0090-9556/82/1004-0429$02.00/0
DRUG METABOLISM AND DISPOSITION
Vol. 10, No. 4
Copyright © 1982 by The American Society for Pharmacology and Experimental Therapeutics
Printed in U.S.A.

URINARY METABOLITES OF 2,6-DIISOPROPYLNAPHTHALENE IN RATS

SHOJI KOJIMA, TOSHIYA HONDA, MITSUO NAKAGAWA, MORIO KIYOZUMI, AND AKIRA TAKADATE

Faculty of Pharmaceutical Sciences, Kumamoto University

(Received December 21, 1981, and in revised form March 19, 1982)

ABSTRACT:

Metabolism of 2,6-diisopropylnaphthalene (2,6-DIPN) in rats was found to proceed exclusively through oxidation of the isopropyl chain of the molecule. Five unconjugated metabolites were isolated from the urine and identified: 2,6-naphthalenedi(2-propan)-2-ol, 2-[6-(1-hydroxy-1-methyl)ethylnaphthalen-2-yl]-2-propionic acid, 2,6-naphthalenedi-2-propionic acid, 2-[6-(1-hydroxy-1-methyl)ethylnaphthalen-2-yl]-2-hydroxypropionic acid, and 2-[6-(1-hydroxy-1-methyl)ethylnaphthalen-2-yl]-1,2-propanediol, together with a small amount of the unchanged compound. The identification of these metabolites was made by means of mass, infrared, and nuclear magnetic resonance spectrometry. The presence of glucuronides of five metabolites described above was also suggested by thin-layer and gas-liquid chromatography of the extract that was obtained after hydrolysis by β-glucuronidase. In addition, the quantitative determination of metabolites indicated that these five were the major urinary metabolites of 2,6-DIPN, and that the total urinary excretion of these metabolites in 24 hr after administration was about 23% of the dose.

Isopropylnaphthalenes have recently come into use in duplicating papers and heat-transfer media as substitutes for polychlorinated biphenyls, which have been recognized as dangerous environmental contaminants (1,2). Kawai reported that diisopropylnaphthalene, which has been the most frequently employed of the isopropylnaphthalenes, caused a decrease in body weight gain and a slight hepatic disorder in mice fed relatively massive doses (960 mg/kg/day) of the compound for a month (3). We have studied the biological fate and toxicity of isopropylnaphthalenes. Recently, we reported that upon single and continuous oral administration of 2,6-diisopropylnaphthalene (2,6-DIPN)[1] to rats, the compound undergoes rapid gastrointestinal absorption, and is mainly deposited in the adipose tissue (4,5).

The present study was performed in rats after oral administration of 2,6-DIPN to obtain an insight into the metabolism of the compound.

