

Fetotoxic Effects of Mono-2-ethylhexyl Phthalate (MEHP) in Mice

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Mono(2-ethylhexyl) phthalate (MEHP), one of the main metabolites of di(2-ethylhexyl) phthalate (DEHP), exerted embryo/fetotoxic effects similar to those of DEHP at lower doses. Oral administration of MEHP (1 mL/kg) to the mice of 8 days gestation resulted in less than 32% of live fetuses, all of which were deformed. When DEHP (10 mL/kg) was given to the pregnant mice of 8 days gestation, approximately 0.03% and 0.003% of the administered dose was found in fetuses as DEHP and MEHP, respectively, after 12 hr.

The presence of the MEHP in fetuses is probably due to the transplacental crossing of the MEHP formed in the maternal body, since the fetuses of mice up to day 9 of pregnancy showed no hydrolytic activity of DEHP to MEHP. Crossing of MEHP through the placenta was proven by an experiment in which MEHP was administered in pregnant mice.

A single injection of MEHP (25 or 50 mg/kg), but not DEHP (500 mg/kg) into pregnant mice, induced a significantly high incidence of somatic mutations in the coat hair of offspring of mice (KYG, ♀ × PW, ♂; C57BL/6Crj, ♀ × PW, ♂). All these data suggest that MEHP could be responsible for the embryotoxic/fetotoxic effects observed with DEHP.

Introduction

A number of investigators have demonstrated that administration of di(2-ethylhexyl) phthalate (DEHP) induced embryotoxic fetotoxic effects in rats (1,2) and mice (3-5). The effects depend on the route, dose, and time when DEHP was given; the oral administration of DEHP (10 mL/kg) to mice on day 7 or 8 of gestation, for example, resulted in a high incidence of dead or malformed fetuses. However, treatment of the mice before or after this sensitive stage, e.g., day 6, 9, or 10 of gestation, resulted in fewer defective fetuses even when higher doses of DEHP were administered (4).

Similar and more toxic effects have been observed when mono(2-ethylhexyl) phthalate (MEHP), an intermediary metabolite of DEHP, was given orally to mice. The administration of MEHP (1 mL/kg) to mice of day 8 of gestation resulted in less than 32% live fetuses, all of which showed gross and skeletal abnormalities (4).

The present investigation was undertaken to determine

whether the embryotoxic/fetotoxic effects of DEHP are related to the metabolic formation of MEHP in the maternal body and/or in fetuses. DEHP is known to be hydrolyzed to MEHP and 2-ethylhexanol (6-8). The hydrolysis is catalyzed by pancreatic lipase (9), acid and alkaline esterases in intestinal mucosa (10,11), plasma (12), and liver (6). Tissue distribution of MEHP after the oral administration of DEHP (or MEHP) has been reported by several investigators in pregnant or nonpregnant rats (7,13,14). The study with ¹⁴C-DEHP in pregnant Sprague-Dawley rats indicated that most tissues and fluids, including maternal blood, fetal tissue, amniotic fluid, and placenta, all contained radiolabel (15). The concentration of MEHP, however, is not clear in this study. We have examined the concentrations of both MEHP and DEHP in tissues including fetuses by giving DEHP (or MEHP) to the mice (ddY-SLC, ♀ × CBA, ♂) on different days of pregnancy. Both DEHP and MEHP were found in fetuses, and their concentrations varied depending on the period of pregnancy.

In our previous paper (16), we demonstrated that MEHP induced genotoxic effects in microbial and mammalian assay systems. The IP administration of MEHP to the KYG or C57BL/6NCrj female mice mated with a PW male resulted in a significantly higher incidence of the appearance of recessive spots.

These data may suggest that MEHP as a metabolite of DEHP is acting as a toxic principle for DEHP.

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Experimental

Virgin mice of the ddY-SLC (SPF) strain (Shizuoka Experimental Animal Corp.) weighing 27–28 g (8–9 weeks old) were kept in a room at $25 \pm 2^\circ\text{C}$ with humidity of $50 \pm 5\%$ for 1 week, after which they were mated with mice of the CBA strain (National Institute of Genetics). A laboratory diet (Funahashi Farm Co.) and tap water were provided *ad libitum*.

MEHP was obtained from the method of Kenyon (17). The crude materials were purified through a silica gel (Wakogel) column (50 mm \times 300 mm) by eluting with chloroform. The material showed one spot on aluminum-TLC coated with silica-gel 60F 254. It revealed a single peak in ECD-GLC on a column packed with 1.5% OV-1.

