Comparison of Diisononyl Phthalate Migration from Polyvinyl Chloride Products into Human Saliva *in Vivo* and into Saliva Simulant *in Vitro*

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Human volunteers chewed polyvinyl chloride (PVC) products under controlled conditions for 60 min (four consecutive periods of 15 min), and then the amount of diisononyl phthalate (DINP) migration into the saliva was determined by HPLC. The PVC products consisted of a molded plate containing added DINP (500 mg/g) and five types of commercial toy containing 160–583 mg/g DINP (mean value 372 mg/g). The DINP migration rates ranged from 3.8 to 32.6 μg/cm^2/hr (mean value 16.4 μg/cm^2/hr). The DINP contents in the toy products did not correlate with the amount of *in vivo* migration. The DINP migration rates during the last 15-min period (45–60 min) decreased by 38–75% compared with the first session (0–15 min). In addition, *in vitro* DINP migration rates from the PVC products into saliva simulant during 15 min of rotary shaking were about 3.6–4.1-fold higher than the rates *in vivo* over 60 min, and remained essentially fixed for each sample.

Key words —— diisononyl phthalate, *in vivo* migration, human saliva, *in vitro* migration, saliva simulant, polyvinyl chloride product

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INTRODUCTION

Diisononyl phthalate (DINP) is a mixture of about 100 primarily branched dialkyl chain isomers (Fig. 1.)[1,2] and many structurally dissimilar compounds known as peroxisome proliferators that induce tumors in the rodent liver.[3] In addition to DINP, other dialkyl phthalate esters are frequently used as plasticizers to impart softness and flexibility to normally rigid polyvinyl chloride (PVC) products, such as toys, construction materials, and general consumer products. DINP increases liver weight and changes liver cell histopathology in rodents exposed to chronic high doses.[4] The rodent kidney is also a target for prolonged high-level exposure to DINP.[5]

Toys represent a unique source of childhood exposure to DINP since it is the major plasticizer used in these products.[6–8] The rate of DINP migration from PVC toys chewed by children has been measured using two general migration tests. An *in vivo* test in which adult volunteers chew PVC toys under controlled conditions was developed by Meuling and Rijk in the Netherlands[9] and by Chen in the U.S.A. (US Consumer Product Safety Commission [USCPSC]).[10] The effect of shaking or impacting PVC toys in simulated saliva *in vitro* has been also tested, but the release rates did not correlate well with the inherent total DINP content.[10,11]

At present, there are no standard *in vitro* methods with good reproducibility for measuring DINP migration. In addition, little data can be obtained from *in vivo* migration tests from the viewpoints of time and ethics. Therefore a rapid, simple, and reproducible *in vitro* migration test is required. We present more detailed findings on DINP migration *in vivo* from PVC products and *in vitro* after three types of mechanical agitation. These findings provide background information for the development of a standard method with which to mimic exposure during chewing.

MATERIALS AND METHODS

Reagents —— We purchased DINP from Kanto Chemical Co., Inc. (Tokyo, Japan; over 97.9% pure) and from Wako Pure Chemical Ind. (Osaka, Japan; 98.0% pure). All other solvents and reagents were of analytical grade, and confirmed to be free of phthalate esters. The composition of the simulated saliva corresponded to the British Standard Specification for Safety Harnesses.[12] Glassware was heated

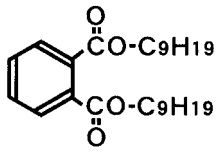

Fig. 1. Structure of DINP

at temperatures over 230°C for at least 5 hr before use.

HPLC Analysis —— The HPLC conditions were as follows: apparatus, LC-10A (Shimadzu, Kyoto, Japan); column, Inertsil C8-3 (0.46 mmϕ × 250 mm); column oven temperature, 40°C; mobile phase, (A) acetonitrile, (B) water; gradient programming, A% 70–100 (15 min, linear gradient); detector, UV (254 nm); injection volume, 10 μl. The quantity of DINP was determined using an absolute calibration curve after the total area of 6 isomer peaks was summed.