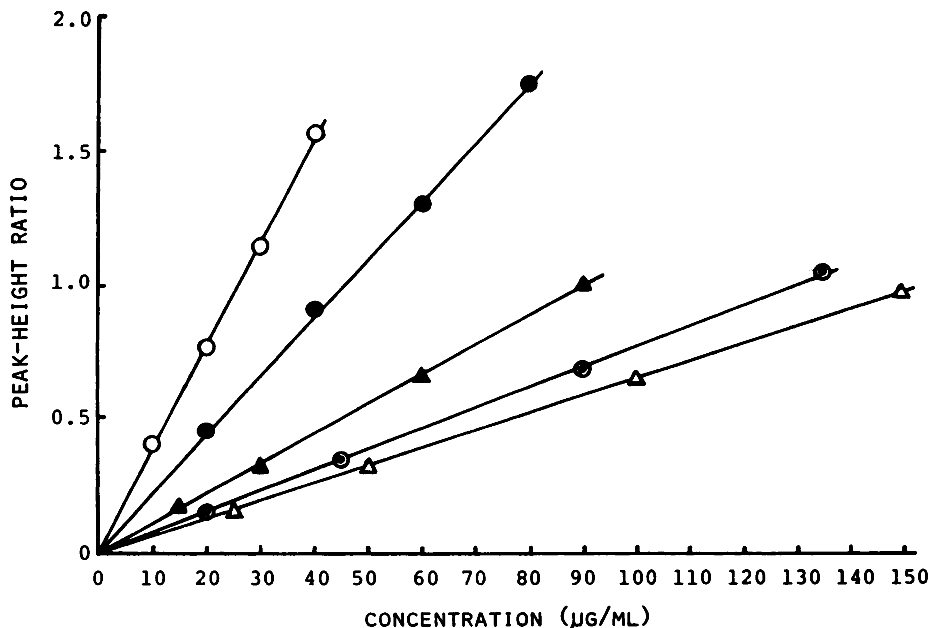

FIG. 1. *Calibration curves for metabolites* **B** (○), **C** (⊙), **D** (△), **E** (▲), and **F** (●).

[1] Abbreviations used are: 2,6-DIPN, 2,6-diisopropylnaphthalene; DMSO, dimethyl sulfoxide.

Send reprint requests to: Shoji Kojima, Ph.D., Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862, Japan.

Materials and Methods

Chemicals. Pure 2,6-DIPN (m.p. 69.5°C) was a gift from Kureha Chemical Co. (Tokyo). Hexamethyldisilazane and trimethylchlorosilane in anhydrous pyridine were purchased from Tokyo Kasei Chemical Co. (Tokyo). β-Glucuronidase (Type IX) was obtained from Sigma Chemical Co. (St. Louis, Mo.). 5-Dimethylaminonaphthalene-1-(2-hydroxyethyl) sulfone was supplied by the Department of Pharmaceutical Analytical Chemistry in this Faculty. Silica gel was purchased from Mallinckrodt Chemical Works (St. Louis, Mo.). Amberlite XAD-2 was purchased from Rohm and Haas Co. (Philadelphia, Pa.). All other chemicals and solvents used in this study were of reagent grade.

DRUG METABOLISM & DISPOSITION
Downloaded from dmd.aspetjournals.org at ASPET Journals on May 17, 2016

TABLE 1

R_F values of metabolites of 2,6-DIPN

Metabolite	R_F in Solvent System[a]	
	I	II
A	0.87	—
B	0.42	—
C	0.42	0.38
D	0.35	0.32
E	0.22	0.15
F	0.21	—
G	0.17[b]	—
H	0.07[b]	—

[a] Solvent system I, $CHCl_3$/*n*-hexane/acetic acid (10:2:1, v/v); II, benzene/pyridine (85:15, v/v).
[b] Diazotized sulfanilic acid-positive spots.

Extraction of Urinary Metabolites. 2,6-DIPN dissolved in olive oil was administered orally at a dose of 240 mg/kg/day to six male Wistar rats (weighing 200–270 g) for 15 days. The animals were housed in individual metabolic cages with diet and water *ad lib*. The urine was collected in flasks containing 1 ml of toluene to prevent putrefaction and stored at −15°C until used. The urine (about 1 liter) was adjusted to pH 1.0–2.0 with concentrated HCl and extracted three times with 300-ml portions of $CHCl_3$. The extract was concentrated to about 50 ml *in vacuo* at about 40° C (Extract 1). Extract 1 was shaken three times with 100-ml portions of 5% $NaHCO_3$ solution. The bicarbonate layer was acidified with concentrated HCl and re-extracted three times with 100-ml portions of $CHCl_3$. The extract was dried over anhydrous Na_2SO_4 and concentrated to about 20 ml, then applied onto a silica gel column (30 mm i.d. × 36 cm) and eluted with $CHCl_3$ to isolate metabolites C, D, and E. The washed Extract 1 was dried over anhydrous Na_2SO_4 and concentrated to 20 ml, and was then chromatographed similarly to isolate metabolites B and F.

The aqueous layer that remained after extraction at pH 1.0–2.0 with $CHCl_3$ was adjusted to pH 6.0–7.0 with 2 N NaOH and poured through a glass column (45 mm i.d. × 50 cm) packed with Amberlite XAD-2. The column was then washed with 1 liter of water and the conjugated metabolites absorbed onto the column were eluted with 1 liter of methanol. The methanol eluate was concentrated to about 50 ml *in vacuo* (Extract 2). Extract 2 was used for the qualitative analysis of metabolites of 2,6-DIPN.