MEHP was dissolved in olive oil and given to mice in a single oral dose of 0.1, 0.5, or 1.0 mL/kg on day 7, 8, or 9 of gestation. Body weight changes were recorded daily for 18 days after gestation. The animals were then sacrificed by dislocating the vertebrae. The uterine horns and ovaries were exposed to count the number of implantations and live fetuses. Malformations (gross and skeletal) and body weight were noted for all live fetuses.

MEHP was determined by ECD-GLC with diazomethane as follows: N_2 gas was passed through tube A containing diethyl ether (10 mL) and tube B containing diethyleneglycol monoethyl ether (10 mL), 5 KOH pellets in 2 mL water and 10% diethyl ether solution of *p*-toluenesulfonyl-*N*-methyl-*N*-nitrosamide, and was introduced to tube C containing MEHP in diethyl ether (5 mL). The methylated MEHP (MeEHP) was passed through a Florisil column containing 2–2.5% water and eluted by 4% diethyl ether-*n*-hexane (200 mL). The 20% diethyl ether-*n*-hexane eluate was used for MeEHP analysis and DIBP (diisobutyl phthalate) was used as a reference compound. The analytical method of the MeEHP employed is essentially the same as that of DEHP and other phthalic acid esters (18). When mouse tissue containing MEHP and DEHP was used, it was homogenized with four volumes of 0.25 M sucrose and acidified with 4 N sulfuric acid followed by two extractions

with diethyl ether (20 mL). The diethyl ether extract was then methylated as described above and analyzed by ECD-GLC.

Radioactivity determination of mouse tissue after the oral administration of ^{14}C -labeled DEHP (10 mL/kg - 1.26 $\mu\text{Ci/mL}$) to 8th day gestation mice was carried out as follows: mouse tissue which was homogenized with four volumes of 0.25 M sucrose and acidified with 4 N sulfuric acid was extracted three times with diethyl ether (10 mL). The ether extract was then concentrated and applied to TLC (Kiesel gel 60F 254). After it was developed with a solvent mixture of chloroform:methanol:acetic acid = 147:7:2, the spots corresponding to DEHP ($R_f = 0.93$) and MEHP ($R_f = 0.46$) were scraped and extracted with chloroform. The radioactivity was determined by liquid scintillation counting. Carbonyl (^{14}C)-labeled DEHP was synthesized according to the method of Williams et al. (19).

The hydrolysis of DEHP to MEHP in mouse tissue homogenate was performed by incubating a mixture of 0.5 mM DEHP (0.1 mL) in 0.1 M Tris-HCl buffer (pH 8.0, 0.2) and tissue homogenate (0.1 mL) at 37°C for 30 min. The MEHP formed was then extracted with 6 mL of *n*-hexane-ethanol-3 N sulfuric acid mixture (300:200:1), and the *n*-hexane layer (2 mL) was reacted with Rhodamine 6G reagent (1 mL) according to the method of Hirayama et al. (20). The absorbancy at 514 nm was read by the Hitachi spectrophotometer (Model 100-60). The hydrolysis of palmitic acid ethyl ester for the hydrolytic activity determination was conducted in the same manner as described for that of DEHP. The hydrolytic activity of mouse tissue homogenate was expressed as an absorbancy reading at 514 nm/mg protein. The protein concentration was determined according to the method of Lowry et al. (21). The mammalian spot test using pregnant mice (KYG or C57BL/6Ncrj mated with PW males) was conducted according to Russell (22). DEHP or MEHP, dissolved in olive oil, was given to the mice on the 10.5th day of gestation and the appearance of white midventral and recessive spots in the coat of the F_1 mice was observed. The genotype of the embryos in

Table 1. Fetotoxic effects of MEHP on mice.

Groups	Day of gestation	Dose administered, orally, mL/kg	Number of implantations ^a	Number of dead fetuses ^b		Number of live fetuses ^b	Numbers of abnormalities ^c		Average body weight of fetuses
				Early death	Late death		Gross	Skeletal	
Untreated control	–	–	56(5)	0	0	56(100)	0	0	1.41 \pm 0.06
Olive oil	7	10.0	31(4)	0	0	31(100)	0	0	1.47 \pm 0.06
MEHP	7	0.1	57(6)	8(14.0)	2(3.5)	47(82.5)	1(2.1)	3(6.4)	1.36 \pm 0.09 *
		1.0	27(4)	17(63.0)	5(18.5)	5(18.5)	0	0	1.28 \pm 0.09 *
	8	0.1	64(8)	1(1.6)	2(3.1)	61(95.3)	4(6.6)	11(18.1)	1.40 \pm 0.08
		0.5	44(5)	1(2.3)	7(15.9)	36(81.8)	4(11.1)	7(19.4)	1.34 \pm 0.11 *
		1.0	22(2)	1(4.5)	14(63.6)	7(31.8)	7(100)	7(100)	1.02 \pm 0.02 *
	9	1.0	30(3)	2(6.7)	1(3.3)	27(90.0)	8(29.6)	0	1.20 \pm 0.09 *

^a Values in parentheses are numbers of pregnant mice used.