DINP Migration Test from PVC Plate and Toys —— The PVC plate was molded at Dainippon Resin Laboratory Co. (Yokohama, Japan) as follows. A mixture of PVC 50 g, dibutyl tin mercuprate 2 g, calcium stearate 0.5 g, and DINP 50 g was heated at 180°C, and then the compounds were molded into 1.0-mm-thick plates containing DINP 462 mg/g. The PVC toys included a pacifier, a teething ring (teether), a rattle, a ball, and a soft doll that contained 538, 389, 380, 255, and 160 mg/g DINP, respectively. The plate and toys were punched over a 2.5 × 3.0 cm area (total surface area was approximately 15 cm^2).

Migration Test in Vivo: One female and three male volunteers gently chewed each of the six samples and phthalate ester-free polypropylene disks as a negative control for 15 min. They performed this task on each sample four times for 15 min each (four sessions), with 5-min breaks. During the experimental periods, saliva was collected in labeled 50-ml glass centrifuge tubes[9,10,13] and the total volume and pH were recorded. All saliva samples were diluted to 10 ml with distilled water, mixed with 10 ml of acetonitrile, and then sedimented by centrifugation at 3000 rpm for 10 min. The *in vivo* migration of the pacifier, teether, and rattle was tested by three of the four volunteers, and the amount of DINP which migrated into the supernatant was quantified by HPLC.

Migration Test in Vitro: Migration was examined after rotary (VR-36, TAITEC Co., Saitama, Japan), vertical (SR-IIW, TAITEC Co., Saitama, Japan), and horizontal (NTS-2000, Tokyo Rika Co., Tokyo, Japan) shaking. Samples were placed in 50-ml glass centrifuge tubes containing 30 ml of simulated saliva, positioned on the rotary shaker in an incubator at 35°C, and shaken for 15 min at 300 rpm. Samples in 50-ml glass centrifuge tubes or 100-ml glass Erlenmeyer flasks containing 30 ml of simulated saliva were agitated on the vertical and horizontal shakers for 15 min at 300 and 120 rpm, respectively. Samples of the solutions (5 ml) were mixed with 5 ml of acetonitrile, and then the amount of DINP which migrated from the samples was quantified by HPLC.

RESULTS AND DISCUSSION

In Vivo Migration of DINP

Table 1 shows the DINP release rates into human saliva (*in vivo*) from the PVC plate and from five toy samples when volunteers mouthed and gently chewed (referred to below as "chewing") them for 1 hr (15 min × 4 sessions). The rates of *in vivo* migration from the PVC plates ranged from 3.8 ± 0.9 to 32.6 ± 2.6 μg/cm^2/hr, and the coefficients of variation ranged from 8% to 37%. The *in vivo* migration rates of DINP from PVC commercial products (toy ducks) has been tested by volunteers in the Netherlands[9] and in the U.S.A.[10] The Dutch consensus study indicated a mean DINP release rate of 10.8 μg/cm^2/hr from samples containing DINP 430 mg/g, which was considerably less than the USCPSC values of 21.9 μg/cm^2/hr. The DINP migration rates from the pacifier and rattle were almost identical to those found in the US study, and that from the teether was similar to that found in the Dutch consensus study.

The Product Safety Laboratory of Health Canada[11] found no correlation between the DINP content of PVC products and the *in vitro* release rate. We found similar results with respect to the *in vivo* release rates into human saliva from six products during chewing. In addition, the *in vivo* migration rate from the plate molded in the laboratory was significantly faster than that from the five toys, which are commercial products with a complex surface structure. Furthermore, the coefficient of variation was minimal on the plate. The *in vivo* migration rate might be more related to the surface design of PVC products.