Enzymatic Hydrolysis of the Conjugated Metabolites. Extract 2 was evaporated to dryness *in vacuo*. About 500 mg of the residue was dissolved in 30 ml of 1/15 M phosphate buffer (pH 6.8) and incubated with 50 mg of β-glucuronidase (438 Fishman units/mg) for 24 hr at 37°C after the addition of 2 drops of $CHCl_3$. The mixture was adjusted to pH 1.0–2.0 with concentrated HCl and extracted three times with 10-ml portions of $CHCl_3$. The extract was dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and subjected to TLC and GLC.

Chromatography. For TLC, Wakogel plates, 0.25 mm thick (Wako Pure Chemical Industries, Osaka) were used. Spots were detected with concentrated HNO_3 or diazotized sulfanilic acid reagent. For GLC, a Hitachi model 163 gas chromatograph equipped with a hydrogen flame-ionization detector was used. The column was a 3-mm i.d. × 3-m glass tube containing 80/100-mesh Shimalite W coated with 3% SE-30. The column temperature was maintained at 210°C. The flow rate of carrier gas (N_2) was 40 ml/min. The retention times for trimethylsilyl derivatives of metabolites **B, C, D, F,** and **E** were 7.8, 9.8, 12.5, 14.4, and 15.6 min, respectively.

Quantitative Determination of Urinary Metabolites. 2,6-DIPN (100 mg/kg) was administered orally to rats. The animals were housed in Bollman cages with diet and water *ad lib.* and the 24-hr urine was collected. The urine (about 20 ml) was adjusted to pH 1.0-2.0 with concentrated HCl and extracted three times with 20-ml portions of $CHCl_3$. The extract was evaporated to dryness *in vacuo* and the residue was dissolved in 10 ml of $CHCl_3$ (sample solution 1 for the determination of unconjugated metabolites).

Each aqueous layer that remained after extraction at pH 1.0–2.0 with $CHCl_3$ was adjusted to pH 6.8 with 2 N NaOH and incubated with 10 mg of β-glucuronidase for 24 hr at 37°C after the addition of 2 drops of $CHCl_3$. The mixture was adjusted to pH 1.0–2.0 with concentrated HCl and extracted three times with 20-ml portions of $CHCl_3$. After removal of the $CHCl_3$ *in vacuo*, the residue was dissolved in 10 ml of $CHCl_3$ (sample solution 2 for the determination of conjugated metabolites).

To 1 ml of Sample solution 1 or 4 ml of Sample solution 2 was added 0.2 ml of 5-dimethylaminonaphthalene-1-(2-hydroxyethyl) sulfone solution (500 μg/ml of $CHCl_3$) as an internal standard and evaporated to dryness. After adding 0.1 ml of hexamethyldisilazane and trimethylchlorosilane in anhydrous pyridine to the residue, the mixture was heated for 5 min in a boiling water-bath and was then subjected to GLC. When the metabolites (**B**, 33 μg; **C** and **D**, 720 μg; **E**, 1200 μg; **F**, 500 μg) of 2,6-DIPN were added to the control urine and the compounds were analyzed by the method described above, mean recoveries of these compounds were 99% for **B**, 96% for **C**, **D**, and **F**, and 84% for **E**. The calibration curves for these metabolites are shown in fig. 1.

Determination of Spectra. IR, mass, and NMR spectra were measured as reported previously from our laboratory (6).

