Bis-(2-ethylhexyl) phthalate, An Ubiquitous Environmental Contaminant

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ABSTRACT

Bis-(2-ethylhexyl) phthalate (DEHP) is the most commonly used plasticizing agent for the widely used plastic polyvinylchloride (PVC). Consequently, this compound is found everywhere in the environment of civilization, where it is in frequent contact with every person. Blood storage bags and tubing, food wrappers, and many children's toys contain appreciable amounts of DEHP.

Given this frequency of exposure, the toxic potential of the compound has become a major concern. Many workers have demonstrated its exceedingly low acute toxicity, while results from chronic exposure studies have been mixed. However, in 1982 the National Toxicology Program reported a significantly increased incidence of hepatocellular carcinoma in rats and mice exposed to high doses of DEHP over a period of two years. The significance of these studies remains in question.

Bis-(2-ethylhexyl) phthalate is metabolized extensively by mammals, but reports of the direct study of the toxic effects of its metabolites are few. Efficient methods for analysis of biological samples for DEHP are available, but they are complicated by the constant presence of this compound as a contaminant.

Introduction

Bis-(2-ethylhexyl) phthalate (DEHP), the most common plasticizer for the ubiquitous polyvinylchloride, has attracted increasing attention in recent years as an environmental and biomedical pollutant. The acute toxicity of DEHP is vanishingly low, but long-term exposure to this plasticizer has been associated with biological effects, particularly liver changes, in a variety of laboratory animals. Recently, liver carcinogenicity has been demonstrated in mice and rats. Bis-(2-ethylhexyl) phthalate is a major component of blood bank bags and surgical tubing, among other products, and the toxicity findings present possible serious complications for the practice of health care.

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The initial step in metabolism of DEHP is a partial hydrolysis to ethylhexyl phthalate, followed by oxidation of the remaining side chain, the extent of which is species dependent. It is rapidly cleared from mammalian systems after acute administration. Reports of residual concentrations in human blood and tissues is probably due to continued environmental exposure. They could also be due to artefacts in the analytical procedure, or to a difference in DEHP elimination after long-term or repeated exposure.

Bis-(2-ethylhexyl) phthalate has been identified and quantified in stored blood and in the plasma of hemodialysis patients, where it appears to be associated with lipoproteins. This association with lipoprotein makes DEHP somewhat difficult to extract from serum; many early reports of DEHP concentrations in stored blood are erroneously low. Gas chromatographic analysis of the extracts shows that stored blood bank plasma may achieve DEHP concentrations in the order of 200 mg per liter before it becomes outdated. Monitoring human exposure and estimating residual body burdens have become important concerns.

Metabolism of Bis-(2-ethylhexyl) phthalate

Following single dose, oral administration in rats, bis-(2-ethylhexyl) phthalate (DEHP) is cleared rapidly. In feeding studies using $^{14}$C-DEHP, rats were fed 500 mg per kg through a stomach tube. About 80 percent of the dose was excreted in the urine and feces within five to seven days. Concomitant results were obtained for rats receiving 50 mg per kg i.v. In rats fed 1,000 and 5,000 ppm continuously, a steady-state concentration in liver and abdominal fat is rapidly achieved, and there is no evidence of accumulation. Excretion becomes independent of dose, up to 180 mg per kg per day, in rats allowed an adaptation period of about four days. Beyond this feeding regimen, there is evidence of saturation of the metabolic mechanism.

Rapid clearance from primates has also been described. More than 50 percent of the infused dose given two cancer patients (94.7 mg in four hours and 174.3 mg in 1.5 hours, respectively) appeared in the urine within six hours. Similar observations were made using the African Green monkey.

