Testicular Toxicity of Mono-*n*-Butyl Phthalate and Related Phthalates on Bound Iron in Rat Red Blood Cells

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It was previously reported that free iron was released from rat red blood cells in both an *in vitro* study with a metabolite, mono-*n*-butylphthalate (MBP), of di-*n*-butylphthalate (DBP) and an *in vivo* study with oral DBP, inducing rat testicular atrophy in hypoxic conditions due to MBP-induced hemoglobin deprivation. This study investigated whether mono-*n*-alkylphthalates (alkyl-carbon C_4-C_6 ; MAP), which result in rat testicular damage, induce iron release from hemoglobin by incubation with rat red blood cells. Iron release was observed with C_3-C_6 MAP incubation at lower doses and with C_1 and C_2 MAP incubation at higher doses, but not with C_7, C_8, and $C_{4(6)}$ MAP.

Key words —— di-*n*-butylphthalate, mono-*n*-alkylphthalate, testicular damage, hemoglobin, hypoxia

INTRODUCTION

Phthalates are used in the manufacture of flexible plastics. Recent interest in phthalates has focused on both the toxic effects to male gonads and hemolysis of red blood cells (RBC) during blood storage. The hemolysis might be caused by lipid peroxidation[1] on the membrane following a depletion[2] of vitamin E in the blood as well as the liver and testis. It was proposed that the testicular toxicity might be associated with hypoxia due to hemoglobin deprivation induced by the metabolite of di-*n*-butylphthalate (DBP).[3]

Foster *et al*.,[4] Fukuoka *et al*.,[5–7] and Oishi and Hiraga[8] have addressed the strong evidence that the monoester is the active principle for induction of reproductive toxicity. The toxic effects in animal models have been characterized by changes in blood cell and sperm counts and in testicular enzyme activities, followed by testicular atrophy. We know relatively little about the time course of the toxic mechanism of DBP and/or its metabolite, monobutylphthalate (MBP), although a decrease was observed in iron levels in both the blood and RBC and the interstitial tissue of the testis prior to the decrease in succinate dehydrogenase (SUDH) activity of Sertoli cells, and finally testicular damage.[5–7,9] However, questions remain on whether the iron release from RBC is caused by mono-*n*-butylphthalate to mono-*n*-hexylphthalate (C_4-C_6 alkylphthalate; MAP) inducing testicular atrophy.[4,8] As a preliminary step in resolving this, the toxic mechanisms were determined as detected by changes in adenine triphosphate (ATP), reduced glutathione (GSH), iron, and hemoglobin in RBC.

MATERIALS AND METHODS

Chemicals —— MBP (purity 99.9%) and other MAP (purity 99.8%) were synthesized by heating an equivalent mixture of phthalic anhydride and the corresponding alcohols, respectively, at 120°C for 40 min, according to the known method.[10] A Fe-Mono Kainos iron assay kit was purchased from Kainos (Tokyo, Japan). DBP (purity 99.9%) and other reagents and other assay kits were from Wako Pure Chemicals (Tokyo, Japan).

Animals —— Adult male Wistar rats (Charles River, Yokohama, 8 weeks old, weight range 300–350 g) were treated after acclimatization for 1 week in this study. Rats anesthetized with pentobarbital were killed and blood and testis homogenates were obtained.

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Concentration of MBP (C_4-MAP) in the Testis Homogenates —— Rat testis homogenates were prepared with 10 mM phosphate buffer (1 ml/1 g testis) from 8-week-old rats. MBP was dissolved in 10 mM phosphate buffer (pH 7.4) at various concentrations of 0 to 60 mM for the homogenates (Fig. 1). It was necessary for the MBP concentration in the homogenates to be at least more than 10-fold the MBP concentration in the testis.[3,9] The partition coefficient of MBP was 0.12 at pH 7.4,[3] and MBP concentrations of 805.8 ± 39.1 pmol in the RBC[6] and 114.5 ± 5.4 nmol/g in the testis[9] were observed 3 hr after an oral toxic dose of 8.6 mmol DBP/kg. The total volume of blood in the rat testis was about 10 μl/g.[11] Therefore the MBP concentration was adjusted to less than 100 mM in the testis homogenates.