Results

Isolation of Unchanged Metabolites. Extract 1 was analyzed by TLC to examine the metabolites of 2,6-DIPN. Eight metabolites were detected and were designated as metabolites A-H. The R_F values are given in table 1. Six metabolites (not including G and

TABLE 3

Selected mass-spectral data for metabolites of 2,6-DIPN

Metabolite	Main Peaks, *m/z* (Relative Intensity)
A	212 (M^+) (66), 211 (33), 197 (100), 169 (39)
B	244 (M^+) (34), 229 (100), 169 (78), 141 (12)
C	258 (M^+) (34), 240 (46), 195 (100), 155 (71)
D	272 (M^+) (58), 227 (100), 181 (30)
E	274 (M^+) (2), 256 (30), 229 (8), 211 (100), 195 (11), 169 (16)
F	260 (M^+) (12), 229 (100), 169 (50), 141 (8)

TABLE 2

IR-spectral data for metabolites of 2,6-DIPN

Metabolite	IR Bands[a]							
	ν_{O-H}	ν_{asCH3}	ν_{sCH3}	$\nu_{C=O}$	Ring $\nu_{C=C}$	$\delta_{C(CH3)2}$	ν_{C-O}	Ring δ_{C-H}
					cm^{-1}			
A		2960	2860		1600	1380, 1355		885, 810, 680
B	3260 (broad)	2960	2860		1600	1380, 1355		885, 830, 690
C	3420	2960	2860	1710–1680	1600	1380, 1360	1140	880, 810, 680
D		2960	2860	1710–1680	1600	1380, 1360	1060	880, 820, 690
E	3300 (broad)	2960	2860	1710–1695	1600	1380, 1360	1130	895, 820, 685
F	3300 (broad)	2960	2860		1600	1380, 1360		900, 820, 685

[a] Determined in KBr pellets.

Downloaded from dmd.aspetjournals.org at ASPET Journals on May 17, 2016

TABLE 4

NMR-spectral data for metabolites of 2,6-DIPN

Metabolite	Chemical Shifts[a]				
	Naphthalene Ring	CH_3	CH	CH_2	OH
			ppm		
A	7.40–8.00 (m)	12H, 1.30 (d)	2.98 (m)		
B	7.45–8.00 (m)	12H, 1.67 (s)			1.85 (s)
C	7.40–8.00 (m)	3H, 1.52 (d)	3.85 (q)		
		6H, 1.65 (s)			
D	7.40–8.10 (m)	6H, 1.53 (d)	3.90 (q)		
E	7.40–8.10 (m)	6H, 1.50 (s)			3.00–7.00 (broad)
		3H, 1.70 (s)			
F	7.40–8.10 (m)	9H, 1.50 (s)		3.50 (d)	4.60 (t)
					4.90 (s)
					5.05 (s)

[a] δ-Values in $CDCl_3$ (metabolites A, B, C, and D) or DMSO-d_6 (metabolites E and F) with reference to tetramethylsilane; *s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *m*, multiplet.

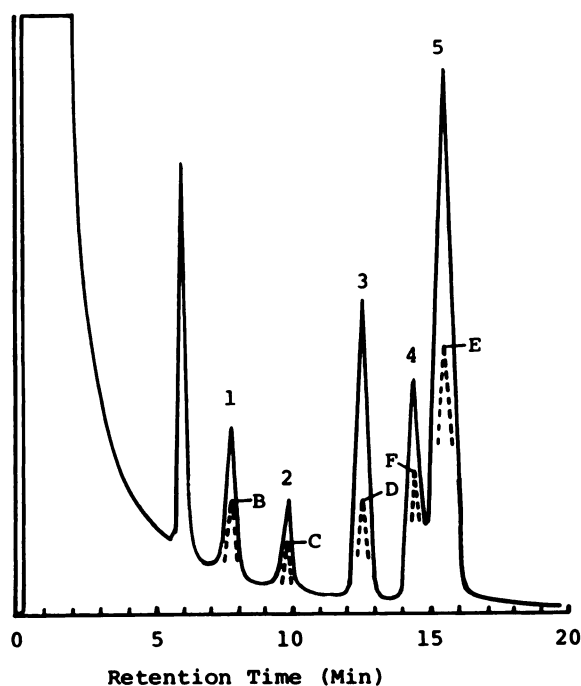

FIG. 2. *Gas chromatogram of metabolites obtained by enzymatic hydrolysis of Extract 2.*

This chromatogram was obtained after the trimethylsilyl derivatization of metabolites. *Peaks 1–5*, metabolites from Extract 2.

