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3-METHYL-4-NITROPHENOL METABOLISM BY URIDINE DIPHOSPHATE GLUCURONOSYLTRANSFERASE AND SULFOTRANSFERASE IN LIVER MICROSOMES OF MICE, RATS, AND JAPANESE QUAIL (*COTURNIX JAPONICA*)

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Abstract—3-Methyl-4-nitrophenol (PNMC) is a component of diesel exhaust particles and one of the major breakdown products of the insecticide fenitrothion. This chemical has a high potential for reproductive toxicity in Japanese quail (*Coturnix japonica*) and rats. Because PNMC inhaled by the body is metabolized by uridine diphosphate glucuronosyltransferase (UGT) and sulfotransferase, we investigated these enzyme activities in the hepatic microsomes and cytosols of quail (as a model of wild birds) and compared these activities with those of rats and mice as models of ecological and human risk assessment. The maximum velocity of the UGT for PNMC in quail was 12.7 nmol/min/mg, which was one third and one fourth those of rats and mice, respectively. The Michaelis–Menten constant of UGT for PNMC in quail was 0.29 mM, which was 1.3- and 1.8-fold higher than that in mice and rats, respectively, but not significantly different. In accordance with these results, UGT activities for PNMC were lowest in quail, with those in mice and rats being 4.4- and 2.7-fold higher, respectively. Sulfotransferase activity for PNMC was considerably less than that of UGT in all animals, including quail; no significant differences in the activities were found among mice, rats, and quail. These results suggest that glucuronidation may be involved primarily in PNMC elimination from wild birds as well as mammals and that the UGT activity in quail is less than that in the rodents.

Keywords—3-Methyl-4-nitrophenol Diesel exhaust particles Fenitrothion Glucuronosyltransferase activity Sulfotransferase activity

INTRODUCTION

The use of diesel engines is steadily increasing because of their superior fuel efficiency and efforts toward decreasing engine exhaust emissions over the past 20 years [1]. Diesel exhaust emissions, however, contain fine particulate matter composed of carbon-based particles that adsorb various organic compounds, including polycyclic aromatic hydrocarbons (PAHs), quinones, and nitro-PAHs [2]. Many of these organic compounds associated with diesel exhaust particles (DEPs) are known to be carcinogenic [3] and mutagenic in rodents [4]. Diesel exhaust particles also can lead to potential adverse effects on both the male and female reproductive systems in rodents [5–8]. Thus, air pollution caused by DEPs may be associated with adverse health effects in humans, including lung cancer [9,10], allergic rhinitis [11,12], and bronchial asthma–like diseases [13,14]. Recently, four nitrophenol derivatives—4-nitrophenol, 2-methyl-4-nitrophenol, 3-methyl-4-nitrophenol (4-nitro-*m*-cresol [PNMC]), and 4-nitro-3-phenylphenol—were isolated from DEPs [15,16]. It is of genuine interest to understand which chemicals can potentially lead to adverse effects in rodents or humans.

3-Methyl-4-nitrophenol also is formed via both metabolism and environmental degradation of fenitrothion [17]. Fenitrothion is widely used as an organophosphorous insecticide, mainly in agriculture for controlling chewing and sucking insects on rice, cereals, fruits, vegetables, stored grains, and cotton. Fenitrothion also is used in public health programs as well as indoors for the control of flies, mosquitoes, and cockroaches [18]. After exposure and subsequent absorption into the body, this pesticide is rapidly metabolized. The main metabolites for this compound are alkyl phosphates, such as dimethyl thiophosphate and dimethyl phosphate, and the phenolic compound PNMC [19]. 3-Methyl-4-nitrophenol is excreted in urine in the form of conjugates with glucuronic or sulfuric acid, producing the corresponding glucuronide (3-methyl-4-nitrophenol glucuronide [PNMCG]) and sulfate (3-methyl-4-nitrophenol sulfate [PNMCS]). The conjugations of PNMC are catalyzed by uridine diphosphate glucuronosyltransferase (UGT) [20] and sulfotransferase (SULT), respectively [21].