The rates of saliva produced by four volunteers chewing the six samples ranged from 37.6 ± 7.7 to 53.6 ± 5.1 ml/hr (Table 1). The pH of the saliva

Table 1. DINP Contents in PVC Samples and *in Vivo* Migration Rates into Human Saliva after Mouthing and Gentle Chewing

PVC sample	Contents[a] (mg/g)	*In vivo* migration[b] μg/cm^2/h)	Saliva produced	
			Rate (ml/h)	pH
Plate	462	32.6 ± 2.6	49.2 ± 5.4	6.9–7.4
Toys				
Pacifier	583	20.0 ± 6.0	37.6 ± 7.7	7.1–7.4
Teether	389	12.5 ± 1.9	41.0 ± 3.7	7.2–7.6
Rattle	380	21.9 ± 2.6	40.1 ± 4.0	7.1–7.5
Ball	255	7.8 ± 2.9	45.2 ± 3.7	6.7–7.6
Soft doll	160	3.8 ± 0.9	53.6 ± 5.1	6.7–7.2
Mean value	372	16.4 ± 2.8	—	—

a) DINP contents in PVC samples were measured by extraction with acetone after rotary shaking at 300 rpm for 3 hr. *b*) Release rates obtained from *in vivo* migration tests after four volunteers chewed PVC samples for 60 min (15 min ×4 sessions). Values are means ± S.D. (pacifier, teether, and rattle, $n = 3$; plate, ball, and soft doll, $n = 4$).

ranged from 6.7–7.6. The normal salivation rate in humans is 60–120 ml/hr when gum is chewed, and the fluctuation range is wide.[14] The rate of saliva production induced by chewing the samples seemed low and was affected by each sample. However, the total release of DINP into the saliva was not altered by the salivation rate and pH of the saliva.

Figure 2 shows the effect of chewing time on the DINP release and rate of saliva produced by four volunteers chewing the six samples. The migration rate of DINP was the most rapid during the first 15 min and thereafter decreased with repetition of the task. The migration rates at the fourth session (45–60 min of chewing time) from the plate, pacifier, teether, rattle, ball, and soft doll were 71, 75, 75, 69, 47, and 38% respectively, of those at the first session (0–15 min). This indicates that DINP gradually migrated from the surface of PVC polymer, because the rate decreased over time. Our findings showed that DINP initially migrated into the saliva through chewing, indicating that the molecular forces that bound DINP and PVC polymer in the samples are weak.[15] The rates of saliva produced during each session were almost identical, and the quantity did not appear to affect DINP migration significantly.

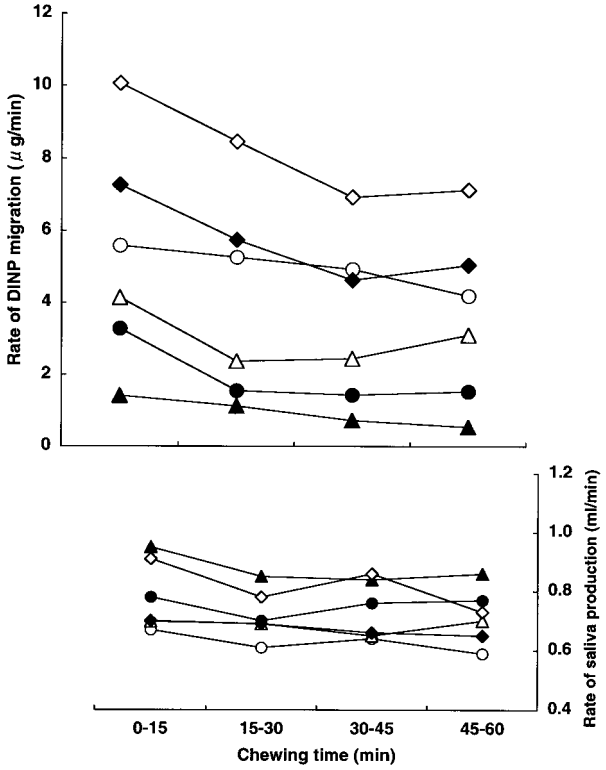

Fig. 2. Effect of Mouthing and Chewing Period on the Rate of DINP Migration from Polyvinyl Chloride Plate and Toy Samples (Surface Area, 15 cm^2) and Saliva Production by Four Volunteers

◇, plate; ○, pacifier; △, teether; ◆, rattle; ●, ball; ▲, soft doll.