Metabolism of DEHP begins with hydrolysis to the monoester, mono-(2-ethylhexyl) phthalate (MEHP). Albro and Thomas present results describing this step using a large variety of rat tissue lipases exhibiting DEHP-hydrolase activity. Monoglyceride and lipoprotein lipases which are adipose hormone sensitive, as well as plasma lipoprotein lipases, lung lipases, mucosal, liver and intestinal lipases (including those from the pancreatic juice and mucosa), were all able to hydrolyse the diester. According to this study, DEHP administered orally would have little opportunity to be absorbed intact owing to hydrolysis by intestinal enzymes. However, injected DEHP would survive long enough for all tested tissue lipases and lipoprotein lipases to play some role in its metabolism. Only glycerol-ester hydrolase (EC 3.1.1.3) and steroleser hydrolase (EC 3.1.1.13) were unable to hydrolyze DEHP. However, in the presence of bile salts, the DEHP-hydrolase activity of the former was enhanced. Although some activity was found in every tissue examined, the bulk of the activity was in the pancreas, liver, and intestinal mucosa. Out of 15 different preparations, only the alkaline liver lipase further hydrolyzed MEHP to the diacid. Oral administration of MEHP to rats by cannula, gavage, or stomach tube demonstrates very rapid metabolism. After a 69 mg per kg dose, more than 80 percent was excreted in 24
hours: 72 ± 2 percent in urine and 8 ± 4 percent in feces.

Mono-(2-ethylhexyl) phthalate undergoes (ω) and (ω-1) oxidation. The alcohol intermediates may then be oxidized to the level of ketone after (ω-1) oxidation or acid after ω-oxidation. Subsequently, the acid may undergo β-oxidation. Recent studies describing the detection of several heretofore unknown polar urinary metabolites of DEHP in the rat, suggest that this description of the metabolic pathway of DEHP be modified. It is argued that a dual modification be made to include the possibility of both a more distant hydroxylation than ω-1, from the methyl terminus, and either simultaneous oxidation at two sites on MEHP or a recycling of an already oxidized product for a second oxidative attack. These arguments are based upon the identification of metabolites which are more polar than previously described whose structures cannot be adequately explained by the original metabolic sequence. These findings warrant further investigation.

Species dependent metabolic differences are prominent, particularly between rodents and primates. The principal point of difference is in the formation of glucuronide conjugates and the extent of oxidative metabolism. In six species tested, five excrete glucuronide conjugates and one does not. The rat is the only species that excretes free metabolites exclusively. Of the other five, the green monkey and man excrete the highest percentage of conjugated metabolites. In a somewhat compensatory mechanism, the predominant metabolites excreted by rats have carboxyl groups on their side chains (diacids); these metabolites require three to six oxidative steps for their formation. In contrast, glucuronide conjugates require no oxidative steps and alcohols require only one. Thus, the requirements for oxidative metabolism by the liver in rats and man, in the instance of a high dose of DEHP, would be very different. This point has been used to question the validity of the rat as a homologous system to man in which to test DEHP toxicity.

The urinary metabolites of DEHP and MEHP have been isolated and identified for rat, mouse, guinea pig, Green monkey, hamster and man, using thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography/mass spectrometry (GC/MS), infrared spectrophotometry (IR), and nuclear magnetic resonance (NMR). Early work identified four major metabolites of DEHP. However, upon acid hydrolysis, the only substituent found was phthalic acid, indicating that the aromatic ring had remained intact throughout the metabolic process. The proposed metabolic pathway of DEHP suggesting MEHP as the intermediate was supported by Chu et al in which rats were fed MEHP and the metabolites isolated. It was found that MEHP is responsible for the metabolites formed via oxidative metabolism. Mono-(2-ethylhexyl) phthalate, given orally, yields the same metabolites as DEHP suggesting MEHP as the intermediary product in DEHP metabolism.

Later studies in rodents and primates have identified several more metabolites found in urine, the structure, distribution and forms of excretion products of which have been clearly described. The prominent differences in oxidative metabolism between rats and primates are evident from this data. For rats, 75 percent of their total urinary metabolites are diacids (principally mono-(4-carboxy-
2-ethylbutyl) phthalate), whereas these compounds account for less than 10 percent of the total for man and Green monkey combined. In contrast, the formation of alcohols and ketones predominate in man and monkey (68.3 percent and 56.9 percent, respectively; particularly, mono-(2-ethyl-5-hydroxyhexyl) phthalate), but only amount to 19 percent of total DEHP metabolites in rat. The simple end products of DEHP oxidative metabolism for primates together with a propensity toward glucuronide conjugation, contrast with the extensive oxidative processes the rat must operate to eliminate DEHP.