Incubation of MBP and Rat Testis Homogenates —— After incubation at 37°C for 10 min, the homogenates were centrifuged at 960 × *g* at 4°C for 15 min to give a supernatant. The supernatant was used to measure enzyme activity and levels of ATP, iron, and protein. Part of the supernatant was filtered (Centrisarto SM 13249, porous to proteins of molecular weight less than 20000, Sartorius, Japan) to measure levels of low molecular protein-bound and unbound (free) irons.

Incubation of MAP and Rat Erythrocytes —— Since testicular atrophy was induced by C_4- to C_6 MAP esters (butyl-, pentyl-, hexyl-, and 2-ethylhexylphthalate),[4,8] it was necessary to determine whether MAP induced iron release from the RBC. Blood samples (about 5 ml) were collected from the jugular vein and subjected to biochemical analysis. Erythrocytes (RBC) from control rats were isolated from the plasma by centrifugation at 1000 × *g* for 10 min and washed three times with saline (15 ml). A suspension (20 μl) of washed RBC (9.0×10^6 μl) from the control rats was incubated with MAP (0, 10, 50, 100, 250, and 500, and 1000, 2500, and 5000 nmol) in Hanks buffer solution (500 μl, pH 7.4) at 37°C for 2 hr.

The incubation mixture was centrifuged at 1000 × *g* for 10 min to give sediment and supernatant fractions. The sediment was lysed with distilled water (500 μl), yielding a lysed solution and ghost pellets. The lysed solution was used to measure levels of ATP, Ca^{2+} ion, GSH, hemoglobin (Hb), and iron (bound and unbound to lower molecular weight proteins filtered through Centrisarto SM13249, molecular weight 20000, Sartorius, Japan).

Hematologic Examination of RBC —— The number of erythrocytes and mean corpuscular volume (MCV) were measured using a Sysmex M-2000 equipped with SysmexDA-1000 (Sysmex Co. Ltd, Japan).

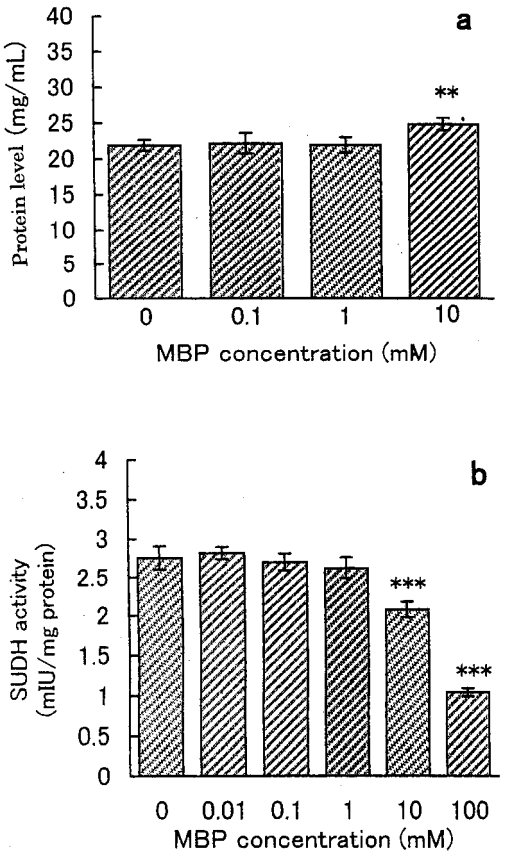

Fig. 1. Effects of MBP on Protein Levels and SUDH Activity in the Testis Homogenates

Rat testis homogenates were incubated with MBP [0 mM (control), 0.1, 1, and 10 mM for protein levels, and 0, 0.01, 0.1, 1, 10, and 100 mM for SUDH activities] at 37°C for 10 min: Protein levels (a) and SUDH activities (b). Values are mean ± S.D. ($n = 5$), $^{**}p < 0.01$ and $^{***}p < 0.005$ compared with the respective control (0 mM MBP).