Comparison of *in Vitro* Migration of DINP with *in Vivo* Migration

Table 2 shows the *in vitro* migration rates of DINP into saliva simulant agitated on three mechanical shakers for 15 min. The DINP release rates from the PVC products ranged from 22.4 to 148.5 μg/cm^2/hr. The amount of DINP migration from the rattle after rotation, vertical, shaking and *in vivo* chewing were equivalent to the mean values of each of the four products, but the amount that migrated after horizontal shaking was half of these values. The USCPSC reported that the DINP release rate obtained by impaction using an air-driven piston ranged from 0.1 to 4.4 μg/cm^2/hr.[11] The release rates obtained *in vivo* are higher than those obtained *in vitro*.[16] Considerably more DINP seems to have migrated due to mechanical shaking than either to

Table 2. Migration of DINP *in Vitro* PVC Plate and Toy Samples were Mechanically Shaken with Saliva Simulant

PVC sample	DINP migration rate[a] (μg/cm^2/hr)			
	in vivo[b]	Rotation[c]	Vertical[d]	Horizontal[e]
Plate	32.6 (7)	124.8 (6)	148.5 (10)	88.3 (14)
Toys				
Pacifier	20.0 (30)	73.3 (8)	117.3 (8)	68.3 (14)
Teether	12.5 (15)	51.7 (7)	93.1 (15)	22.4 (25)
Rattle	21.9 (12)	83.5 (3)	112.5 (10)	25.1 (22)
Mean value	21.8 (16)	83.3 (6)	117.9 (11)	51.0 (19)
y=a+bx[f]				
a	—	3.2151	58.656	−15.91
b	—	3.6832	2.7216	3.0775
r[g]	—	0.9962	0.9833	0.7841

a) Release rates obtained from *in vitro* migration tests of simulated saliva after mechanical shaking for 15 min. Values are means (coefficient of variations) ($n = 5$). *b*) Release rates obtained from *in vivo* migration tests after 4 volunteers chewed PVC samples for 60 min (15 min × 4 sessions). Values are means ± S.D. (pacifier, teether, and rattle, $n = 3$; plate, ball and soft doll, $n = 4$). *c*) Glass centrifuge tubes (50 ml) were rotated at 300 rpm at 35°C. *d*) Glass centrifuge tubes (50 ml) were vertically shaken at 300 shakes/min at room temperature. *e*) Glass Ehlrenmyer flasks (100 ml) were horizontally shaken at 120 shakes/min at room temperature. *f*) These equations showed the regression line of the *in vitro* migration rates to that of the *in vivo* rates. *g*) Product-moment correlation coefficient.

the type of impaction used at the USCPSC or from chewing by adult volunteers.

The *in vitro* migration rates from samples agitated by rotary, vertical, and horizontal shaking for 15 min were 3.6–4.1, 4.6–7.4, and 1.1–3.4-fold higher, respectively, than those of *in vivo* migration for 60 min. The *in vitro* release rates obtained by rotary shaking compared with the *in vivo* migration rates were almost fixed for each sample. The regression lines of the migration rates *in vitro* to those *in vivo* showed linearity, and the correlation coefficients (r) were ranged from 0.7841 to 0.9962 (Table 2). We found that there was a highly positive correlation between the migration rates after rotation shaking *in vitro* and chewing *in vivo*. In addition, the coefficient of variation in the migration rates caused by rotary shaking was smaller than that caused by either vertical or horizontal shaking.

On the bases of comprehensive reviews of data relating to the toxicology of DINP and its rate of migration from PVC toys during the mouthing activities of children, Wilkinson and Lamb concluded that DINP in PVC toys does not present a significant risk to the health of children.[16] The present study found that the *in vivo* migration test results were equivalent to those obtained in the U.S.A. and in the Netherlands. In addition, the migration test using a rotary shaker may be useful as *in vitro* standard method of mimicking exposure while chewing. Both the *in vivo* and *in vitro* migration rates into saliva are used as partial means of estimating exposure to DINP derived from toys in Japan.

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