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# Metabolism of N-phenyl-2-naphthylamine and N-phenyl-1-naphthylamine by rat hepatic microsomes and hepatocytes

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**Abstract** — The carcinogenic antioxidants, N-phenyl-1-naphthylamine (P1NA) and N-phenyl-2-naphthylamine (P2NA) were examined in vitro for biotransformation by rat hepatic microsomes and in freshly isolated hepatocytes. HPLC-analysis of hepatocyte incubations with revealed that phenols were the major metabolites in both cases. P1NA formed one phenolic metabolite only, while incubation with P2NA yielded two phenols identified as 6-hydroxy-P2NA and 4'-hydroxy-P2NA by cochromatography with authentic samples. $\beta$-naphthylamine, a metabolite indicating dephenylation of P2NA was not detectable.

Metabolism studies with microsomes revealed that the phenols were formed by cytochrome P-450 dependent monooxygenases. Pretreatment of animals with phenobarbital and 3-methylcholanthrene both increased the rate of microsomal metabolism of P1NA and P2NA, indicating that more than one P-450 enzyme mediate the oxygenation reaction. Animal pretreatment with single and repeated doses of P1NA and P2NA did not markedly stimulate metabolism, but induced ethylmorphine demethylation in males and females and benzo (a) pyrene hydroxylation in females.

**Keywords**: N-phenyl-2-naphthylamine; N-phenyl-1-naphthylamine; hepatocytes.

## INTRODUCTION

At present there is no conclusive evidence on the mechanism of the carcinogenic action of the two compounds. Genotoxic DNA damaging metabolites initiating the first step of carcinogenis for many chemical carcinogens are not known so far for P2NA and P1NA. There are some concern that P2NA may be metabolized by dephenylation to the human carcinogen 2-naphthylamine. This was suggested by the observation of Kummer and Tordoir (1975) who found that human volunteers excreted 2-naphthylamine after intake of P2NA.

Moore *et al.* (1977) reported P2NA dephenylation in human and Batten and Hathaway (1977) noted this reaction in dogs. However, Anderson *et al.* (1982) could not detect 2-naphthylamine or the putative precursor, N-hydroxy-P2NA in rat liver microsomes.

We are not aware of a study on the metabolism of the carcinogenic isomer, P1NA. In this case, dephenylation would lead to the noncarcinogenic compound 1-naphthylamine, suggesting that the metabolic activation process is not likely to occur via dephenylation of P1NA.

In this study we wish to report the results of a study on in vitro metabolism of P2NA and P1NA. Oxidative metabolism of both compounds and the effect of the two compounds as inducers of their own metabolism and of other typical monooxygenase reactions was studied in microsomes. To analyze which metabolites are formed under conditions more related to the in vivo situation, rat hepatocytes were prepared as an in vitro system containing both activating and deactivating enzymes and the metabolites of P1NA and P2NA were analyzed by HPLC. As a result, phenolic metabolites were formed as reaction products. No evidence was obtained that N-hydroxylated or dephenylated metabolites are formed during oxidative metabolism.

## MATERIALS AND METHODS

*Chemicals*

P2NA and P1NA were obtained from Fluka AG and Riedel-de Haen AG and recrystallized from hexane; Enzyme and co-enzymes were purchased from Boehringer (Mannheim, Ger.); aldrin (1, 2, 3, 4, 10, 10-hexachloro-1, 4, 4α, 5, 8, 8α-hexahydro-1, 4-endo-5, 8-exo-dimethano-naphthalene) and dieldrin (1, 2, 3, 4, 10, 10-hexachloro-6, 7-epoxy-1, 4, 4α, 5, 6, 7, 8, 8α-octahydro-1, 4-endo-5, 8-exo-dimethanonaphthalene), purity 99%, from Riedel-de Haen (Seelze Ger.); sodium phenobarbital and 3-methylcholanthrene were purchased from Fluka (Neu-Ulm, Ger.); 4, 7-diphenyl-1, 10-phenan-throline (bathophenanthroline) was obtained from EGA-Chemie, Ger.; 4'-hydroxy-P2NA was obtained from Aldrich Co.

*Instruments*

Mass spectrum were obtained in Finnigan mat system 8222, Tektronix 4010; UV spectra was acquired using a Perkin-Elmer, Lambda 5, UV/vis spectrophotometer equipped with a Gynkotek constant flow pump; HPLC, Model 600/200, a Gynkotek Gradient Former Model 250, a Kratos, SF76 detector, and an Altex ultrasphere ODS column (30cm x 4.6mm); Gas-chromatographic determinations were carried out with a Hewlett-Packard model 5750G gas chromatography equipped with a $^{63}$Ni-electron-capture detector.