Measurement of ATP, Ca^{2+}, Enzyme Activity, GSH, Hb, Iron, and Protein ——

ATP Assay: A lysed solution of RBC was centrifuged at 960 × *g* for 15 min followed by recentrifugation at 8000 × *g* for 10 min at 4°C to give a supernatant, respectively. ATP levels in the supernatant were assayed using a TOA ATP Analyzer AF 100 (TOA Electronics Ltd., Japan) equipped with a TOA Auto Dispenser DF 10.

Ca^{2+} Assay: The content was determined using a Ca^{2+} assay kit (Calcium E-Test, Wako Pure Chemicals).

Enzyme Activity Assay: The targeted enzyme was SUDH that showed a change in activity before dissociation of germ cells.[5] SUDH activity was assayed

by the known method.[12] Enzyme activity in each sample was assayed in duplicate to obtain the mean value.

GSH Assay: The above sample (0.2 ml) of RBC lysed with water was added to a solution (0.3 ml) of 1.67 g of metaphosphoric acid, 0.2 g of EDTA-Na_2, and 30 g of NaCl in 100 ml of water. The mixture was allowed to stand for 5 min and centrifuged at $9000 \times g$ for 10 min at 4°C. The supernatant (0.2 ml) was diluted with 0.5 M sodium phosphate buffer (0.6 ml, pH 9.15). A solution of 0.1% *o*-phthalic aldehyde in methanol (0.1 ml) was added to the diluted solution, and the fluorescence was determined after 1 min at 420 nm (emission) and 350 nm (excitation) using a Hitachi F-3010 fluorescence spectrophotometer, according to the two known methods.[13,14]

Hb Assay: The levels in Hb were determined using an assay kit (Hemoglobin B Test, Wako, sodium lauryl sulfate method, Wako Pure Chemicalså).

Iron Assay: Total iron was determined from the mean obtained using both an assay kit (Fe-Mono Kainos) and a Hitachi atomic absorption spectrometer (Model 170-50) at 248.3 nm after complete digestion with concentrated nitric acid (1.0 ml; specific gravity 1.38 available for metal ions) at 110°C for 2 days.

Protein Assay: The protein assay was carried out using a Bio-Rad potein assay kit.

Statistical Analyses —— Data are expressed as mean ± S.D. ($n = 5$). Data for control and treatment groups were compared using the unpaired Student's *t*-test (two-tailed) or the Dunnett-type mean rank test.[15]

RESULTS

Levels in Protein, Enzyme Activity, and Iron in Testis Homogenates

In previous investigations of toxic mechanisms,[5,6,9] questions remained whether MBP induced the direct release of iron from the testis as well as RBC.[9] To resolve that question, changes in levels of proteins, enzyme activity, and iron in the homogenates were examined.

Changes in protein levels were not observed up to 1 mM MBP in the homogenates (Fig. 1a), but increased at 10 mM MBP. Decreases in SUDH activity were observed in an MBP concentration-dependent manner in the homogenates (Fig. 1b). The decrease was supported by the *in vivo* results of the orally toxic dose of DBP.[3,5,6]

Iron levels did not change. They were 19.08 ± 1.08 μg/dl for 0 mM MBP (control), 21.19 ± 2.05 μg/dl for 1 mM MBP, 19.64 ± 1.09 μg/dl for 10 mM MBP, and 19.08 ± 2.00 μg/dl for 100 mM MBP.

Effects of MAP on Levels of ATP, Ca^{2+}, GSH, Hb, and Iron in Rat RBC

The MCV usually remains within normal limits. Table 1 shows the effects of MAP on Hb and iron levels and ratios of ATP, Ca^{2+}, or GSH to Hb. The concentrations of 10, 50, 100, 250, and 500 nmol/medium corresponded to 55 atto mole (amol), 270 amol, 550 amol, 1.39 femto mol (fmol), and 2.75 fmol/RBC, respectively. No change in Hb level was observed.