^b Numbers in parentheses indicate percent dead or live fetuses based on total numbers of implantations.

^c Numbers in parentheses indicate percent abnormalities based on total numbers of live fetuses.

* Significantly different from untreated controls at 99% level ($p < 0.01$).

Table 2. Distribution of DEHP in pregnant mice after the oral administration of DEHP on day 8 of gestation.

Groups		Distribution of DEHP, $\mu\text{g/g}$ of tissue ^{a,b}				
Time after DEHP administration, hr	Number of pregnant mice	Maternal tissues				Fetal tissue
		Liver	Kidney	Pancreas	Blood ^c	
0 ^d	6	1.5 \pm 0.3 (0.9 \pm 0.2)	3.0 \pm 0.9 (0.4 \pm 0.1)	24.0 \pm 10.6 (0.8 \pm 0.3)	<0.1 (3.9 \pm 2.1)	6.2 \pm 2.0 (0.4 \pm 0.1)
1	7	1060 \pm 538 (809 \pm 277)	112 \pm 47.3 (12.7 \pm 5.4)	3660 \pm 2340 (218 \pm 140)	3.9 \pm 2.1 (3.3 \pm 1.8)	274 \pm 120 (17.3 \pm 9.0)
3	6	1480 \pm 620 (726 \pm 317)	58.6 \pm 28.4 (6.8 \pm 3.5)	11100 \pm 5680 (343 \pm 212)	<0.1	522 \pm 291 (22.8 \pm 12.0)
12	5	1260 \pm 803 (662 \pm 416)	144 \pm 83.4 (17.9 \pm 10.3)	4910 \pm 3320 (249 \pm 169)	<0.1	426 \pm 290 (32.5 \pm 19.8)

^a Each value represents the mean \pm SE.

^b Numbers in parentheses represent mg% of administered DEHP.

^c Total blood volume of a mouse was assumed to be 8.5% of its body weight.

^d Untreated control.

KYG \times PW were a/a B/b CP/c^{ch}p D/d s/S, while those in C57BL/6NCRj \times PW were a/a B/b CP/c^{ch}p D/d.

Results

As shown in Table 1, the incidence of death and malformation of fetuses caused by MEHP was dose- and time-dependent. At a dosage of 0.1 mL, the highest incidence of fetal death (81%) was observed among mice in which MEHP was given on day 7 of gestation, while the incidences were 68% and 10% when MEHP was administered on day 8 and 9 respectively. As for the gross and skeletal abnormalities, all live fetuses were malformed when MEHP was given on the 8th day of gestation, while a lower incidence of malformation or no malformation was observed on administration of MEHP on day 7 or 9 of gestation. The results of the time dependence closely resembles those of DEHP administration. Furthermore, the data on day 8 of gestation suggested that teratogenic effects of MEHP are related to the dose administered as was previously seen in DEHP: the higher the dose, the larger the numbers of malformed fetuses observed. Average body weights of most live fetuses in MEHP-treated mice were significantly lower than that of the control group, regardless of the day of administration.

Tables 2 and 3 show the distribution of DEHP and could be altered according to the gestation period for

MEHP in pregnant mice (8th day of gestation) when DEHP was given orally (10 mL/kg). The distribution of DEHP and MEHP are expressed as micrograms per gram of tissue weight and milligram-percentage of the dose administered. Though DEHP in the blood was minimal, it was detected in the liver, kidney, pancreas, and fetal tissue. The highest concentrations of both DEHP and MEHP were observed in the pancreas. The concentration of DEHP in fetal tissue increased with time after its administration; the amounts were 522 $\mu\text{g/g}$ (22.8 mg-%) and 426 $\mu\text{g/g}$ (32.5 mg-%), 3 and 12 hr, respectively, after DEHP administration. MEHP was also detected in fetuses. The level of MEHP in fetal tissue was approximately 1% that of DEHP and corresponded to 4.6 $\mu\text{g/g}$ tissue weight 12 hr after DEHP administration. The presence of DEHP and MEHP in fetuses was confirmed by an experiment with ¹⁴C-DEHP. The total amounts of DEHP and MEHP detected by ECD-GLC and by the method of radioactivity determination were almost same (data not shown).