TABLE 5

Metabolites of 2,6-DIPN in 24-hr urine after a single oral administration

The values represent means ± SD for three to six animals.

Metabolite	Unconjugated	Conjugated
	% of dose	
A	< 0.1	
B	0.61 ± 0.08	0.13 ± 0.03
C	1.27 ± 0.01	< 0.1
D	0.78 ± 0.24	0.63 ± 0.21
E	14.83 ± 1.46	2.67 ± 0.42
F	1.83 ± 0.15	0.31 ± 0.04
Total	19.40 ± 1.31	3.73 ± 0.42

H) were isolated by silica gel column chromatography and were purified by recrystallization from $CHCl_3$. Twelve milligrams of metabolite B (m.p. 156–157.5°C), 20 mg of metabolite C (m.p. 140–143°C), 12 mg of metabolite D (m.p. 190–193°C), 200 mg of metabolite E (dec.p. 115–118°C), and 40 mg of metabolite F (m.p. 139–141°C) were obtained in crystalline form.

Structural Determination of Unconjugated Metabolites. The structures of unconjugated metabolites were determined by means of IR, NMR, and mass spectrometry. Tables 2, 3, and 4 summarize the spectral data for the metabolites.

Metabolite A. As shown in tables 1 and 2, R_F values and IR spectrum of metabolite A showed good agreement with those of an authentic sample of 2,6-DIPN.

Metabolite B. The mass spectrum (table 3) showed that the compound contains two more oxygen atoms than does 2,6-DIPN. A fragment-ion peak at *m/z* 229, due to the loss of CH_3 from the isopropyl group, is noteworthy. The IR spectrum (table 2) showed, in addition to the bands characteristic of 2,6-DIPN, a band at 3260 cm^{-1} characteristic of an OH group. The NMR spectrum (table 4) showed the presence of a mobile proton at 1.85 ppm attributable to an OH group and a singlet (12H) at 1.67 ppm due to the $(CH_3)_2C<$ group. These data enabled us to assign its structure as 2,6-naphthalenedi(2-propan)-2-ol.

Metabolite C. The mass spectrum (table 3) of metabolite C afforded a molecular ion at *m/z* 258. The IR spectrum (table 2) showed the presence of bands for OH and COOH groups. The NMR spectrum (table 4) showed the presence of one CH_3 group (1.52 ppm, 3H), one $(CH_3)_2C<$ group (1.65 ppm, 6H), and one CH group (3.85 ppm). However, the distinct signals attributable to the OH group were not observed in the spectrum, because of the overlap with the signals attributable to the CH_3 and $(CH_3)_2C<$ groups. These data confirmed the proposed structure as 2-[6-(1-hydroxy-1-methyl)ethylnaphthalen-2-yl]-2-propionic acid.

Metabolite D. The mass spectrum (table 3) of metabolite D afforded a molecular ion at *m/z* 272 and a fragment-ion peak at *m/z* 227 due to the loss of the COOH group. The IR spectrum (table 2) showed bands attributable to a COOH group. The NMR spectrum (table 4) showed the presence of the CH_3-$CH<$ moiety.

