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INTERACTION OF DI-(2-ETHYLHEXYL) PHTHALATE WITH THE PHARMACOLOGICAL RESPONSE AND METABOLIC ASPECTS OF ETHANOL IN MICE

DEEPAK K. AGARWAL*, SARITA AGARWAL and PRAHLAD K. SETH
Industrial Toxicology Research Center, Mahatma Gandhi Marg, Lucknow 226001, India

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Abstract—The interactions of di-(2-ethylhexyl) phthalate (DEHP) with the pharmacological response and metabolic aspects of ethanol in mice were investigated at oral doses of DEHP of 1.5, 3.0 and 7.5 g/kg or intraperitoneal doses of 3.7, 7.5 and 18.9 g/kg, administered once or daily for 7 days. A single oral or intraperitoneal administration of DEHP resulted in a significant increase in the ethanol-induced sleeping time, associated with an inhibition of alcohol dehydrogenase activity in liver; the effect of intraperitoneal administration was significant only at the highest dose. The activities of high and low K_m aldehyde dehydrogenases in mouse liver were not affected by a single dose of DEHP by either route. Repeated oral doses of DEHP produced significant reductions in the ethanol-induced sleeping time and increases in the activities of alcohol and aldehyde dehydrogenases, whereas repeated intraperitoneal doses of DEHP significantly increased the sleeping time and decreased the activity of alcohol dehydrogenase, without any perceptible effect on the activities of aldehyde dehydrogenases. *In vitro* studies with mouse liver preparations revealed significant inhibition of alcohol dehydrogenase activity by mono-(2-ethylhexyl) phthalate and 2-ethylhexanol and of high and low K_m aldehyde dehydrogenase activities by DEHP and mono-(2-ethylhexyl) phthalate at concentrations ranging from 0.03 to 1.00 mM. In all cases, *in vitro* enzyme inhibition by mono-(2-ethylhexyl) phthalate was most pronounced.

Di-(2-ethylhexyl) phthalate (DEHP)†, a widely used plasticizer, is known to leach from finished polyvinyl chloride (PVC) products into blood, physiological fluids, commercial solvents and food materials. Entry of plasticizers into the human system during transfusion and hemodialysis, and through the food chain, as well as their ubiquitous presence in the environment, has aroused concern over possible health hazards. Recent years have witnessed a rapid growth of the literature on the toxicity of phthalic acid esters (PAEs); some of these studies reveal their potential for producing a wide range of toxic effects when exposed to mammals and aquatic invertebrates [1, 2].

One of the factors that may significantly affect the biological response to xenobiotics is their interaction with variables in the internal and external environment and/or pharmacological agents. Information on the interactions of PAEs with other xenobiotics is of importance in assessing their toxicities, for a person may be exposed to several simultaneously. Our previous work in this direction and the reports from other laboratories have shown that pretreatment with DEHP can alter the duration of action of sedative-hypnotic drugs, e.g. barbiturates and methaqualone [3–5], and can modify the biological responses of parathion, an organophosphorus pesticide [6], and carbon tetrachloride [7]. The present paper reports the interaction of DEHP with the pharmacological response and metabolic aspects of ethanol, a common solvent and a social drink.

* Send correspondence to Dr. Agarwal at his present address: Materials Science Toxicology Laboratories, University of Tennessee Center for the Health Sciences, 26 South Dunlap, Memphis, TN 38163, U.S.A.

† Abbreviations: DEHP, di-(2-ethylhexyl) phthalate; MEHP, mono-(2-ethylhexyl) phthalate; 2-EH, 2-ethylhexanol; PAEs, phthalic acid esters; PVC, polyvinyl chloride; ADH, alcohol dehydrogenase; and AlDH(s), aldehyde dehydrogenase(s).

MATERIALS AND METHODS

Animals and treatment. Adult male Swiss mice from the Industrial Toxicology Research Center colony were maintained under standard laboratory conditions on a pellet diet (Hind Lever Laboratory Animal Feeds, Bombay, India) and water *ad lib.* Two separate batches of animals were treated in the same manner, as follows, for the enzymatic and sleeping time studies. Undiluted DEHP was administered to the animals as oral doses of 1.5, 3.0 and 7.5 g/kg or intraperitoneal doses of 3.7, 7.5 and 18.9 g/kg, once or daily for 7 days. Control animals, run in parallel with each group, received equal volumes of normal saline in place of DEHP. The doses of DEHP were selected on the basis of their known pharmacologic [8] and biochemical [9, 10] effects and were extended to the higher levels in order to accentuate the differences between the biological responses to the plasticizer when administered orally and intraperitoneally.