Recently, PNMC has been reported to exhibit estrogenic and antiandrogenic activity in vitro and in vivo in rats [15,22–24]. In addition, this compound induced impairment of testicular function in adult male Japanese quail (*Coturnix japonica*) [25] as well as male rats [26]. In the quail, a 78-mg/kg dose administered singly significantly decreased plasma testosterone levels by half compared with those in the untreated group, whereas in the rats, a 100-mg dose given consecutively for 5 days decreased plasma testosterone level by the same level as

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in quail. Although the direct comparison of the adverse effects of PNMC in the two methodologically different studies is difficult, quail may be more vulnerable to the adverse effects on the reproductive system than rats. Here, one question may be raised about the species difference seen in the reproductive toxicity for PNMC. Because species differences were noted in UGT and SULT activities [27], it should be clarified whether those also are seen in these enzyme activities for PNMC among other species, such as mice, rats, and quail. Humans are at risk of exposure to PNMC as well; therefore, it is very important to determine the kinetic parameters as well as the species difference in quail as well as in rats or mice, which often are used as experimental animals for ecological and human risk assessment.

The aim of the present study was to determine UGT and SULT activities for PNMC in liver microsomes and cytosols in Japanese quail and in mice and in rats, respectively. The results of this investigation served to elucidate whether the differences in UGT activity for PNMC among quail and mice or rats might reflect varying degrees of susceptibility to reproductive toxicity in birds and/or mammals.

MATERIALS AND METHODS

Chemicals

3-Methyl-4-nitrophenol was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Acetonitrile, methanol, and acetic acid (high-performance liquid chromatography [HPLC] grade), and uridine 5′-diphosphate glucuronic acid (UDPGA) were purchased from Wako Pure Chemical (Osaka, Japan) and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) and β-glucuronidase from Sigma-Aldrich Co. (St. Louis, MO, USA).

Experimental animals

In the present study, mice and rats were used according to the Guidelines for Animal Experiments of the Nagoya University Animal Center, and the use of quail was approved by the Animal Care and Use Committee of the Japanese National Institute for Environmental Studies. Eight-week-old male C57BL6/N mice and eight-week-old male Sprague-Dawley rats were purchased from Clea Japan (Tokyo, Japan). Six-week-old male Japanese quail (*C. japonica*) and their food were provided by Kanematsu Quail Diet (Kanematsu Agritech, Ibaraki, Japan). Mice and rats were housed in cages in a clean room under controlled temperature (23–25°C), relative humidity (57–60%), and light (lights-on, 9:00 AM to 11:00 PM). Quail (age, six to nine weeks) were housed in metal cages in a controlled environment (lights-on, 5:00 AM to 7:00 PM; temperature, 23 ± 2°C; humidity, 50 ± 10%; air exchanged 20 times hourly). Rodents and quail were killed at 10 and 9 weeks of age, respectively. Liver was removed and stored at −85°C until used. Mean body and liver weights were 23.9 and 1.22, 351.2 and 13.3, and 133.6 and 2.15 g in mice, rats, and quail, respectively.

Preparation of microsomes and cytosol from liver

The livers were homogenized in three volumes (w/v) of 0.25 M sucrose and 10 mM phosphate buffer (pH 7.4) with an ultrasonic cell disrupter (Yamato Scientific, Tokyo, Japan). Enzyme fractions were prepared at 4°C by differential ultracentrifugation: The supernatant of the first centrifugation at 10,000 *g* for 10 min was further centrifuged at 105,000 *g* for 1 h to obtain the microsomal and cytosolic fractions. The cytosols were used for the measurement of SULT activity, and the microsomal pellets were resuspended in the buffer to provide a protein content of 10 mg/ml and then used to determine UGT activity. Protein contents were measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard.

