issue to come out of this review is the conclusion of the Revised Technical Review (EPA, 2005) that, given the asserted absence of data that spongiosis hepatitis is a species-specific lesion, spongiosis hepatitis is (or may be) relevant to humans. As stated in the Conclusions on Chronic Toxicity, Section 1, pg.66 "In the absence of information that clearly indicates a species-specific mode of action for development of spongiosis hepatitis, the occurrence of this lesion in rats is assumed to be relevant for humans." It is clear that the pathogenesis of spongiosis hepatitis is unknown and that the specific mode of causality has not been identified. However, a review of the literature (see above) leads to the conclusion that spongiosis hepatitis is a lesion confined almost entirely to one species and with a marked predominance in males. I could find no citations in PubMed for the last 20 years identifying a similar lesion (search terms: spongiosis hepatitis, cystic degeneration, microcystic degeneration, spongiotic pericytoma) in humans or other species with the exception of teleost fish. Given the distinctive appearance of the lesion histologically, it is unlikely that this lesion would go unreported. While it is not possible to prove a negative, the virtual absence of reports in the literature should be interpreted as strong support for the species specificity of the lesion. It is my expert judgment that, the weight of evidence strongly supports the conclusion that spongiosis hepatitis is primarily a male rat-specific response and is not indicative of a human health threat. In that regard, spongiosis hepatitis is analogous to another male rat-restricted response, alpha 2u-microglobulin nephropathy, which is not regarded as

indicative of human health threats. Spongiosis hepatitis is recognized as an age-related spontaneous lesion and poses no significant health threat to affected animals, particularly where the lesions present are almost entirely minimal to mild in severity, as was the case for DINP. Given this perspective, the conclusion that DINP poses or can be reasonably anticipated to cause serious or irreversible hepatic toxicity in humans is not supported by the DINP findings or the literature (or lack of literature) on the biology of spongiosis hepatitis. The relationship to human health is, at most, unclear, and based on the scientific evidence discussed here, it is highly doubtful that there is any relationship. As such, the presence of spongiosis hepatitis in the livers of DINP-treated male rats is not, in my opinion, sufficient evidence to conclude that DINP can be reasonably anticipated to cause serious or irreversible liver toxicity in humans.

Increased Liver Weight/Peroxisome Proliferation in Rats and Marmosets

Increases in liver weights were cited in the Revised Technical Review of DINP as a one of the indications for severe liver disease. In the Moore study liver weight to body weight ratios were significantly increased in male rats in the 600 ppm, the 1200 ppm, and the 1200 ppm recovery dose groups and in female rats in the 600 ppm and 1200 ppm dose groups. Significant body weight reductions were observed in these dose groups for male rats and for the 1200 ppm dose group in the females. This decrease in the denominator of the ratio can lead to an apparent increase in liver weight, but in all cases the liver to brain weight ratio was also increased confirming a relative increase in liver weight for these groups. In the Lington study 300 ppm and 600 ppm male and female DINP-treated rats had increased liver weight/body weight ratios starting at 6 months of treatment and continuing until 24 months of treatment, although there were some body weight decreases in the male 600 ppm group from 12 months on and in the female 300 ppm group at 24 months. The liver to brain weight ratio was not provided in this study. However, the mechanism leading to the liver weight increases is not clear. The cause for the increases in liver weight for the male rats is likely to be multifactorial. Given the presence of mononuclear cell leukemia and hepatocellular neoplasia, liver weight data is difficult to interpret. It should be noted that liver weights in male and female DINP-treated marmosets, which may be more representative of the human response, were not affected by 13 weeks of treatment with 2500 mg/kg/day of DINP (Webley, 1998).

