Diisononyl phthalate (DINP) is a complex mixture of predominantly nine-carbon branched-chain dialkyl phthalate isomers, predominantly containing nine carbons in the alkyl chain. DINP is used primarily as a plasticizer in polyvinyl chloride plastics (Abe et al. 2003) and is widely used in automotive, building materials, consumer products, and toys [Center for the Evaluation of Risks to Human Reproduction (CERHR) 2000; Kavlock et al. 2002].

In rats, DINP shows antiandrogenic activity (Gray et al. 2000). Specifically, nipple atrophy and testis atrophy after perinatal exposure to 750 mg/kg DINP have been observed in male rats (Gray et al. 2000). It appears that DINP, like di(2-ethylhexyl) phthalate (DEHP), a widely used phthalate, alters sexual differentiation of the male rat by inhibiting testicular testosterone synthesis (Gray et al. 2000). Specifically, nipple retenosity of the male rat by inhibiting testicular testosterone synthesis (Gray et al. 2000).

DINP biomonitoring to measure exposure in humans is of interest because of the potential adverse health effects of DINP. More important, children may be exposed to higher levels of DINP than adults because infants and small children mouth toys and other articles that can contain DINP (Kavlock et al. 2002). Because DINP is not covalently bound to the plastics, it can migrate into saliva and be swallowed (Kavlock et al. 2002). In previous studies the hydrolytic monoester of DINP, mono(oxoisononyl) phthalate (MCIOP), has been used for human exposure assessment of DINP [Centers for Disease Control and Prevention (CDC) 2005; Silva et al. 2004a]. However, the frequency of detection of MCIOP was very low compared with other phthalate metabolites. The low frequency of detection of MCIOP in human populations may be attributable, at least in part, to the fact that MCIOP further metabolizes to unidentified oxidative metabolites before being excreted in urine. Although the metabolism of DEHP (Albro 1986; Koch et al. 2004, 2005a; Silva et al. 2006a) and di-n-octyl phthalate (DnOP) (Albro and Moore 1974; Calafat et al. 2006; Silva et al. 2005) in rodents and humans is relatively well known, the metabolism of DINP has been less studied (McKee et al. 2002).

In rodents, MCIOP was found to metabolize to unidentified oxidative products (McKee et al. 2002). Recently, several urinary oxidative metabolites of DINP were identified and detected at much higher concentrations than MCIOP in DINP-dosed rats (Silva et al. 2006a).

It was postulated that these metabolites could be used as biomarkers of exposure to DINP in humans (Silva et al. 2006a). In this study, MNP and three of these oxidative metabolites, mono(carboxylisoctyl) phthalate (MOINP), mono(hydroxyisononyl) phthalate (MHINP), and mono(oxoisononyl) phthalate (MCOINP) (Figure 1), were measured in 129 human urine samples from adults with no known exposure to DINP. As in rodents, in this group of adults the frequency and the magnitude of detection were significantly higher for the oxidative metabolites than for MNP.
prescreened urine cup. The analytical method for measuring DINP oxidative metabolites in urine was adapted from previously published methods (Blount et al. 2000; Silva et al. 2003, 2004b). Briefly, the urine samples (1 mL) were spiked with an internal standard solution containing $^{13}$C$_2$-MEHP, $^{13}$C$_4$-MEOHP, $^{13}$C$_4$-MEHHP, $^{13}$C$_5$-MECPP, $^{13}$C$_5$-MINP, and 4-MeUmb. MeUmb-glu was added to evaluate the completion of the deglucuronidation reaction with β-glucuronidase. Phthalate monoester metabolites were extracted by automated solid-phase extraction (SPE) using a commercial SPE system (Zymark Corp., Hopkinton, MA) after enzymatic hydrolysis. The metabolites in the urine extract were chromatographically resolved by high-performance liquid chromatography (HPLC) using a Surveyor HPLC system (ThermoFinnigan, San Jose, CA) equipped with a Betasil phenyl HPLC column (3 µm, 100 mm × 2.1 mm; ThermoHyperil-Keystone, Bellefonte, PA) using a nonlinear water:acetonitrile solvent gradient. The metabolites were detected by negative ion electrospray ionization tandem mass spectrometry using a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer (ThermoFinnigan). For the measurement of the unconjugated metabolites, we eliminated (ThermoFinnigan). For the measurement of Quantum triple quadrupole mass spectrometry using a ThermoFinnigan TSQ treat the β-glucuronidase. Under our experimental conditions, the isomeric metabolites of DINP were not chromatographically resolved and eluted as broad peaks. The entire area under the peak encompassing all isomers was integrated for quantification. The limits of detection (LODs) were 0.25 ng/mL for MOINP, MHINP, and MCIOP. The LOD for MINP was 0.36 ng/mL. Oxidative metabolism is an enzymatically mediated reaction. Therefore, oxidative metabolites cannot result from potential contamination with DINP during sampling, storage, or analysis.