Processing of tissue. The animals were fasted overnight and killed by cervical dislocation, 18 hr after a single or seven consecutive daily doses of DEHP. The livers were quickly removed and homogenized individually in ice-cold 0.25 M sucrose, using a Potter–Elvehjem-type glass homogenizer fitted with a Teflon pestle to yield suitable homogenates. A part

Table 1. Effect of DEHP on ethanol-induced sleeping time in mice*

Group	Sleeping time (min)	
	Single dose	Seven consecutive daily doses
Control	34.2 ± 5.0	34.9 ± 2.8
DEHP-treated, p.o.		
1.5 g/kg	39.6 ± 4.9	30.4 ± 5.4
3.0 g/kg	50.5 ± 4.7†	23.0 ± 3.0‡
7.5 g/kg	55.6 ± 4.8‡	17.5 ± 3.0§
Control	33.2 ± 2.2	33.3 ± 3.3
DEHP-treated, i.p.		
3.7 g/kg	38.2 ± 2.2	43.6 ± 2.1†
7.5 g/kg	40.9 ± 3.4	54.5 ± 3.7§
18.9 g/kg	41.2 ± 2.7†	64.6 ± 3.0‖

* Each value is the mean ± S.E. for five observations.
† $P < 0.05$, when compared with the respective control.
‡ $P < 0.02$, when compared with the respective control.
§ $P < 0.01$, when compared with the respective control.
‖ $P < 0.001$, when compared with the respective control.

of each homogenate was individually processed to isolate mitochondria and post-mitochondrial supernatant fraction by the method of Johnson and Lardy [11].

Enzymatic studies. The activities of the high and low K_m aldehyde dehydrogenases (AlDH, aldehyde:NAD oxidoreductase, EC 1.2.1.3) were assayed in whole homogenates and mitochondria, respectively, treated with Triton X-100 (final concentration 0.02%, v/v) to expose total enzyme activity as described by Tottmar and Marchner [12]. The activity of alcohol dehydrogenase (ADH, alcohol:NAD oxidoreductase, EC 1.1.1.1) was assayed in the post-mitochondrial supernatant fraction according to the procedure of Koivula *et al.* [13]. The units of dehydrogenase activity were calculated by using the molar extinction coefficient of NADH, i.e. $E_{340} = 6.22 \times 10^6\ M^{-1}\ cm^{-1}$.

In vitro *studies.* The *in vitro* effects of DEHP and of two of its major metabolites, i.e. mono-(2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol (2-EH), were studied on the activities of ADH and AlDHs in mouse liver preparations. All the compounds were sonicated in the buffer solutions to be used for the enzyme assay, followed by their quantitative transfer into the enzyme preparations and thorough mixing in a vortex mixer. Suitable aliquots of such preparations were used for the enzyme assay, representing concentrations of DEHP, MEHP and 2-EH from 0.03 to 1.00 mM.

Sleeping time studies. DEHP-treated and control animals were fasted overnight, prior to the sleeping time studies, to maintain uniformity with the enzymatic studies. All the animals were given a single intraperitoneal injection of ethanol [3.0 g/kg in normal saline as 15% (w/v) solution], 18 hr after the last treatment with DEHP or normal saline. The time that elapsed between the loss and return of the

Table 2. *In vivo* effect of DEHP on the activity of alcohol dehydrogenase in mouse liver*

Group	Alcohol dehydrogenase [nmoles NAD reduced·min^{-1}·(mg protein)$^{-1}$]	
	Single dose	Seven consecutive daily doses
Control	7.14 ± 0.31	7.83 ± 0.67
DEHP-treated, p.o.		
1.5 g/kg	6.43 ± 0.82	13.85 ± 1.93†
3.0 g/kg	5.69 ± 0.44‡	18.75 ± 2.35§
7.5 g/kg	3.90 ± 0.23†	21.27 ± 3.25§
Control	7.76 ± 0.86	6.23 ± 0.68
DEHP-treated, i.p.		
3.7 g/kg	7.55 ± 0.19	4.80 ± 0.90
7.5 g/kg	7.05 ± 0.22	4.43 ± 0.23‡
18.9 g/kg	4.22 ± 0.13§	3.85 ± 0.27†

* ADH activity was measured in the post-mitochondrial supernatant fractions of mouse liver homogenates. Each value is the mean ± S.E. for five observations.
† $P < 0.02$, when compared with the respective control.
‡ $P < 0.05$, when compared with the respective control.
§ $P < 0.001$, when compared with the respective control.