Peroxisome proliferators have the ability to increase liver weight through stimulation of peroxisome proliferator-activated receptor (PPAR) alpha causing an increase in cell proliferation as well as expansion of cell volume through increased formation of peroxisomes (Cattley et al., 1998). This activity may provide an explanation for increases in liver weight. There are several publications demonstrating that DINP has peroxisome proliferator activity in mice (Kaufmann et al, 2002; Valles et al., 2003). DINP has also been shown to have modest peroxisomal proliferative capacity in rats (Smith et al., 2000; Moore, 1998).

In the Moore Study (Moore, 1998) significant elevations in palmitoyl-CoA oxidase were observed in the 12,000 ppm DINP dose group male rats at all time points (weeks 1,2,13,104) and female rats at 6,000 ppm at 104 weeks. The main histologic

finding in hepatocytes in the Moore study related to increased hepatocyte eosinophilia and increased hepatocyte size in the majority of male and female rats treated with 12,000 ppm, both of which are characteristic responses to peroxisome proliferators. These findings lead to the conclusion that peroxisome proliferation in the 12,000 ppm dose group is present. It is possible that peroxisome proliferation may play a role at the 6,000 ppm dose as well given the increase in palmitoyl-CoA oxidase levels in the liver of female rats in the Moore study. Therefore, in the high dose group (12,000 ppm) the increased liver weight may likely be attributed to peroxisome proliferation given the lack of significant vacuolization, congestion or other disturbances to account for liver mass increases. It should be noted that DINP has been shown to induce smooth endoplasmic reticulum (microsomes), which could contribute to the increased liver weight and can lead to increased cytoplasmic eosinophilia and modestly increase cell size (Bird et al. 1986). It is not clear that peroxisome proliferation affected the lower dose groups, as there was no evidence of peroxisome proliferation by morphological evaluation in the Lington (Lington et al., 1997) study (maximum dose 6,000 ppm DINP) in any of the DINP-treated rats. Additional studies, provided as unpublished reports (Certa, 1993; Jansen et al., 1992) add some support for peroxisomal proliferation activity in rats treated with lower doses of DINP, but the changes in enzyme activity are relatively small and are not consistent between studies. In a study by Certa (Certa, 1993), dodecanoic acid 12-hydroxylase was statistically increased in female Fischer-344 rats gavaged with DINP for 14 days at doses that were approximately 500 and 1500 ppm of DINP in one study, but not another. An additional study (Jansen et al., 1992) showed modest elevations in peroxisome-related enzymes with enoyl CoA hydratase statistically elevated at a dose of approximately 4000 ppm of DINP. This and other enzymes were elevated in rats gavaged with approximately 12,000 ppm, as well in this study. These data raise the possibility, but do not confirm that DINP may be associated with increased peroxisomal enzymes at doses around 3000 ppm. DINP stimulation of PPAR alpha may have a more potent effect on cell proliferation than peroxisome proliferation at these doses.

The main point, however, is that the liver weight data from the two chronic studies of DINP do not, by themselves, support a conclusion of "serious" liver injury in the rats. Further, because no changes in liver weights were seen in primates following 13 weeks of DINP exposure at the dramatically higher level of 2500 mg/kg/day (Webley, 1998), the data as a whole do not support the conclusion that DINP can be reasonably anticipated to cause significant liver weight changes in humans, and do not support the conclusion that DINP can reasonably be anticipated to cause any serious liver effects in humans.

Hepatic Transaminase Levels

EPA cites the liver enzyme data from the studies conducted by Lington (Lington et al., 1997) and by Moore (Moore, 1998) as evidence of DINP-exposure-related hepatotoxicity in rats. Overall, the increases in serum transaminases are modest. The interpretive emphasis should be placed on ALT, as it is the most specific transaminase to assess liver injury in the rat. AST is regarded as not specific for liver injury in the rat because it is found in a number of other tissues (Loeb and Quimby, 1999; Boone et al., 2005). In a preclinical setting, recommended guidelines indicate that ALT levels are not

typical levels in rats and similar increases in humans. It is noteworthy that marmosets exposed up to and including 2500 mg/kg/d for 90 days did not have increased liver weights or any liver enzyme elevations at week 4 or week 13 (Webley, 1998).