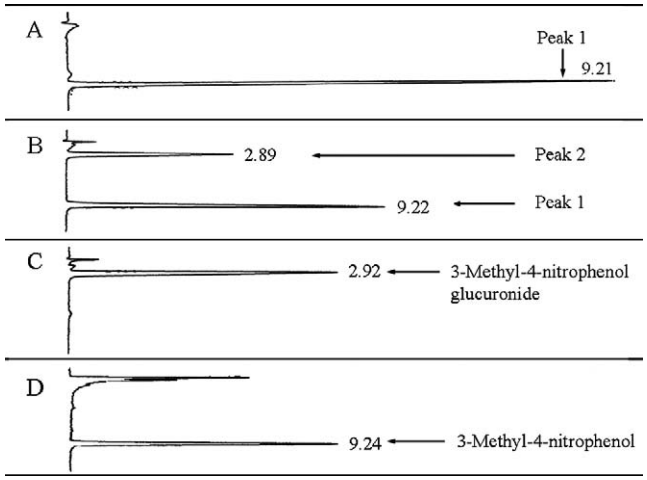

Fig. 1. Chromatograms of 3-methyl-4-nitrophenol and 3-methyl-4-nitrophenol glucuronide reaction products with and without uridine 5′-diphosphate glucuronic acid and with and without β-glucuronidase, respectively, in mouse liver microsome. **(A)** Chromatogram of 3-methyl-4-nitrophenol reaction products in incubation mixture without uridine 5′-diphosphate glucuronic acid for 10 min. **(B)** Chromatogram of 3-methyl-4-nitrophenol reaction products in incubation mixture with uridine 5′-diphosphate glucuronic acid for 10 min. Peak 1 is 3-methyl-4-nitrophenol, and peak 2 is a new metabolite peak thought to be 3-methyl-4-nitrophenol glucuronide. **(C)** Chromatogram after incubation of 3-methyl-4-nitrophenol glucuronide without β-glucuronidase for 1 h. **(D)** Chromatogram after incubation of 3-methyl-4-nitrophenol glucuronide with β-glucuronidase for 1 h.

Analysis of UGT activity

The UGT activity for PNMC was determined according to the method described by Elovaara et al. [28] with a slight modification. A metabolite of PNMC with UGT was determined by HPLC. Fifty micrograms of hepatic microsomal protein were preincubated with 10 μl of 0.125% Triton X-100 at 37°C for 5 min. The incubation mixture (final volume, 250 μl) consisted of 50 mM Tris-HCl buffer (pH 7.4), 4 mM $MgCl_2$, 0.12 mM KCl, 0.5 mM PNMC, and preincubated microsomes. The reaction was started with 10 μl of 50 mM UDPGA (sample vial) or autoclaved distilled water (reference vial). After incubation for 10 min at 37°C, the reaction was stopped by adding 250 μl of ice-cold acetonitrile. The reaction samples were then placed in an ice bath for 10 min before centrifugation at 10,000 *g* for 5 min. The resulting supernatants were injected into a HPLC system (Hitachi, Tokyo, Japan) equipped with a Wakopak Wakosil-II 5C18-100 column (film thickness, 5 μm; inner diameter, 4.6 mm; length, 150 mm; Wako Pure Chemical, Osaka, Japan), L6000 pump, AS-2000 autosampler, L7300 column oven, L4200 UV-Vis detector (at 300 nm), and D-2500 integrator. Acetonitrile with 0.2% acetic acid in Millipore water (35:65 v/v) was used as the mobile phase at a flow rate of 1 ml/min. Under these conditions, the peaks of PNMCG (a metabolite of PNMC) and PNMC appeared at 2.9 and 9.2 min, respectively (Fig. 1A and B), and the formation of PNMCG as well as the amount of reduced PNMC were linearly increased with the incubation time (≤30 min) and protein concentration (0–200 μg/250 μl). The UGT activity (nmol/min/

mg protein) was calculated as follows: Substrate amounts added (PNMC [nmol])·(peak area for PNMC in the reference vial − that in sample vial)/peak area for PNMC in reference vial/incubation time (10 min)/microsomal protein (50 μg). In this equation, the difference between PNMC amount in the reference vial and in the sample vial corresponds to that of PNMCG produced.

To identify the PNMC metabolite peak on the chromatogram in Figure 1B as PNMCG, HPLC elutions from 2.5 to 3.5 min after the sample injection were collected. The collected fraction was dried and resolved in an aliquot of Tris-HCl buffer (pH 7.4). Then, the resolved solution was buffered with sodium acetate buffer (pH 5.0) and hydrolyzed enzymatically using β-glucuronidase at 37°C for 1 h (hydrolyzed sample). As a reference, another resolved solution without the β-glucuronidase was incubated under the same conditions (nonhydrolyzed sample). After ice-cold acetonitrile was added to the hydrolyzed and nonhydrolyzed samples, both samples were centrifuged at 10,000 *g* for 10 min and then analyzed using the HPLC method described above.