Iron bound and unbound to lower molecular weight proteins (less than 20000) in the RBC showed a significantly dose–dependent increase in C_1-C_6 MAP, that is, mono-*n*-pentylphthalate at 50 amol, mono-*n*-propyl-, mono-*n*-butyl-, and mono-*n*-hexylphthalate at 270 amol, mono-*n*-methylphthalate at 1.39 fmol and mono-*n*-ethylphthalate at 2.75 fmol, respectively, but not in $C_{4(6)}$, C_7, and C_8 MAP, that is, mono-2-ethylhexyl-, mono-*n*-heptyl-, and mono-*n*-octylphthalate, respectively.

Depletion of ATP was observed, but not in inverse proportion to the doses of monoesters, from butyl to hexyl esters at 50 amol, methyl at 270 amol, and ethyl, propyl, heptyl, octyl, and 2-ethylhexyl esters at 550 amol.

Depletion of Ca^{2+} levels was not observed with MAP except for pentyl at 2.75 fmol and butyl at 5.5 fmol.

No change in GSH levels was observed in esters of MAP C_1 to C_6 but its depletion was observed for heptyl at 1.39 fmol and for octyl and 2-ethylhexyl esters at 2.75 fmol.

DISCUSSION

The effects of MAP on RBC should be discussed in association with the reproductive toxicity of DBP. Fukuoka *et al*.[5,6] and Zhou *et al*.[9] proposed a possible mechanism and time sequences of toxic events by which DBP is metabolized to MBP and that MBP might pass through the erythrocyte membrane to accelerate deprivation of Hb, depletion of GSH, and release of iron in RBC, followed by depletion of SUDH in Sertoli cells. Three hours after oral dosing of DBP, MBP levels in the RBC were 805.8 ±

Table 1. Effects of Phthalates on ATP, Ca^{2+}, GSH, Hb, and Iron in Rat Erythrocytes (RBC)