Statistical analysis of the data was performed using the Statistical Analysis System (SAS) software (SAS Institute Inc., Cary, NC). Samples with values below the LOD were assigned a concentration equal to the LOD divided by the square root of 2 for the statistical analyses (Hornung and Reed 1990). Statistical significance was set at $p < 0.05$.

**Subjects.** The urine samples analyzed for this study were collected specifically for analysis of phthalate metabolites in 2005 from a demographically diverse group of 129 U.S. adults of both sexes with no documented exposure to DINP. No personal information from the subjects was available. Samples were collected between 0800 hr and 1700 hr and were not necessarily first morning voids. The study protocol was reviewed and approved by the CDC Human Subjects Institutional Review Board. A waiver for informed consent for this project was requested under 45 CFR 46.116(d) (Code of Federal Regulations 2005).

**Results and Discussion**

Although DINP is a less potent inducer of peroxisomal proliferation than DEHP (McKee et al. 2000), DINP exerts antiandrogenic effects similar to that of DEHP in DNP-dosed rats (Gray et al. 2000). The effects of DINP exposure in humans are not currently known.

Phthalates with long alkyl side chains, such as DEHP and DnOP, metabolize extensively before being excreted in urine both in rodents and humans (Albro 1986; Albro and Moore 1974; Koch et al. 2004, 2005b; Silva et al. 2005). Similarly, in rats administered DINP, some MINP was excreted in urine, but oxidative metabolites of MINP, MHINP, MCIOP, and MOINP were excreted as the major urinary metabolites (Silva et al. 2006a).

We measured the urinary concentrations of MINP, MHINP, MCIOP, and MOINP in 129 human adults. We observed a wide range of exposures to DINP (Table 1). MHINP was present in all samples tested at concentrations ranging from 1.4 to 202.7 ng/mL, with 5% of the samples having > 43.7 ng/mL (Table 1).

![Figure 1. DINP metabolites proposed as biomarkers for exposure assessment to DINP in humans.](image)

**Table 1. Urinary levels (ng/mL) of DINP metabolites in a group of 129 U.S. adults.**

| Urinary DNP metabolite | n | 10th | 25th | 50th | 75th | 90th | 95th | Geometric mean | Frequency of detection (%) |
|------------------------|---|------|------|------|------|------|------|-----------------|--------------------------|
| MCIOP Total            | 129| 2.0  | 3.9  | 8.4  | 18.3 | 27.3 | 46.2 | 7.8            | 97                      |
| Freec                  | 82 | 2.0  | 2.9  | 5.1  | 11.6 | 22.8 | 15.5 | 6.1            | 98                      |
| MHINP Total            | 129| 2.6  | 5.4  | 13.2 | 23.2 | 40.2 | 43.7 | 11.4           | 100                     |
| Freec                  | 82 | 1.8  | 2.9  | 5.8  | 9.1  | 15.5 | 20.1 | 5.4            | 100                     |
| MOINP Total            | 129| < LOD | 0.5  | 1.2  | 2.4  | 5.0  | 6.6  | 1.2            | 87                      |
| Freec                  | 82 | < LOD | < LOD | < LOD | 0.3  | 0.7  | 1.3  | NA             | 30                      |
| MINP Free              | 129| < LOD | < LOD | < LOD | < LOD | < LOD | < LOD | < LOD          | 0                       |
| Total                  | 82 | < LOD | < LOD | < LOD | < LOD | < LOD | < LOD | < LOD          | 0                       |

NA, applicable; the geometric mean was calculated only if the frequency of detection was ≥ 60%.