Analysis of SULT activity

The SULT activity for PNMC was determined according to the method described by Yodogawa et al. [29] with a slight modification. The reaction mixture consisted of 100 μg of hepatic cytosolic protein, 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, and 200 μM PAPS in a final volume of 250 μl (sample vial). 3′-Phosphoadenosine 5′-phosphosulfate was omitted from the mixture for controls (reference vial). The reaction was started with 2.5 μl of PNMC (0.25 mM) and incubated at 37°C for 10 min. After the reaction was stopped by the addition of 250 μl of ice-cold acetonitrile, the reaction tubes were placed in an ice bath for 10 min, followed by centrifugation at 10,000 *g* for 5 min. The resulting supernatants were analyzed using the HPLC method described above. Because the SULT activity for PNMC was barely discernible, we could not detect the PNMCS peak on the chromatogram. Therefore, the presence of SULT activity was analyzed only by measuring the disappearance of PNMC concentrations after incubation (i.e., the difference between the PNMC peak area of reference vial and that of samples after incubation). Thus, the activity (nmol/10 min/mg protein) was calculated as the amount of substrate that disappeared during the incubation time (10 min) per cytosol protein content by a method similar to the one described in connection with UGT activity.

UGT kinetics

Uridine diphosphate glucuronosyltransferase activity for PNMC was measured in duplicate determination using three individual liver microsomes of mice, rats, and quail and different concentrations of PNMC (0.1, 0.2, 0.25, 0.33, 0.5, 1.0, and 2.5 mM). Incubation conditions were chosen so that the product formation was linear with respect to both the amount of microsomal protein (50 μg) and the incubation time (10 min). Using the mean values of the UGT activity obtained against each substrate concentration, the maximum velocity (V_{max}), and Michaelis constant (K_m), the values for PNMC were calculated from Lineweaver-Burk plots.

Statistical analysis

Statistical differences between group mean values were determined by the Kruskall-Wallis test or Wilcoxon rank-sum test. Differences with p values of less than 0.05 were considered to be statistically significant.

RESULTS

PNMCG characterization

When comparing the chromatograms of the reaction mixtures for measuring the UGT activity of PNMC with and without UDPGA, a new metabolite peak appeared only in the former at a retention time of 2.9 min (Fig. 1B). For identification of the new peak, the fraction from 2.5 to 3.5 min with a peak at 2.9 min was collected and measured as described in *Materials and Methods.* The chromatograms of the fraction incubated without and with β-glucuronidase are shown in Fig. 1C and D, respectively. After hydrolysis, the peak at 2.9 min completely disappeared, to be replaced by the appearance of a peak corresponding to PNMC at 9.2 min. These results suggest that the new peak at 2.9 min is the glucuronidation product of PNMC (i.e., PNMCG).

UGT kinetics

The K_m and V_{max} of UGT activity for PNMC were calculated from Lineweaver-Burk plots (Fig. 2 and Table 1). The V_{max} in quail was 12.7 nmol/min/mg, which was one third and one fourth that in rats and mice, respectively. The mean K_m of UGT for PNMC in quail was 0.29 mM, which was 1.3- and 1.8-fold higher than the mean K_m in mice and rats, respectively. No significant difference, however, was found between them. As a result, the V_{max} to K_m ratio for PNMC was the smallest in quail, followed by rats and then mice.

UGT activity for PNMC

The UGT activities for PNMC in hepatic microsomes of mice, rats, and quail are shown in Table 2. The activity was the lowest in quail, whereas the activities in mice and rats were 4.4- and 2.7-fold higher, respectively.

SULT activity for PNMC

The SULT activity for PNMC in the hepatic cytosols of mice, rats, and quail shown in Table 3 was considerably less than that of UGT in all the animals, including quail. No significant differences in that activity were found among mice, rats, and quail.

DISCUSSION

A statistical difference in UGT activity for PNMC was seen among mice, rats, and quail. The fact that the K_m values did not differ among them, whereas the V_{max} values were lower in birds than in rodents, suggested that the affinity of PNMC for UGT is no different between rodents and birds but that the constitutive expression of the enzyme is lower in birds than in rodents. Thus, the V_{max} to K_m ratio (often referred to as an index of intrinsic clearance) was the smallest in quail, but the ratios were almost the same between mice and rats. Short et al. [27] reported species differences in UGT activity: For *p*-nitrophenol, the activity was the highest in goats (14.28 ± 6.73 nmol/mg protein/min), followed by rats (2.61 ± 1.26 nmol/mg protein/min) and then chickens (1.65 ± 1.01 nmol/mg protein/min). These results suggest that there may be a species difference in UGT activity for another nitrophenol. The above report concerning species difference in activity is so limited, however, that it is doubtful whether this difference can be generalized to all substrates of UGT. In contrast with