Mono-phthalates	Concentration in medium	Hb (pg/RBC)	ATP/Hb (μmol/g)	GSH/Hb (μg/g)	Ca2+/Hb (mg/g)	Fe (ng/ml)
Methyl phthalate	0 nmol	21.5 ± 1.75	2.3 ± 0.1*	3.15 ± 0.25	3.61 ± 0.31	3.56 ± 0.3
R = CH_3	10 nmol	21.4 ± 1.4	2.0 ± 0.2	3.19 ± 0.31	3.49 ± 0.33	3.45 ± 0.3
	50 nmol	22.3 ± 2.2	1.3 ± 0.08*	3.29 ± 0.15	3.44 ± 0.29	3.89 ± 0.4
	100 nmol	20.3 ± 1.4	0.8 ± 0.1*	2.66 ± 0.23	3.23 ± 0.32	5.29 ± 0.5
	250 nmol	19.8 ± 0.9	0.9 ± 0.1*	2.49 ± 0.31	3.33 ± 0.29	10.7 ± 1.1*
	500 nmol	23.6 ± 1.2	0.8 ± 0.1*	2.36 ± 0.49	3.21 ± 0.27	39.8 ± 4.1**
Ethyl phthalate	0 nmol	21.5 ± 1.75	2.3 ± 0.1*	3.15 ± 0.25	3.61 ± 0.31	3.56 ± 0.3
R = C_2H_5	10 nmol	22.1 ± 1.1	2.5 ± 0.2	3.11 ± 0.23	3.23 ± 0.30	3.66 ± 0.4
	50 nmol	24.0 ± 3.7	2.0 ± 0.2	3.00 ± 0.18	3.22 ± 0.32	4.31 ± 0.5
	100 nmol	22.6 ± 1.4	1.6 ± 0.02*	3.01 ± 0.20	3.49 ± 0.29	3.55 ± 0.4
	250 nmol	22.8 ± 1.6	1.6 ± 0.1*	2.86 ± 0.17	3.26 ± 0.32	5.87 ± 0.9
	500 nmol	22.0 ± 0.7	1.7 ± 0.1*	2.81 ± 0.16	3.33 ± 0.31	49.9 ± 4.8**
Propyl phthalate	0 nmol	21.5 ± 1.75	2.3 ± 0.1*	3.15 ± 0.25	3.61 ± 0.31	3.56 ± 0.3
R = C_3H_7	10 nmol	22.75 ± 1.9	2.2 ± 0.2	3.05 ± 0.23	2.99 ± 0.33	4.99 ± 0.6
	50 nmol	24.4 ± 0.7	1.6 ± 0.2	3.00 ± 0.11	2.95 ± 0.15	58.8 ± 5.4**
	100 nmol	22.9 ± 1.4	1.2 ± 0.06*	3.00 ± 0.12	2.93 ± 0.20	57.6 ± 9.9**
	250 nmol	22.4 ± 0.5	1.5 ± 0.1*	2.86 ± 0.15	2.95 ± 0.19	85.6 ± 12.5**
	500 nmol	23.9 ± 1.0	1.2 ± 0.1*	2.81 ± 0.17	3.02 ± 0.25	83.8 ± 8.0**
Butyl phthalate	0 nmol	21.5 ± 1.75	2.3 ± 0.1*	3.15 ± 0.25	3.61 ± 0.31	3.56 ± 0.3
R = C_4H_9	10 nmol	21.4 ± 1.8	1.9 ± 0.1*	3.08 ± 0.31	3.01 ± 0.29	5.66 ± 0.6
	50 nmol	23.4 ± 1.4	1.4 ± 0.1*	3.09 ± 0.44	3.21 ± 0.18	15.7 ± 1.3**
	100 nmol	22.7 ± 1.5	1.1 ± 0.1*	3.11 ± 0.13	2.78 ± 0.26	20.0 ± 2.1**
	250 nmol	21.3 ± 1.9	1.4 ± 0.1*	2.98 ± 0.19	3.39 ± 0.26	32.3 ± 6.4**
	500 nmol	23.1 ± 2.3	1.1 ± 0.1*	2.95 ± 0.02	3.23 ± 0.12	41.9 ± 6.2**
	1000 nmol	21.3 ± 0.2	0.5 ± 0.02**	3.00 ± 0.25	3.06 ± 0.08*	61.8 ± 10.8**
	2500 nmol	21.8 ± 1.4	0.6 ± 0.03**	3.05 ± 0.34	2.90 ± 0.11*	—
	5000 nmol	22.8 ± 0.9	0.5 ± 0.01**	3.02 ± 0.26	2.85 ± 0.15*	—
Pentyl phthalate	0 nmol	21.5 ± 1.75	2.3 ± 0.1*	3.15 ± 0.25	3.61 ± 0.31	3.56 ± 0.3
R = C_5H_{11}	10 nmol	21.3 ± 1.8	2.6 ± 0.1*	3.12 ± 0.13	3.22 ± 0.32	9.85 ± 1.2*
	50 nmol	21.7 ± 1.2	1.8 ± 0.1*	3.34 ± 0.12	3.27 ± 0.09	78.4 ± 10.7*
	100 nmol	20.9 ± 1.7	1.7 ± 0.1*	3.21 ± 0.09	3.28 ± 0.04	63.3 ± 10.2**
	250 nmol	22.8 ± 1.3	1.8 ± 0.1*	2.93 ± 0.26	3.26 ± 0.08	66.0 ± 5.4**
	500 nmol	21.2 ± 2.2	1.7 ± 0.1*	2.95 ± 0.07	2.65 ± 0.05*	82.1 ± 14.3**

*Significant for $p < 0.05$, ** for $p < 0.01$, compared with the respective controls; Dunnett-type mean rank test. Each concentration of MAP, 10, 50, 100, 250, and 500, corresponds to 0.05, 0.27, 0.55, 1.39, and 2.75 fmol/RBC, respectively. R, alkyl residue of mono-alkyl phthalates. Fe, free and bound iron to lower molecular protein than MW 20000. n.d, not detectable. The MBP levels in the plasma were 6.45 ± 0.15 μmol/ml 3 hr after oral dosing of 8.6 mmol DBP/kg.