The published descriptions of the metabolism of DEHP in various mammals are quite consistent, but with the expected progressively increasing number of metabolites identified in most systems as more recent papers are presented. The differences in metabolism demonstrated by the various species raises serious doubts about extrapolation from one to another. Only the non-human primates studied metabolize DEHP along a pathway similar to that of man.

Methods of Assay

In the early 1970's, several reports were published describing methods for the determination of bis-(2-ethylhexyl) phthalate (DEHP) in the tissue of human subjects who had received transfusion of polyvinylchloride (PVC) bag stored blood or who had undergone hemodialysis. However, a follow-up letter by one of the reporting groups indicated that they had found the same prevalence of DEHP in the tissues of patients who had not undergone transfusion. The same letter explained that their analytical technique did not permit accurate comparison of the quantity of the plasticizer in the tissues of the treated and untreated subjects. This called into immediate question the reliability of these early procedures for assaying DEHP. Based on the observation that existing methods for assay of DEHP were in many cases unreliable, a series of new procedures was described for analysis of biologic material (table I). Several of the previously reported methods give erratic results and low and erratic recovery values in our laboratory. Many of the methods employ methanol in the extracting solvent, probably causing some transesterification of the DEHP. Experiments in our laboratory show that DEHP in the presence of methanol is extensively transformed to methyl 2-ethylhexyl phthalate at gas chromatography injection port temperatures. Because of the possibility of residual methanol in the sample, the many methods employing it in the extracting solvent should be avoided or used with extreme care. Also the plasticizer, DEHP, is bound to lipoprotein and extraction requires denaturation of the sample protein during the procedure. Supporting this contention is our observation that the relatively low recovery of DEHP from plasma by simple chloroform extraction can be increased by pre-treating the plasma with sodium dodecyl sulfate.

Variability of the assay methods may explain some of the differences in reported concentrations of DEHP in bag stored whole blood. These concentrations are substantial, approaching 120 g per liter after three weeks of storage. In analyzing the blood and tissues, where the concentrations of DEHP may be three orders of magnitude lower, another variability factor becomes important. This plasticizer is a widely distributed environmental contaminant. In our laboratory, a jar of clean alumina, previously heated for 12 hours at 500°F will pick up extractable amounts of DEHP from the ambient air within one week. All glassware must be freshly acid washed for use in the assay.
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In our hands, the method described by Rock and co-workers has proven quite reliable when used with these precautions in mind. The method uses water, acetonitrile, and hexane as an extraction solvent, with flame ionization gas chromatography for the analysis. The recovery of DEHP from plasma is between 95 percent and 98 percent in our laboratory. The sample background from the extraction of plasma is low, and no interfering peaks have been found from human sam-
pies. No back-extraction or solid phase sample cleaning is required. The method can easily quantitate 20 ng of DEHP in a one ml plasma sample, and it has been confirmed by computerized gas chromatography-mass spectrometry in our laboratory that the sample peak interpreted as DEHP consists of only this compound. The within-run coefficient of variation of this method in our hands is eight percent at 100 ng per ml.

**Acute and Chronic Toxicity**

The literature is replete with information regarding the very low order of acute toxicity of bis-(2-ethylhexyl) phthalate, the earliest published report being that of Hodge in 1943. Doses of up to 34 g per kg body weight given via stomach tube to 50 rats failed to produce any deaths. Intraperitoneal injections of up to 128 g per kg to 65 mice resulted in only seven deaths. These early results were soon corroborated by Shaffer et al with information being reported for the rabbit also (oral LD 50 = 33.9 g per kg). Other workers have further substantiated these findings. 29