Table 1. Mean Liver Enzyme Values (IU/L) in Male DINP-Treated Rats (Lington Study)

Liver Enzyme	Month	0	0.03	0.3	0.6
AST	6	68 ± 8 [#]	71 ± 15	102 ± 32 [*]	137 ± 95 [*]
	12	102 ± 17	101 ± 22	145 ± 36 [*]	185 ± 86 [*]
	18	69 ± 7	68 ± 12	113 ± 76	146 ± 105 [*]
	24	92 ± 42	93 ± 72	112 ± 58	206 ± 220
ALT	6	37 ± 8	38 ± 7	81 ± 52	128 ± 145 [*]
	12	71 ± 13	70 ± 21	113 ± 56	158 ± 101
	18	42 ± 10	39 ± 7	69 ± 39	128 ± 126 [*]
	24	42 ± 23	45 ± 23	89 ± 76 [*]	74 ± 68
ALK Phos	6	56 ± 10	54 ± 6	59 ± 6	72 ± 16 [*]
	12	49 ± 9	51 ± 13	54 ± 13	60 ± 19
	18	49 ± 6	41 ± 6	65 ± 48	63 ± 18
	24	41 ± 14	47 ± 38	65 ± 47 [*]	116 ± 132 [*]

= Standard Deviation

Table 2. Mean Liver Enzyme Values (IU/L) in Male DINP-Treated Rats (Moore Study)

Liver Enzyme	Month	0	500 ppm	1500 ppm	6000 ppm	1200 ppm	1200 ppm/stop
AST	6	102 ± 49 [#]	94 ± 19	93 ± 17	105 ± 37	93 ± 46	80 ± 12
	12	99 ± 25	106 ± 23	125 ± 47	210 ± 97 [*]	132 ± 40 [*]	181 ± 60 [*]
	18	83 ± 11	96 ± 29	86 ± 12	128 ± 55	124 ± 70	107 ± 79
	24	86 ± 32	94 ± 38	76 ± 16	203 ± 249 [*]	175 ± 162	225 ± 224 [*]
ALT	6	75 ± 41	65 ± 10	66 ± 13	70 ± 27	64 ± 19	58 ± 9
	12	75 ± 24	79 ± 17	97 ± 44	180 ± 91 [*]	122 ± 42 [*]	177 ± 86 [*]
	18	54 ± 6	69 ± 16	58 ± 9	90 ± 37 [*]	93 ± 56	83 ± 59
	24	53 ± 16	60 ± 18	51 ± 10	118 ± 107	113 ± 103	118 ± 111

= Standard Deviation

In summary, a review of the liver transaminase levels, with an emphasis on serum ALT, shows that there is a DINP treatment-related enzyme increase in male rats. The proportion of enzyme elevations is modest. It would be more appropriate to interpret the enzyme elevations as evidence of, at most, moderate liver injury at 6000 ppm and 12000 ppm dose groups.

It is important to note that the effects seen in rats were not duplicated in primates dosed at levels up to 2500 mg/kg/day. No significant liver enzyme changes or liver weight changes were evident in treated marmosets, casting doubt on the relevance of the rodent data for human health effects (Webley, 1998).

Overall Conclusions

The main histologic lesion, spongiosis hepatitis, can be regarded as a rat-specific lesion without a counterpart in human hepatic pathology. The enzyme and liver weight data from the two studies of DINP administration in rats do not support a conclusion of "serious" liver injury induced by DINP in rats. Notably, primates did not develop any hepatic or clinical pathology abnormalities following 13 weeks of DINP exposure at levels up to 2500 mg/kg/day. Under the conditions of the reviewed studies my conclusion is that DINP should not be considered to pose a significant hazard to humans for serious chronic liver injury. In my expert opinion, the available data, considered as a whole, do not support the conclusion that DINP is known to cause or can reasonably be anticipated to cause serious or irreversible liver toxicity in humans

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