The next experiment was performed to study the extent to which the concentration of DEHP and MEHP DEHP administration. Figure 1 shows the fluctuation of DEHP and MEHP in tissue 1 hr after the oral administration of DEHP (10 mg/kg) or MEHP (1 mg/kg) to pregnant and nonpregnant mice. The concentration of

Table 3. Distribution of MEHP in pregnant mice after the oral administration of DEHP on day 8 of gestation.

Groups		Distribution of DEHP, $\mu\text{g/g}$ of tissue ^{a,b}				
Time after DEHP administration, hr	Number of pregnant mice	Maternal tissues				Fetal tissue
		Liver	Kidney	Pancreas	Blood ^c	
0 ^d	6	1.4 \pm 0.9 ^c (1.1 \pm 0.7) ^d	1.0 \pm 0.6 (0.3 \pm 0.1)	1.5 \pm 0.6 (0.1 \pm 0.0)	<0.1	1.2 \pm 0.4 (0.1 \pm 0.0)
1	7	29.1 \pm 7.7 (20.2 \pm 5.6)	6.8 \pm 2.7 (2.0 \pm 0.8)	381 \pm 142 (30.6 \pm 11.0)	83.8 \pm 12.0 (71.8 \pm 10.3)	4.3 \pm 1.1 (0.3 \pm 0.1)
3	6	79.3 \pm 24.4 (54.1 \pm 16.9)	5.0 \pm 1.0 (0.8 \pm 0.1)	454 \pm 224 (32.2 \pm 13.5)	27.5 \pm 4.5 (22.4 \pm 3.7)	8.2 \pm 1.4 (0.6 \pm 0.1)
12	5	32.1 \pm 12.6 (24.2 \pm 9.8)	4.8 \pm 1.1 (0.9 \pm 0.2)	436 \pm 243 (30.7 \pm 17.5)	33.3 \pm 5.3 (27.9 \pm 4.4)	4.6 \pm 1.0 (0.3 \pm 0.1)

^a Each value represents the mean \pm SE.

^b Numbers in parentheses represent mg% of administered DEHP.

^c Total blood volume of a mouse was assumed to be 8.5% of its body weight.

^d Untreated control.

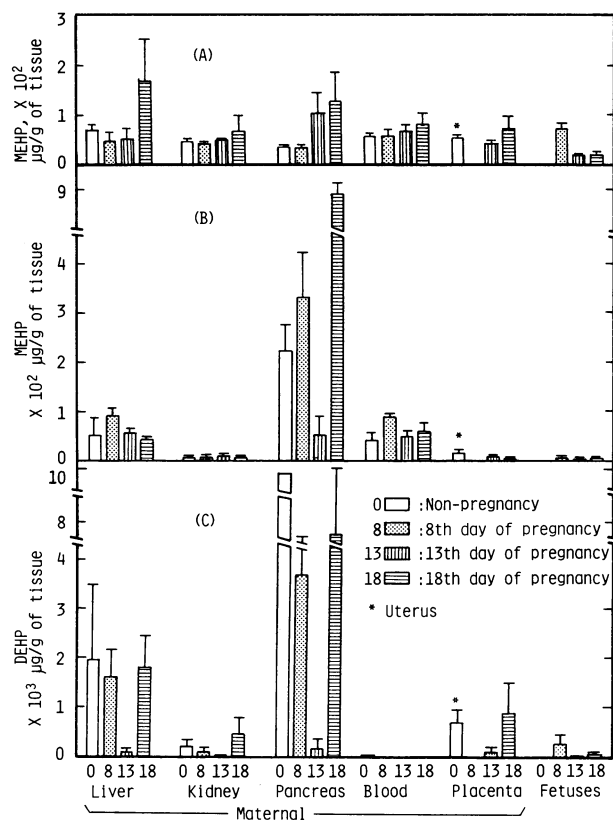


FIGURE 1. Concentration of DEHP or MEHP in tissues 1 hr. after the oral administration of DEHP or MEHP to pregnant and non-pregnant mice: (A) distribution of MEHP after the oral administration of MEHP; (B), (C) the distributions of MEHP and DEHP respectively, after the oral administration of DEHP. DEHP (10 mL/kg) or MEHP (1 mL/kg) was given to the mice and their concentration in various tissues and fluid were determined by GLC. The analytical procedure is as described in the Experimental section.