One observation of note is that a comparison of acute LD 50 to chronic LD 50 for DEHP in mice reveals an increase in toxicity when these animals are exposed over a long period. Intraperitoneal injections of the undiluted compound reveals an acute LD 50 of 38.35 ml per kg which decreases to 6.40 ml per kg at the end of the first week of repeated administration. Although the acute toxicity is exceedingly low, the question of the potentially harmful effects of chronic exposure has lead to numerous investigations. 10,29,45

Carcinogenicity

Numerous studies of the potential carcinogenicity of bis-(2-ethylhexyl) phthalate and other phthalate esters have been carried out in rats and mice. A short review of this work through 1977 has been published. All of these studies suffer various limitations ranging from too few animals and poor survival to limited pathology examinations and result reporting. These experiments do not allow an evaluation of the carcinogenicity of these compounds in rats and mice.

Kluwe et al, in a two-year carcinogenesis bioassay performed with Fisher 344 rats and B6C3F1 mice to study the effects of chronic exposure to DEHP, reports a positive correlation between exposure and increased incidence of hepatocellular carcinoma. This is the first
study of the chronic exposure to DEHP to report these findings.

Subjected to very high doses of dietary DEHP (six or 12 g DEHP per kg feed for rats; three or six g DEHP per g feed for mice) for an extended period of time (103 consecutive weeks), animals of both sexes developed tumors of the liver at a significantly increased incidence compared with a concurrent control population. Twenty of the 57 diagnosed hepatocellular carcinomas in the DEHP treated mice (sexes and doses combined) had metastasized to the lung. Pulmonary metastases were not observed in the control mice nor in any of the rats. Male rats ingesting the higher dose (12 g per kg diet) exhibited a 90 percent incidence of severe seminiferous tubular degeneration and testicular atrophy in comparison to a two percent incidence in controls. There was also evidence of pituitary hypertrophy in this population. These were the only non-neoplastic lesions detected in the rat. Male mice on the higher dose (six g per kg diet) also exhibited testicular atrophy as well as a higher incidence of chronic inflammation of the kidney.

The conclusions from this study have been called into question on the basis of several points in experimental procedure and result interpretation (e.g., control group/test group comparisons, exceeding the “Maximum Tolerated Dose”, species dependent metabolic differences between rodents and primates, etc.). Each of these points has been adequately dealt with in a later paper by the author of the original study. The official report from the National Toxicology Program, in reviewing the results of this study, states that “under the conditions of the bioassay, DEHP was carcinogenic for F344 rats and B6C3F1 mice of either sex, causing an increased incidence of hepatocellular carcinoma.” The definitive significance of this data, however, to the potential carcinogenicity of DEHP in humans will require further study; a situation this compound shares with many others demonstrated to be carcinogenic in rodents. The question of projecting the results of DEHP carcinogenicity testing in rats to humans is particularly acute because of the considerable difference in the way these species metabolize this compound.

**DEHP Induced Hepatic Changes**

Together with progressive enlargement of the liver and proliferation of the smooth endoplasmic reticulum, clearly demonstrable changes in hepatic enzyme activity associated with DEHP administration have been reported. Lake et al report a marked initial increase in alcohol dehydrogenase activity and microsomal protein and hepatic cytochrome p-450 content followed by a downward trend as treatment progressed in rats fed two g per kg per day for up to 21 days. Succinate dehydrogenase (SDH), glucose-6-phosphatase (G-6-P), as well as aniline-4-hydroxylase activities were consistently inhibited in this study to about 60 percent of the control values. In confirmation of the biochemical data, histological studies showed a reduction in enzyme activity in the portal area after four days, extending to the mid zonal region of the liver with continued treatment. Increased microsomal p-450 contents have also been reported by other workers along with markedly stimulated microsomal lauric acid hydroxylase activity.