Downloaded from dmd.aspetjournals.org at ASPET Journals on May 17, 2016

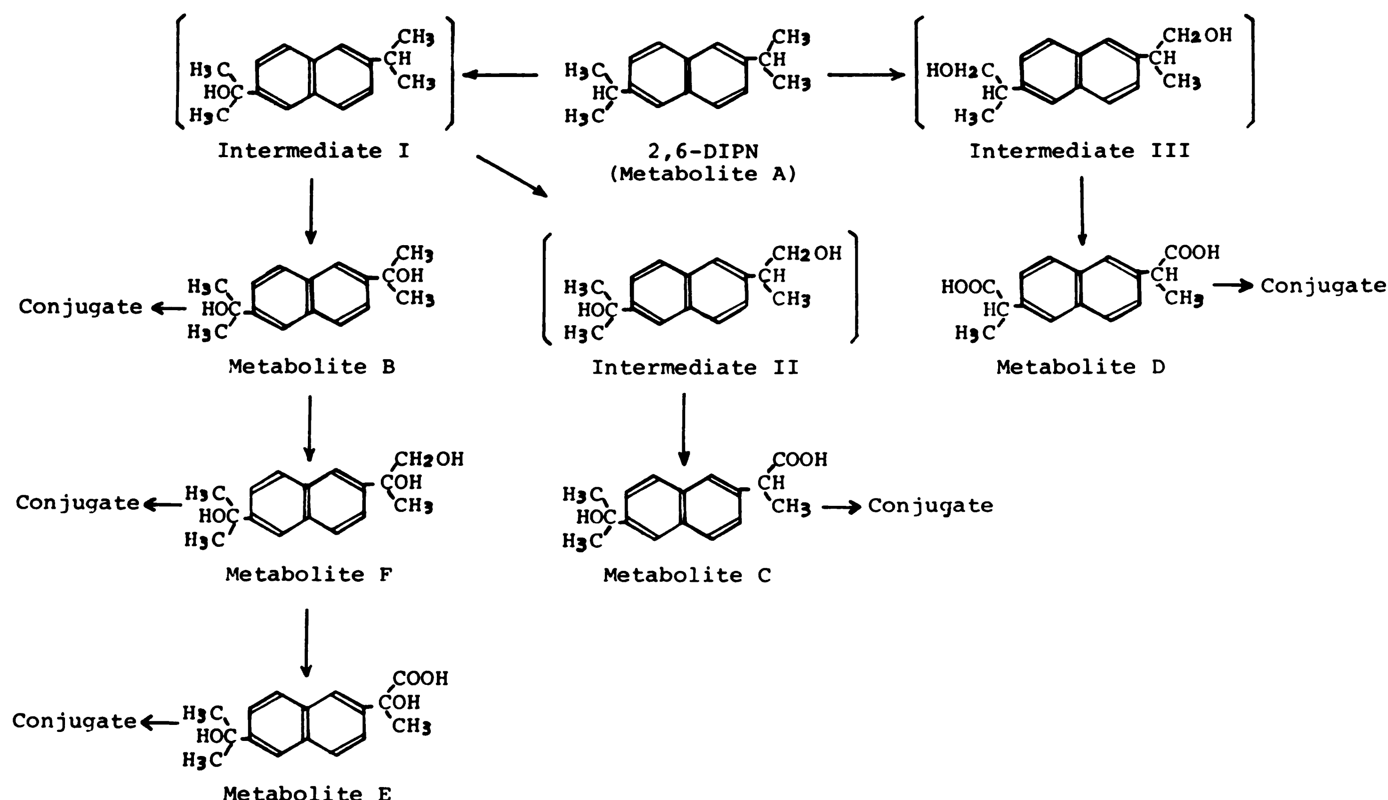

FIG. 3. *Proposed metabolic pathways of 2,6-DIPN in the rat.*

These data confirmed the proposed structure as 2,6-naphthalenedi-2-propionic acid.

Metabolite E. The mass spectrum (table 3) afforded a molecular ion at *m/z* 274. The IR spectrum (table 2) showed the presence of OH and COOH groups. The NMR spectrum (table 4) showed the presence of a singlet (3H) at 1.70 ppm due to a CH_3 group, a singlet (6H) at 1.50 ppm due to a $(CH_3)_2C<$ group, and of mobile protons at 3.00-7.00 ppm attributable to OH groups. These data enabled us to assign the structure as 2-[6-(1-hydroxy-1-methyl)ethylnaphthalen-2-yl]-2-hydroxypropionic acid.

Metabolite F. The mass spectrum (table 3) showed the presence of three more oxygen atoms than in 2,6-DIPN. A fragment-ion at *m/z* 229, due to the loss of a CH_2OH group, is noteworthy. The IR spectrum indicated the presence of bands at 3300 cm^{-1} characteristic of an OH group together with those of 2,6-DIPN (table 2). The NMR spectrum (table 4) showed the presence of three CH_3 groups (1.50 ppm), one CH_2 group (3.50 ppm), and three OH groups (4.60, 4.90, 5.05 ppm). These data confirmed the proposed structure as 2-[6-(1-hydroxy-1-methyl)ethylnaphthalen-2-yl]-1,2-propanediol.