Table 3. *In vivo* effects of DEHP on the activities of high and low K_m aldehyde dehydrogenases in mouse liver*

Group	Aldehyde dehydrogenase [nmoles NAD reduced·min^{-1}·(mg protein)$^{-1}$]			
	High K_m		Low K_m	
	Single dose	Seven consecutive daily doses	Single dose	Seven consecutive daily doses
Control	15.28 ± 1.90	18.36 ± 3.12	9.51 ± 1.67	7.76 ± 1.34
DEHP-treated, p.o.				
1.5 g/kg	14.27 ± 2.10	29.54 ± 2.50†	9.85 ± 0.76	17.91 ± 1.61‡
3.0 g/kg	16.21 ± 0.70	34.07 ± 2.65§	9.63 ± 2.24	20.57 ± 2.35‡
7.5 g/kg	16.35 ± 0.80	36.95 ± 2.76§	7.15 ± 1.85	29.23 ± 2.75‡
Control	15.01 ± 2.00	19.09 ± 2.63	9.57 ± 1.62	7.76 ± 1.26
DEHP-treated, i.p.				
3.7 g/kg	15.22 ± 1.50	17.31 ± 2.47	9.64 ± 1.88	6.75 ± 0.96
7.5 g/kg	18.19 ± 2.40	15.15 ± 1.61	10.00 ± 1.91	5.37 ± 0.86
18.9 g/kg	17.14 ± 1.70	14.53 ± 3.76	10.10 ± 2.11	5.41 ± 0.67

* Activities of high and low K_m aldehyde dehydrogenases were measured in the whole homogenates and mitochondria respectively. Each value is the mean ± S.E. for five observations.
† $P < 0.05$, when compared with the respective control.
‡ $P < 0.001$, when compared with the respective control.
§ $P < 0.01$, when compared with the respective control.

righting reflex was recorded as the sleeping time induced by ethanol. The loss and return of the righting reflex were observed by laying the animals on their backs until they returned to their feet.

Estimation of protein content. The total protein content of various tissue preparations was assayed in the trichloroacetic acid-precipitates by Folin phenol reagent as described by Lowry *et al.* [14], using bovine serum albumin as standard.

Evaluation of statistical significance. Statistical significance of the results was evaluated by Student's *t*-test as described by Fisher [15]; P values less than 0.05 were considered to be significant.

RESULTS

The effects of DEHP on ethanol-induced sleeping time in mice are shown in Table 1. A single oral or

Table 4. *In vitro* effect of DEHP, MEHP and 2-EH on the activities of alcohol and aldehyde dehydrogenases in mouse liver*

Group	Alcohol dehydrogenase [nmoles NAD reduced·min^{-1}·(mg protein)$^{-1}$]	Aldehyde dehydrogenase [nmoles NAD reduced·min^{-1}·(mg protein)$^{-1}$]	
		High K_m	Low K_m
Control	8.40 ± 0.43	22.59 ± 1.29	8.15 ± 0.48
DEHP (mM)			
0.25	7.20 ± 0.84	17.95 ± 1.38†	5.43 ± 0.37†
0.50	7.20 ± 0.84	17.42 ± 1.29†	4.75 ± 0.31‡
1.00	7.20 ± 0.80	13.89 ± 1.02‡	4.07 ± 0.26‡
MEHP (mM)			
0.03	3.60 ± 0.31‡	21.70 ± 1.87	8.15 ± 0.21
0.06	3.30 ± 0.26‡	20.25 ± 1.62	6.79 ± 0.42
0.09	4.80 ± 0.35‡	18.66 ± 1.04†	4.75 ± 0.29‡
0.12	2.40 ± 0.17‡	16.70 ± 1.04§	4.66 ± 0.28‡
0.25	1.20 ± 0.11‡	12.31 ± 0.72‡	4.07 ± 0.26‡
0.50	ND‖	3.93 ± 0.22‡	2.71 ± 0.15‡
1.00	ND	1.76 ± 0.14‡	ND
2-EH (mM)			
0.25	8.40 ± 0.50	20.84 ± 1.80	9.87 ± 0.88
0.50	6.60 ± 0.47†	19.89 ± 1.63	8.87 ± 0.68
1.00	6.60 ± 0.44¶	19.81 ± 1.69	7.47 ± 0.55

* ADH activity was determined in the post-mitochondrial supernatant fractions; AlDH activities, high and low K_m, were measured in the whole homogenates and mitochondria respectively. Each value is the mean ± S.E. for five observations.
† $P < 0.05$, when compared with the respective control.
‡ $P < 0.001$, when compared with the respective control.
‖ ND = not detectable.
¶ $P < 0.02$, when compared with the respective control.

intraperitoneal dose of DEHP significantly increased the sleeping time; the effect of the intraperitoneal dose was significant only at the highest dose level. Repeated administration of DEHP, however, produced effects which differed with the route of administration; oral doses of DEHP significantly decreased the sleeping time whereas intraperitoneal doses increased the same. The effects suggest a dose dependence and were more pronounced with the oral administration.

Results presented in Table 2 show significant inhibition of ADH activity after a single exposure to the higher doses of DEHP by the two routes. Repeated oral doses of DEHP significantly increased the ADH activity in mouse liver but repeated intraperitoneal administration decreased it in an apparently dose-dependent manner.