Inhibition of SDH as well as adenosine triphosphatase (ATPase) has also been demonstrated in other vital organs (heart, lung, and kidney) of rats receiving three intraperitoneal injections of 4.92 g DEHP per kg body weight over a period of 21 days. Solubilized with Tween 80, DEHP has also been shown to inhibit SDH at much lower concentrations (5 to
Others have reported the inhibition of hepatic aminopyrine-N-demethylase and aniline hydroxylase, but no effect on G-6-P and NADPH-cytochrome-c-reductase at these doses and routes of administration of DEHP. Others have reported the inhibition of hepatic aminopyrine-N-demethylase and aniline hydroxylase, but no effect on G-6-P and NADPH-cytochrome-c-reductase at these doses and routes of administration of DEHP.

Related to such pronounced changes in hepatic enzyme activity, as measured by *in vitro* assay, is the effect of DEHP administration on barbiturate-induced narcosis. Rubin and Jaeger reported a significant increase in hexobarbital sleeping time in rats and mice pretreated with 250 or 500 mg DEHP per kg (i.p.) when compared with controls. Others report similar findings in mice when pretreated with as little as 30 mg DEHP per kg. In a longer study and at somewhat higher doses (1400 mg per 100 g body weight for 14 days and 2800 mg per 100 g body weight for 19 weeks), Leber reported a decrease in hexobarbital sleeping time owing to an increase in the activity of drug metabolizing enzymes *in vivo*. Earlier work with mice reported similar findings. These discordant data may reflect the differences in the acute interactions between DEHP and the barbiturate-induced narcosis and the chronic effect DEHP may have on the metabolism, tissue sensitivity, distribution, and excretion of the barbiturate.

In 1977, a series of experiments were undertaken to determine the toxic potential of DEHP as administered via plasma transfusion to six month-old Rhesus monkeys. The animals were transfused weekly for one year with platelet-rich (PRP-3 animals) and platelet-poor plasma (PPP-2 animals) from polyvinyl chloride (PVC) storage bags. A non-transfused control group as well as a group which underwent weekly transfusions with plasma stored in polyethylene (PE) storage bags were also included in the study. Four of the five monkeys transfused with PVC-PRP or PVC-PPP included in the study demonstrated histologic abnormalities in liver biopsy specimens. Non-transfused controls and those which underwent PE-plasma transfusions showed no hepatic changes. Tc sulfur colloid liver-spleen scan indicated a reduction in hepatic perfusion in the treated monkeys. Conversely, the control animals and PE transfused monkeys were both normal.

Hepatic peroxisome (microbody) proliferation has also been reported following the administration of DEHP. In addition, a concomitant increase in the hepatic activities of peroxisome associated enzymes (i.e., catalase, carnitine acetyltransferase, carnitine palmitoyltransferase) has also been described. The biological implications of the peroxisome proliferating activity of DEHP are yet to be determined. However, a possible relationship between peroxisome proliferation and hypolipidemia has been suggested.

The hypolipidemic effects of dietary DEHP (zero to four percent DEHP for up to four weeks) have been reported for rats. Rats fed diets containing up to 4.0 percent DEHP for four weeks exhibited decreased plasma triglycerides for the 1.0 and 2.0 percent groups with no change in plasma cholesterol concentration. Plasma free fatty acids and ketones were elevated in all groups and blood glucose and hepatic glycogen stores were significantly decreased. These changes are similar to the hypolipidemic effects of p-chlorophenoxyisobutyrate (CPIB). An apparent decrease in rat hepatic triglyceride concentration was noted after prolonged injection of 0.1 to 0.5 percent DEHP. Inhibition of sterologenesis and squa lene biosynthesis following dietary administration of DEHP has also been reported. In rats fed 0.5 percent DEHP for two to 11 days, there was a significantly decreased conversion of acetate-1-<sup>14</sup>C and mevalonate-5-<sup>3</sup>H into squalene, C-30-sterols, and C-27-sterols by liver minces or slices *in vitro*. The
degree of inhibition was found to increase with the duration of DEHP feeding. The inhibition of \(^{3}\text{H}\)-mevalonate conversion to squalene and sterols developed more slowly, being reduced to 70 percent of control values in 11 days whereas \(^{14}\text{C}\)-acetate conversion was reduced to 35 percent of control values during the same period. It has also been reported that the inhibitory effects of dietary DEHP on lipid metabolism in the mature rat is transmitted across the placental barrier to the developing fetus and that the abnormal pattern of lipid metabolism is only partially restored to normal during the suckling period. These data suggest that DEHP or its metabolites have biological activity and are capable of modifying lipid metabolism in experimental animals.