both phthalate in tissues varied greatly depending on the day of DEHP administration. The fluctuations were greatest in the pancreas, in which the concentration of both DEHP and MEHP were the highest among those tissues examined. A very low concentration was observed when DEHP was given on day 13 of gestation, though the reason for this is not clear. As far as the fetuses are concerned, the concentrations of both DEHP and MEHP were the highest when administered to the mice on day 8 of their gestation. The above results might be correlated to the high incidence of teratogenic effects induced by the administration of DEHP or MEHP on that day. In the administration experiment of DEHP, there are two possibilities to explain the presence of MEHP in fetuses. One explanation is the hydrolysis of DEHP to MEHP in the maternal body followed by the transfer of MEHP across the placenta; the other is direct hydrolysis of DEHP to MEHP in the fetuses. In order to examine the hydrolytic activity of the tissue of pregnant mice, DEHP or ethyl palmitate was incubated at 37°C for 30 min with a homogenate of pancreas, liver, or fetuses obtained from pregnant mice of varying days of gestation. MEHP or palmitate formed in the above reaction was

determined as described in the Experimental section.

Figure 2 shows the results of the hydrolysis of DEHP to MEHP. As expected, the pancreas showed the highest activities among the three organs tested. The activity varied greatly, depending on the day of pregnancy. As it is clear from Figure 2, no hydrolytic activity was detected in fetuses from mice on days 7, 8, or 9 of gestation. Weak activity (0.01 μ mole of MEHP/mg protein), was detected in fetuses from mice of day 10 of gestation. Similar activity changes were observed when ethyl palmitate was used as a substrate; no activity was detected with fetuses from 7th or 8th day pregnant mice. The same weak activity (0.01 μ mole of palmitic acid/mg protein) was detected in both fetuses from 9th or 10th day pregnant mice (The hydrolytic activities of ethyl palmitate of the pancreas and liver were 3.28–5.77 and 0.01–0.11 μ mole of palmitic acid/mg protein, respectively).

From the above experiment, it can be assumed that MEHP found in the fetuses of mice administered DEHP (7th or 8th day of gestation) is of maternal origin. The transplacental crossing of MEHP into mouse fetuses has already been demonstrated by GLC analysis. These findings may further suggest that MEHP is the acting compound which directly interacts with mouse fetuses to produce the teratogenic effects. We have previously reported that MEHP, not DEHP, showed mutagenic effects in a variety of assay systems (16). These systems include: Rec assay using *B. subtilis*, reverse mutation test using *E. coli* WP2 and *S. typhimurium* TA100, forward mutation assay, and chromosome aberration test with V79 Chinese hamster cells and Syrian golden hamster embryonic cells. In addition to the above findings, the results of the spot test using inbred strains of mice (KYG, ♀ × PW ♂ or C57BL/6Crj ♀ × PW ♂) should be noted.

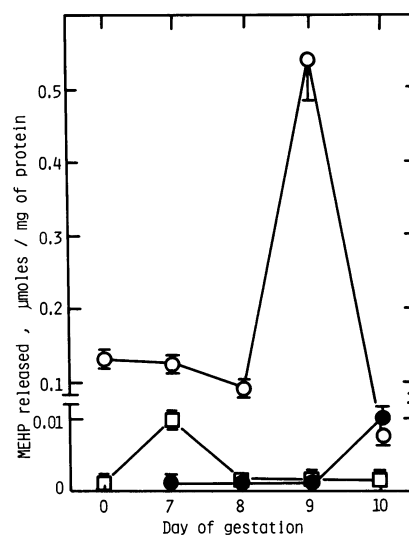


FIGURE 2. DEHP hydrolytic activity in maternal and fetal tissue of pregnant mice: 0 represents a nonpregnant control, (○) pancreas, (□) liver, (●) fetuses. Acyl hydrolase activity was determined with Rhodamine 6G using mice tissue homogenates of various stages of pregnancy. The tissue homogenate was prepared with 4-fold 0.25 M sucrose solution and was incubated at 37°C for 30 min as described in the Experimental section.

Table 4. Spot incidence in offspring of KYG or C57BL females mated with PW males, following injection with DEHP and MEHP on day 10.5 after conception.