$^{13}$C$_2$-MECPP was used as the internal standard for MCIOP. $^{13}$C$_5$-MEDHP was used as the internal standard for MOINP, $^{13}$C$_5$-MEHHP and $^{13}$C$_5$-MINP were used as the internal standards for MHINP and MINP, respectively. LOD/√2 was used for the statistical computations if the concentration was below the LOD. LODs were 0.36 ng/mL (MINP) and 0.25 ng/mL (MCIOP, MHINP, and MOINP). Only 82 samples were available in sufficient quantities to determine the concentrations of free metabolites.

![Figure 2. Median levels of DINP and DEHP metabolites in a group of 129 U.S. adults. For concentrations < LOD, a value of LOD/√2 was used for the statistical computations.](image)
Based on similar physicochemical properties and metabolism between DINP and DEHP (Koch et al. 2004, 2005b; Silva et al. 2006b, 2006c), the lower concentrations of DINP oxidative metabolites than of DEHP oxidative metabolites in this group of adults suggest that environmental exposures to DINP may be lower than the exposure to DEHP. However, because DINP is a mixture of isomers, it is also possible that the prevalence of exposure to DINP is underestimated by measuring only these three oxidative metabolites. Furthermore, the elimination half-life of the oxidative metabolites of DINP is presently unknown. Therefore, the differences in urinary concentrations observed among DINP oxidative metabolites and their DEHP counterparts may also reflect differences in toxicokinetic parameters.

Because all three DINP metabolites result from the same parent compound, their urinary concentrations were highly correlated with each other, with correlation coefficients varying from 0.73 to 0.83 (p < 0.001; Figure 3), similar to previous findings regarding DEHP metabolites (Barr et al. 2003; Kato et al. 2004; Koch et al. 2004, 2005b).

Glucuronidation not only facilitates urinary excretion of phthalate metabolites but may also reduce their potential biological activity if the putative biologically active species is the free metabolite. We measured both total and free urinary concentrations of MHINP, MOINP, and MCIOP and found that although MCIOP mostly excreted in its free form, MOINP excreted mostly glucuronidated. The percentage of MHINP excreted either as a conjugate or free form was similar (Figure 4). Furthermore, the concentration of the glucuronidated form of the metabolites increased with increasing levels of the total metabolite concentrations, indicating the absence of enzyme saturation at environmental exposure levels (Figure 5).

In summary, we measured the urinary concentrations of three oxidative metabolites of DINP (MCIOP, MHINP, and MOINP) and the hydrolytic metabolite MINP in 129 anonymous adults. The oxidative metabolites were present in all samples tested, and their urinary concentrations were highly correlated with each other. By contrast, the hydrolytic monoester MINP was not detected in any of the samples. The most abundant DINP urinary metabolites were the ω and ω-1 oxidative metabolites, MCIOP and MHINP,
respectively. MCIOP was excreted in urine predominantly in its free form, whereas MOINP was excreted glucuronidated. The significantly higher frequency of detection and urinary levels of oxidative metabolites than of MINP confirm the validity of these oxidative metabolites as biomarkers for DINP exposure assessment. More important, these data suggest that exposure to DINP is widespread and that it has been underestimated by using MINP as the sole DINP urinary biomarker.

In Figure 5, values for urinary MOINP glucuronide on the y-axis have been modified from the original manuscript published online. The corrected values are 0.1, 1, 10, 100, and 1,000. The original values were 0.1, 1, 10, and 100.

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