39.1 pmol,[6] and Hb and ATP levels in the RBC were depleted to 83.9% and 82.3%, respectively,[6,7] leading to reduced oxygen transport capacity of the blood. Furthermore, testicular atrophy was induced by restricted mono-*n*-$C_{4\text{-}6}$-alkylphthalates ($C_{4\text{-}6}$-MAP) and mono-2-ethylhexylphthalate ($C_{4(6)}$-MAP), accompanied by depletion of SUDH.[4,8,10,16] Recently, Watanabe *et al*.[3] suggested that the SUDH depletion was caused by a hypoxic condition [20–30% dissolved oxygen (DO)] based on *in situ* testis perfusion results. However, it is unclear whether the release of iron from RBC was caused by restricted MAP at lower concentrations than in *in vivo* conditions.[6,7] We therefore incubated RBC with MAP to examine changes in the levels of ATP, Ca^{2+}, GSH, and Hb.

The hypothesis that the Hb in RBC is deprived by restricted MAP seemed to be supported by an

Table 1. Continued

Mono-phthalates	Concentration in medium	Hb (pg/RBC)	ATP/Hb (μmol/g)	GSH/Hb (μg/g)	Ca2+/Hb (mg/g)	Fe (ng/ml)
Hexyl phthalate	0 nmol	21.5 ± 1.75	2.3 ± 0.1*	3.15 ± 0.25	3.61 ± 0.31	3.56 ± 0.3
R = C_6H_{13}	10 nmol	21.6 ± 1.3	2.6 ± 0.1*	3.08 ± 0.23	3.13 ± 0.08	3.66 ± 0.3
	50 nmol	21.6 ± 1.0	1.9 ± 0.1*	3.10 ± 0.12	2.99 ± 0.13	5.35 ± 0.5*
	100 nmol	22.2 ± 1.4	1.6 ± 0.06**	3.00 ± 0.15	3.06 ± 0.09	21.4 ± 2.5**
	250 nmol	21.4 ± 1.0	1.9 ± 0.1*	2.70 ± 0.13	3.29 ± 0.11	n.d.
	500 nmol	21.1 ± 0.5	1.6 ± 0.1*	2.55 ± 0.32	3.10 ± 0.09	n.d.
Heptyl phthalate	0 nmol	21.5 ± 1.75	2.3 ± 0.1*	3.15 ± 0.25	3.61 ± 0.31	3.56 ± 0.3
R = C_7H_{15}	10 nmol	21.3 ± 1.8	2.2 ± 0.1*	3.11 ± 0.22	3.14 ± 0.21	n.d.
	50 nmol	20.7 ± 0.1	2.2 ± 0.1*	3.09 ± 0.18	3.07 ± 0.32	n.d.
	100 nmol	22.1 ± 1.4	1.6 ± 0.04*	3.00 ± 0.15	2.88 ± 0.24	n.d.
	250 nmol	21.0 ± 0.2	1.4 ± 0.3**	2.50 ± 0.06*	3.30 ± 0.11	n.d.
	500 nmol	21.1 ± 0.5	1.3 ± 0.03**	1.69 ± 0.39*	3.29 ± 0.11	n.d.
Octyl phthalate	0 nmol	21.5 ± 1.75	2.3 ± 0.1*	3.15 ± 0.25	3.61 ± 0.31	3.56 ± 0.3
R = C_8H_{17}	10 nmol	21.5 ± 1.7	2.5 ± 0.1*	3.15 ± 0.17	3.59 ± 0.29	n.d.
	50 nmol	20.8 ± 0.1	2.5 ± 0.1*	3.07 ± 0.17	3.11 ± 0.21	n.d.
	100 nmol	21.2 ± 012	1.8 ± 0.1*	3.11 ± 0.21	3.45 ± 0.19	n.d.
	250 nmol	20.7 ± 0.2	1.3 ± 0.1*	2.99 ± 0.12	3.15 ± 0.22	n.d.
	500 nmol	21.6 ± 0.5	1.2 ± 0.1*	2.37 ± 0.18*	3.07 ± 0.26	n.d.
Ethylhexyl phthalate	0 nmol	21.5 ± 1.75	2.3 ± 0.1*	3.15 ± 0.25	3.61 ± 0.31	3.56 ± 0.3
R = $CH_2CH(C_2H_5)C_4H_6$	10 nmol	24.0 ± 2.2	2.3 ± 0.6	3.21 ± 0.22	3.60 ± 0.26	n.d.
	50 nmol	22.8 ± 1.6	2.3 ± 0.6	3.00 ± 0.1*	3.51 ± 0.21	n.d.
	100 nmol	22.0 ± 0.7	1.6 ± 0.1*	3.03 ± 0.11	3.44 ± 0.19	n.d.
	250 nmol	17.6 ± 1.3	1.6 ± 0.1*	2.95 ± 0.20	3.01 ± 0.26	n.d.
	500 nmol	18.8 ± 1.3	1.8 ± 0.1*	2.51 ± 0.25*	3.02 ± 0.31	n.d.