Conjugated Metabolites of 2,6-DIPN. To examine the presence of possible conjugated metabolites of 2,6-DIPN in the aqueous layer after extraction at pH 1.0-2.0, the conjugated metabolites in Extract 2, which was obtained by the use of Amberlite XAD-2, were hydrolyzed with β-glucuronidase. The R_F values for the aglycones, which were extracted from the above hydrolysate with $CHCl_3$, and the retention times for their trimethylsilyl derivatives (fig. 2) showed good correspondence with those of the metabolites (**B, C, D, E,** and **F**) isolated as the unconjugated metabolites. Thus, these aglycones were identified as metabolites **B, C, D, E,** and **F**.

Other Metabolites. As shown in table 1, metabolites **G** and **H**, which were observed on thin-layer chromatograms of Extract 1, were considered to be phenolic compounds based on positive reactions with diazotized sulfanilic acid reagent. However, the structures of these metabolites have not yet been elucidated.

Quantitative Determination of Metabolites of 2,6-DIPN. We determined the unconjugated and conjugated metabolites in the 24-hr urine of rats receiving a single oral administration of 2,6-DIPN (100 mg/kg) (table 5). The results showed that about 23% of the administered 2,6-DIPN was excreted in the 24-hr urine, and that metabolite **E** was the major metabolite.

Discussion

The metabolic pathway of 2,6-DIPN in the rat, based on the urinary metabolites identified in this study, is summarized in fig. 3. As in the metabolism of 2-isopropylnaphthalene reported previously (6), one of the metabolic routes involved in the biotransformation of 2,6-DIPN is oxidation of the isopropyl sidechains at the tertiary carbon atoms giving rise to metabolite **B**. Metabolite **B** may be hydroxylated to metabolite **F**, which in turn is further metabolized to metabolite **E**. In addition, metabolite **C** may result by way of the supposed intermediates **I** and **II**. Another metabolic route is the direct oxidation of one of the two methyl groups of each isopropyl chain, probably through the formation of the primary alcohol, to a carboxyl group, giving rise to metabolite **D**. Furthermore, it was found that the metabolites **B, C, D, E,** and **F** were present in conjugated forms. It is interesting that the amount of the conjugated metabolites was about ⅙ of that of the uncon-

Downloaded from dmd.aspetjournals.org at ASPET Journals on May 17, 2016

jugated metabolites. Phenolic metabolites may also be present, but were not isolated.

Iwahara reported that the excretion of radioactivity in the urine and feces in rats treated with ^{3}H-diisopropylnaphthalene was approximately 26% and 71% of the dose, respectively (7). In the present study, the total urinary excretion of unconjugated and conjugated metabolites of 2,6-DIPN in 24 hr after administration was about 23% of the dose (table 5). These findings suggest that metabolites **B**, **C**, **D**, **E**, and **F** are the major urinary metabolites of 2,6-DIPN.

References

1. D. B. Peakall and J. L. Lineer, *BioScience* **20**, 958 (1970).
2. R. Edwards, *Chem. Ind. (London)* **20**, 1340 (1971).
3. M. Kawai, *Med. Biol. (Tokyo)* **86**, 217 (1973).
4. S. Kojima, M. Nakagawa, R. Suzuki, M. Horio, and Y. Tanaka, *Chem. Pharm. Bull.* **26**, 3007 (1978).
5. S. Kojima, M. Nakagawa, R. Suzuki, M. Horio, Y. Taniguchi, and Y. Tanaka, *Eisei Kagaku* **25**, 221 (1979).
6. S. Kojima, K. Maruyama, and T. Babasaki, *Drug Metab. Dispos.* **8**, 463 (1980).
7. S. Iwahara, *Nat. Defence Med. J.* **21**, 273 (1974).

Downloaded from dmd.aspetjournals.org at ASPET Journals on May 17, 2016