Data on the effects of DEHP administration on the activities of high and low K_m AlDHs are summarized in Table 3. The activities of AlDHs were not affected after a single oral or intraperitoneal dose, or repeated intraperitoneal doses of DEHP. However, repeated oral doses of DEHP markedly increased the AlDH activity, and the effect on low K_m AlDH was greater than that on high K_m AlDH.

Observations on the *in vitro* effects of DEHP, MEHP and 2-EH on the activities of ADH and AlDHs in mouse liver preparations are presented in Table 4. As evident, DEHP had no effect on ADH activity but significantly inhibited the activities of both high and low K_m AlDHs at concentrations from 0.25 to 1.00 mM. MEHP had a marked inhibitory effect on the activities of ADH and AlDHs at concentrations from 0.03 to 0.25 mM and 0.09 to 1.00 mM respectively. Addition of 2-EH to mouse liver preparations resulted in a significant inhibition of ADH activity at concentrations of 0.50 and 1.00 mM but had no appreciable effect on the activities of high and low K_m AlDHs.

DISCUSSION

Ethanol is primarily oxidized by ADH, and the resultant acetaldehyde is, in turn, converted to acetate by the action of AlDHs. Changes in the activities of ADH and AlDHs may, therefore, lead to alterations in the blood level of ethanol and influence ethanol-induced sleeping time. Such an effect has been reported following treatment with disulfiram [16], analgesics [17], and barbiturates [18]. The complementary changes in ethanol-induced sleeping time and enzyme activities, observed in the present study, suggest that a similar effect of DEHP on ethanol metabolism brings about a modification in the pharmacological response to ethanol. This is supported by a recent report on the rapid clearance of ethanol from blood as a consequence of its increased oxidation after repeated oral administration of DEHP in rats [19].

Inhibition of ADH activity is probably a direct effect of DEHP and/or its metabolites as evident with our *in vitro* observations and those of Albro [20], who reported a competitive inhibition of ADH (yeast) by 2-EH. The marked increases in the activities of ADH and AlDHs on repeated oral doses of DEHP, however, seem to have been substrate-induced effects, primarily by 2-EH which has been shown to stimulate ethanol oxidation [19] and act as a substrate for horse liver ADH [20]. The resultant aldehyde of 2-EH, i.e. 2-ethylhexanal, has also been suggested to be a good substrate for mammalian AlDHs [20]. Further, similar biphasic effects on the activities of ADH [9] and drug-metabolizing enzymes [21] of rat liver have been reported after acute and prolonged exposure to DEHP.

Mitochondrial oxidation [22] and AlDHs [23] are considered to be regulators of ethanol metabolism. The greater sensitivity of mitochondrial low K_m AlDH to DEHP, compared to that of ADH and high K_m AlDH, both under *in vivo* and *in vitro* conditions, indicates that the observed interaction is probably mediated through DEHP-induced changes in the mitochondrial structure and function [9, 10].

It should be noted here, though, that under our experimental conditions DEHP-induced alterations in the ethanol-induced sleeping time and the activities of ADH and AlDHs were not fully accounted for by each other. It is possible, therefore, that the overall effect of DEHP, observed in the present study, may be related to other rate-limiting factors in ethanol oxidation, e.g. availability of oxidizing equivalents [24, 25]. This is consistent with an earlier report suggesting a severe shift in the ratio of redox equivalents (NAD:NADH) due to rapid oxidative turnover of MEHP and 2-EH [20].

Under *in vitro* conditions, the significant inhibition of ADH and AlDHs by MEHP and 2-EH suggests that the effects of these metabolites may contribute substantially to the *in vivo* effects of DEHP, a phenomenon observed in several other studies [9, 19, 21]. These studies were conducted at concentrations known to leach from plastic materials into physiological fluids [26], cause cytotoxicity in cell cultures [27] and inhibit a variety of enzymes, both in purified and crude preparations [28, 10, 21], and are of importance in view of the metabolism of DEHP and the accumulation of the metabolites in human blood and RBC concentrates stored in PVC-blood bags [29].

The observations of the present study and those reported earlier [19, 20] indicate that MEHP and 2-EH are the determining entities in the interaction of DEHP with ethanol. This may, as well, explain the differential effects of the plasticizer when administered orally and intraperitoneally, for DEHP is almost completely absorbed as MEHP and 2-EH from the intestine [30–32], whereas hydrolysis of DEHP by liver is relatively slower. Differential exposures of the liver to the metabolites would, thus, occur by the two routes. Also, differences in the physical state of the plasticizer in the intestine and the peritoneal cavity may affect its pharmacokinetics [33] and result in differential responses of DEHP by the two routes of administration.

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