increase in lower molecular protein bound and unbound iron in the RBCs, accompanied by depletion of ATP at 10 nmol (55 amol/RBC) of C_4-C_6 MAP, at 50 nmol (270 amol/RBC) of C_1-MAP, and at 100 nmol (550 amol/RBC) of C_2-, C_3- C_7-, C_8-, and $C_{4(6)}$-MAP (Table 1). Although a difference in Hb levels between the control and treated RBC was not observed, the levels of lower molecular protein bound and unbound iron increased in the RBC. This suggested a dissociation of Hb to the subunits. Hemoglobin is a tetramer, consisting of two pairs of polypeptide chains. C_4- and C_5-MAP, which resulted in severe testicular damage,[4,8,16] depleted Ca^{2+} (Table 1), which stabilized the tetrameric structure of Hb and fully recovered the tetramer from the four dissociated subunits.[17] Depletion of ATP was also inactivated to maintain membrane integrity[18,19] and to allow Ca^{2+} to permeate into the cells.[20] Since Hb molecules were constrained by both hydrogen bonding and hydrophobic interactions between the four subunits,[21] the dissociation of the subunits was induced by H^+ concentration and led to a decrease in oxygen binding.[22–25] Previous reports pointed to a possibility that the increase in the lower molecular protein-bound and unbound iron might partially result from dissociation of Hb to subunits due to the acidity of MBP[7] (C_1-C_6 MAP in Table 1), indicating a decrease in oxygenation affinity, and low oxygen transport, and then hypoxia. Among the hydrophobic (corresponding to C_7, C_8, and $C_{4(6)}$ MAP) interactions, di-2-ethyhexylphthalate changed the erythrocyte membrane compositions, accompanied by increases in the ratios of protein-glycosaminoglycans, protein-carbohydrate, cholesterol-phospholipid,[26] and then the membrane exhibited the detrimental effects of the increased lipid peroxidation and then hemolysis.[1] Therefore it was suggested that hydrophilic phthalates (C_1-C_6 MAP) might induce a dissociation of low molecular bound iron and free iron from the RBC and hydrophobic phthalates (C_7, C_8, and $C_{4(6)}$ MAP) might induce the membrane to undergo lipid peroxidation by depletion of GSH (Table 1).

Deprivation of Hb by DBP *in vivo*[7] leaves the

open question of how erythrocytes protect. Bieber[27] provided an answer to this question: Erythropoietin was also produced in response to the oxidative stress that might be generated by inflammation, hypoxia-ischaemia, and tissue damage caused by oxygen deprivation. Our previous and unpublished studies provided evidence for the ability to stimulate production of RBCs in bone marrow 6 hr after the decrease in Hb levels that occurred 3 hr after oral dosing of DBP,[6,7] in which Hb contents were 1.4 ± 0.2 mg/ml for the bone marrow in the controls, and 1.4 ± 0.1 and 1.8 ± 0.2 mg/ml ($p < 0.05$) at 3 and 6 hr in DBP treated rats, respectively (unpublished data). Therefore, since the data suggested at least a recovery of RBC from the injury induced by phthalate in the animal model, our results support the possibility that the testicular damage induced by phthalate might result from short-term hypoxia due to its metabolite.

Taken together, the results of this study indicate the possibility that the restricted MAP might cause release of iron from RBC, suggesting hypoxia. However, since the mechanism by which the testicular SUDH activity was reduced by hypoxic conditions[3] and by incubation with MBP (Fig. 1) remains unclear, further studies will be needed to determine the relationship between dissociation of the hemoglobin-tetramer into the subunits and SUDH depletion in the testis homogenates by MAP.

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