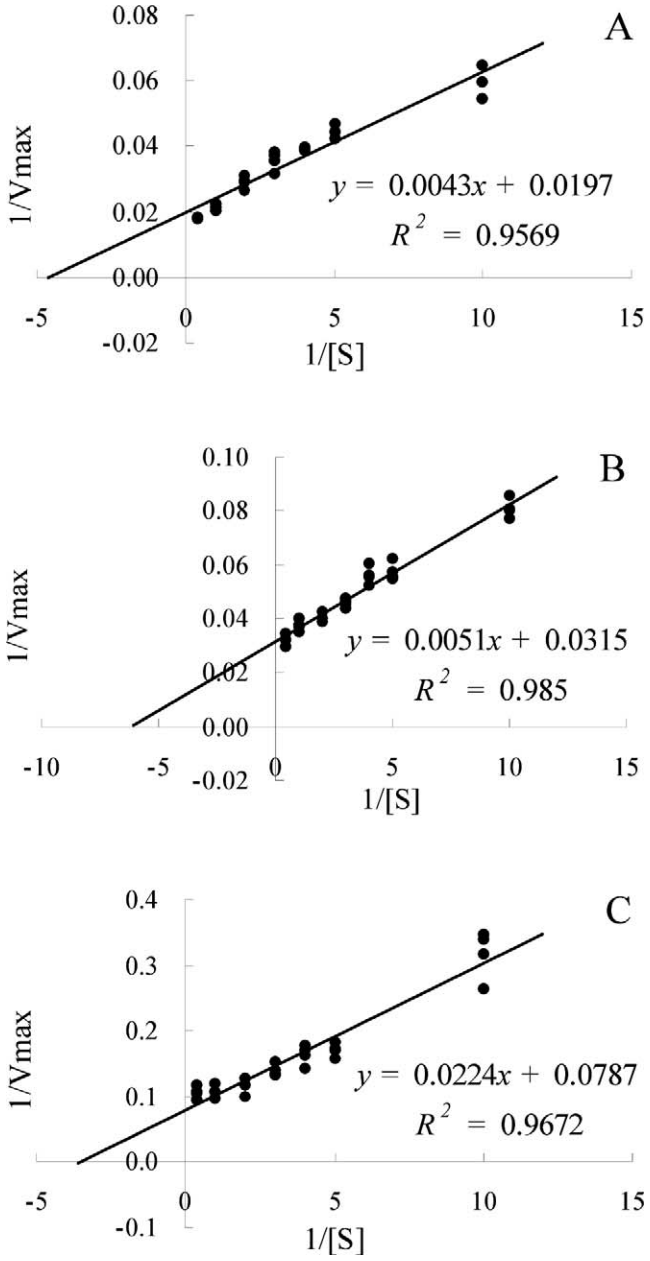

Fig. 2. Lineweaver-Burk plot of uridine diphosphate glucuronosyltransferase activity for 3-methyl-4-nitrophenol using hepatic microsomes from mouse (**A**), rat (**B**), and quail (**C**). V_{max} = maximum velocity; [S] = concentration of 3-methyl-4-nitrophenol.

UGT activity, no significant difference was found in SULT activity among mice, rats, and quail. In addition, the SULT activity was only one tenth the UGT activity, suggesting that the latter is a major conjugation enzyme for PNMC. The PNMC has potentially adverse effects on the reproductive system in quail [25] as well as in rats [26], suggesting the need to examine whether this potential is truly stronger in quail than in rats. Such a study may show the identity of the genuine toxic substance in PNMC.

Alarming levels of DEPs are spewed into the environment in many countries. Each year, 58,902 tons of DEPs in Japan [30], 111,530 tons in the United States [31], 37,000 tons in the United Kingdom [32], and 240,000 tons in the rest of the European Union [33] are emitted. One kilogram of DEP contains 28 mg of PNMC [15,16], suggesting that 1,036 to 6,720 kg of PNMC are potentially emitted into the global environment annually. In fact, Morville et al. [34] reported a PNMC mean concentration of 0.69 ng/m^3 (maximum, 5.03 ng/m^3) in air samples collected in urban, suburban, and rural sites in France. Yoshida et al. [6] reported that a DEP dose of 3 mg/m^3 in ICR mice induced the degeneration of Leydig cells and reduced daily sperm production after 12 h of exposure over 6 months. Watanabe and Oonuki [5] reported that exposure (6 h/d for 5 d/week) to DEP containing 5.63 mg/m^3 of particulate matter, 4.10 ppm of nitrogen dioxide, and 8.10 ppm of nitrogen oxide from birth to three months suppressed testicular function in male rats, as evidenced by a decrease in their sperm production. In contrast, exposure to 1 or 3 mg/m^3 of DEP for eight months did not affect sperm production in Fischer 344 rats but did significantly elevate serum and testicular testosterone [7]. Although estimating the exact PNMC dose is difficult in such experiments, the PNMC contained in the DEPs may be one of the toxicants inducing reproductive toxicity.