Treatment	Dose, mg/kg	No. born	Surviving up to 4 weeks, %	White spot (WMVS), %	Recessive spot (RS), %
DEHP	500	129 ^a (159) ^b	93.8 ^a (97.5) ^b	4.1 ^a (0.6) ^b	0.8 ^a (2.6) ^b
MEHP	25	159	96.9	1.3	2.6*
MEHP	50	172 (117)	95.3 (97.4)	1.2 (0)	3.7** (5.3)*
Control	—	500 (426)	97.4 (99.8)	1.6 (0.5)	0.4 (0.9)

^a Numbers represent the results from F₁ of KYG × PW.

^b Numbers in parentheses represent the results from F₁ of C57BL/6NCrj × PW.

**p* < 0.05.

***p* < 0.01.

Table 4 summarizes the results. DEHP (500 mg/kg) or MEHP (25, 50 mg/kg) was injected once into pregnant mice after 10.5 days gestation. The surviving rate for the offspring up to 4 weeks and the appearance of white midventral spots (WMVS) in treated groups, which probably resulted from killing of melanocyte precursor cells, were not significantly different from those of the control group (except in the case of offspring from KYG females treated with DEHP). The appearance of the numbers of recessive spots (RS) were significantly different in the MEHP-treated groups. The present findings again suggest that MEHP is more genotoxic than DEHP.

Discussion

The effects of DEHP and MEHP on fetotoxicity and on teratogenicity have been reviewed by Peakall (23), Thomas et al. (24,25) and well summarized in the status report on phthalate in consumer products (26).

We have reported in the previous paper that not only DEHP but MEHP could produce fetotoxic and teratotoxic effects on pregnant mice (ddY, ♀ × CBA, ♂) at sufficiently high dosage (4,27).

The effects were closely related to the time and dose for both DEHP and MEHP. Treatment on day 7 of gestation resulted in the early death of embryos/fetuses, whereas on day 8 of gestation, the treatment resulted in later deaths. Moreover, the toxic effects were similar if the results of 10 mL and 1 mL of DEHP and MEHP were compared. The administration of either DEHP or MEHP on day 8 of gestation resulted in both gross (47%, 100%) and skeletal (33%, 100%) abnormalities while on day 9, gross abnormalities (21% for DEHP, 30% for MEHP) were exclusively produced. Their effects on the mortalities of the fetuses versus the day of gestation are also of the same pattern.

It follows that MEHP is probably responsible, at least in part, for the fetotoxicity of DEHP. DEHP is known to be metabolized to MEHP followed by ω or ω-1 oxidation to produce a variety of oxidation products in animals and men (28-30). The hydrolysis of DEHP to MEHP is catalyzed by pancreatic lipase (9) as well as by esterases of serum (12), mucosal epithelium (10,11) and liver (6). The data from the present study on DEHP hydrolysis indicate

that the hydrolytic activities of the fetuses up to the 9th day of gestation were almost negligible compared with those of the pancreas and liver. Only a weak activity was found for the fetuses of 10th day gestating mice. It is assumed that the presence of MEHP in mouse fetuses (0.3-0.6 mg-% of DEHP administered, 1-12 hr after the DEHP administration) in Table 2 is the result of transplacental incorporation of MEHP which was formed in maternal bodies. The possibility of transplacental crossing of MEHP is evident from Figure 1A. In Figure 1A-C, a higher concentration of MEHP (and DEHP) was observed in fetuses of 8th day pregnant mice compared with those from other mice of varying days of pregnancy. A less restrictive placental barrier may be possible in 8th day gestation mice.

The presence of MEHP (or DEHP) in the fetuses may indicate the embryotoxic/fetotoxic effects of MEHP (or DEHP) though other indirect effects such as the metabolic disturbance of maternal bodies could also be an explanation for toxic effects on embryos/fetuses.

We previously reported that MEHP, but not DEHP, showed mutagenic effects directly in bacteria and mammalian cell bioassay system. The effects of MEHP on *S. typhimurium* TA100 disappeared when MEHP was preincubated with rat liver S9 fraction. The metabolic oxidation of MEHP molecule seems more likely to occur in rats than in mice (30). Rapid metabolic oxidation of MEHP to dicarboxylic acid in rats might prevent embryos/fetuses from death or malformation: thus little or less embryotoxic/fetotoxic effects have been observed in more rats than in mice (31-33).

Finally, recessive spot incidences in somatic mutation test with the offsprings of mice from two strains were significantly induced by MEHP. Although this is a preliminary experiment, a genotoxic effect of MEHP has been again demonstrated. It seems probable that higher doses (> 500 mg/kg) of DEHP would produce a similar effect in this test.

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