**Teratogenicity and Related Effects**

The fetoletality of DEHP at high concentrations in the chick embryo has been well established. However, of the numerous studies conducted to investigate its potential teratogenicity, only two investigators have reported a correlation of fetal malformation with its administration. Singh et al reported that in 27.3 percent of fetuses from DEHP-treated rats (10 ml undiluted DEHP per kg, i.p. on days 5, 10, and 15 of gestation), gross abnormalities were noted. Among these were absence of tail, anophthalmia, twisted hind legs, and hematomas. No abnormalities were noted in fetuses from rats receiving a lower dose (5 ml per kg). In another study in which dietary DEHP was administered in doses from 0.2 to 1.0 percent, fetal abnormalities were noted at the 0.2 percent level only. These data suggest that DEHP may be teratogenic at high levels in laboratory animals, but the results are inconclusive. Furthermore, the normalization of experimentation with regard to dose, route of administration, and duration of exposure must be accomplished before a definitive statement can be made.

Bis-(2-ethylhexyl) phthalate has been shown to produce no chromosomal aberrations in human leukocytes and Chinese hamster ovary cells as well as no damage to fetal lung cells \textit{in vitro}. However, transplacental administration of DEHP and its major metabolite, MEHP, resulted in morphological transformations and chromosomal aberrations in the embryonic cells of the Syrian golden hamster. It is suggested that the actual mutagenic agent is MEHP rather than the parent compound. Sex organ effects in rats receiving DEHP are well described. In male and female rats receiving 5 ml per kg of undiluted DEHP i.p. on days 1, 5, and 10 of a 22 day study, histopathological studies revealed focal degeneration of the seminiferous tubules and edema of the interstitium in testes but no detectable alterations in the ovary of treated animals as compared to controls. Other studies reveal similar testicular effects in response to DEHP, MEHP, and other phthalate diesters and their corresponding monoesters.

In cytotoxicological studies, heart cells from seven-day old chick embryos died after being exposed to media which had been kept in polyvinylchloride tubing for 40 minutes at 23°C. Aliquots of the same medium kept in glass produced no untoward effects on embryonic cells over the same time period. Other cellular effects of DEHP include a significant but reversible growth inhibition of human diploid fibroblasts when exposed to DEHP at a concentration of 0.15 μM.

**Discussion**

Bis-(2-ethylhexyl) phthalate (DEHP), known only as a laboratory curiosity just fifty years ago, was patented for use as a plasticizer in 1933. It is the largest vol-
ume plasticizer used in the world with nearly 400 million pounds produced in 1977. It is now an environmental contaminant to which virtually everyone is exposed. Worthy of particular attention to its widespread use in medical and surgical products.

The toxicity of DEHP has been studied extensively and competently by many investigators in several systems. It is clear that its acute toxicity is minuscule by any measure in all mammalian species tested. It is also clear that chronic administration of large amounts of this chemical can cause liver changes in rats, mice, guinea pigs, dogs, and the rhesus monkey. The findings in the monkey are interesting because of the similarity of the model with the human, and because the dosage of DEHP was in the same order as might be expected in the case of a person undergoing long term multiple blood transfusions. However, the findings are in a single report which is now seven years old, and as yet there has been no report of analogous findings in humans.

The hepatic microsome proliferation and probably related hypolipidemic effects in the rat are intriguing. These findings raise questions concerning the possible effect of DEHP on lipid transport and metabolism. Again, there are no reports of effects in man.

The reports of teratogenicity and carcinogenicity for reasons stated cannot be considered definitive. Neither should they be ignored. Clearly, the issue of DEHP and its relationship to man remains open. Further toxicity studies are required, particularly with regard to possible effects of its human metabolites.

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