3-Methyl-4-nitrophenol also is a degraded product of fenitrothion, an organophosphorous insecticide that is used worldwide. The amount of fenitrothion released into the environment in Japan was approximately 1,300 tons in 2002, and roughly half was degraded into PNMC [35]. Asman et al. [36] reported that the median concentrations of PNMC in rainwater were 83 and 89 ng/L at the Danish Roskilde and Oure agricultural stations, respectively, and that fenitrothion was identified as the main source. Baroja et al. [37] also reported that PNMC was detected at a concentration of 24.1 ng/m^3 on the sixth day

Table 2. 3-Methyl-4-nitrophenol glucuronidation activity in hepatic microsomes from mouse, rat, and quail[a]

	n	Glucuronidation activity (nmol/min/mg protein)
Mouse	5	30.2 ± 2.4
Rat	5	18.8 ± 1.2[b]
Quail	6	6.9 ± 1.4[bc]

[a] Values are presented as the mean ± standard deviation for each group.
[b] Significantly different from mouse ($p < 0.01$).
[c] Significantly different from rat ($p < 0.01$).

Table 1. Kinetic parameters for 3-methyl-4-nitrophenol glucuronidation activity in hepatic microsomes from mouse, rat, and quail[a]

	n	Michaelis–Menten constant (mM)	Maximum velocity (nmol/min/mg protein)	Maximum velocity/Michaelis–Menten constant
Mouse	3	0.22 ± 0.05	50.8 ± 4.3	232.6 ± 29.7
Rat	3	0.16 ± 0.02	31.8 ± 2.2	196.1 ± 16.9
Quail	3	0.29 ± 0.04	12.7 ± 0.9	44.6 ± 8.2

[a] Values are presented as the mean ± standard deviation for each group.

Table 3. 3-Methyl-4-nitrophenol sulfation activity in hepatic cytosol from mouse, rat, and quail[a]

	n	Sulfation activity (nmol/10 min/mg protein)
Mouse	5	9.1 ± 3.2
Rat	5	10.3 ± 4.8
Quail	6	6.2 ± 2.2

[a] Values are presented as the mean ± standard deviation for each group.

and 54.7 ng/m^3 in the second week after fenitrothion was sprayed in forests in Spain. These findings clearly indicate that large amounts of PNMC may be emitted into the environment in the form of fenitrothion degradation products. Thus, both DEP- and fenitrothion-derived PNMC need to be taken into consideration when assessing the environmental risk of PNMC.

The present study suggests that the UGT plays an important role in PNMC elimination in vivo not only in quail but also in mice and rats. Whether UGT activity in humans is similar to that in quail, rats, or mice remains unknown. Uridine diphosphate glucuronosyltransferases comprise a multigenetic family. The genetic polymorphisms of UGT isoforms are of potentially toxicological, pharmacological, and physiological significance. At least 14 functional UGT isozymes have been identified in humans. Genetic polymorphisms have been identified for the following 12 isozymes: UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT2A1, UGT2B4, UGT2B7, UGT2B15, and UGT2B28 [38]. The genetic polymorphism may affect the rates of glucuronidation and, thereby, influence the risk of an individual to develop chemical-induced toxicity [39,40]. The activity of PNMC for each isozyme of human UGT and the effect of polymorphisms on the activity have to be investigated to know the risk from PNMC in humans exposed to DEPs or fenitrothion sources.

In conclusion, this article provides the first experimental evidence on the UGT activity for PNMC, an activity that is lowest in quail, followed by rats and then mice. The relevance of different UGT activities to the risk assessment of PNMC-related compounds from the viewpoint of their reproductive toxicity needs to be further explored.

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