*Pretreatment with inducers and preparation of microsomes*

Wistar rats (150 – 200) (inbred strains, Neuherberg, GSF Research Center Ger.) of both

sexes were used. For pretreatment with 3-methylcholanthrene (3-MC), male Wistar rats received i. p. injection of 20 mg / kg b. w. on each of two consecutive days. Phenobarbital (PB) was administered by a single i. p. injection of 80 mg / kg b. w. in saline, and subsequently 0.1% PB in their drinking water for 1 week. Animals were killed 24 hrs after the last dosage. For pretreatment with P2NA or P1NA, male or female Wistar rats received a single i. p. injection of P1NA (200, 500 and 1000 mg / kg b. w.) and P2NA (250, 500 and 750 mg / kg b. w.) or were given the P1NA and P2NA repeatedly (200 mg / kg b. w. daily) for 4 days or for 8 days in 3 weeks by gastric intubation. Control animals were pretreated with the corresponding vehicle.

Preparation of liver microsomes was as general methods (Wolff, 1977). Protein concentrations were determined by the biuret reaction (Szarkowska, 1963). Microsomes were used immediately upon preparation or a few days after storage at −80°C. Enzyme activities did not significantly change during this period.

*Microsomal incubations and determination of hydroxylated arylamines*

Incubation mixtures contained 5.2 mmol/L glucose 6-phosphat, 0.6 mmol/L $NADP^+$, 0.7 units glucose 6-phosphate dehydrogenase, 0.1 mol / L $Na^+$ / $K^+$ phosphate, pH 7.5, 0.5 mmol / L P2NA or P1NA (dissolved in 10 μl 95% ethanol) and approx. 2 mg microsomal protein in a total volume of 1.0 ml. Incubations were conducted at 37℃ under shaking for 15 min and the reaction stopped by placing the tubes on melting ice and shaking with water-saturated amyl acetate. The phases were separated by centrifugation and the total concentration of N- and ring-hydroxylated arylamines was determined by a modification of the reducing equivalent assay of Anderson, Mitchum and Beland (1982). Aliquots (0.5 ml) of the amyl acetate extract were mixed with 0.6 ml 95% ethanol, 0.2 ml 1mol / L sodium acetate (pH 4.6), 0.2 ml bathophenanthroline reagent (3.3 mg / ml in 95% ethanol) and 0.05 ml of 0.01 mol / L ferric nitrate in 0.1 mol / L acetic acid. The colorimetric reaction was allowed to develop for 10 min, then 0.05 ml of 0.02 mol / L phosphoric acid was added, and the absorbance was determined at 535 nm. The hydroxyarylamine concentration was calculated using $\varepsilon_{535} = 39200M^{-1}$.

*Other monooxygenase assays*

The activity of aldrin epoxidase was determined by electron capture gas chromatography according to Wolff *et al.* (1979). N-demethylation of ethylmorphine was measured by the method of Nash (1953) with modifications as described by Wolff (1978). AHH activity was assayed by the fluorimetric determination of hydroxybenzo (α) pyrene according to Wiebel *et al.* (1977).

*Incubations with hepatocytes and digestion with β-glucuronidase*

500 μl of 5mmol / L P2NA suspension or 2.5mmol / L P1NA suspension was added to every one of 8 flasks containing 12 ml liver cell suspension with 8 mg/ml protein. The flasks were shaken for 1h at 37°C and gassed with $O_2$, the control flask was only added with the

substrate but not incubated. One hour later the flasks were removed out to stop the reaction and extracted with ethyl acetate (water-saturated). The combined organic extracts were evaporated and redissolved in methanol. The metabolites were then separated by HPLC and analyzed by mass spectroscopy.

The aqueous phase was digested with $\beta$-glucuronidase, the incubation mixtures contained: concentrated aqueous solution 4 ml, $\beta$-glucuronidase activity ca. 20 u/ml, 0.5 mol/L ammonium acetate buffer, pH 4.8, 10 ml. The mixture were incubated for 24 hrs at 25℃. The reaction stopped, were extracted with ethyl acetate and were treated like organic phases.

*HPLC analysis of metabolites*

The organic extracts from the incubation mixture were evaporated and redissolved in methanol. Aliquots of the solution were injected into a Gynkotek HPLC equipped with a Model 600/200 flow pump, a Model 250 gradient former, a Kratos SF 769 detector and an Altex ultrasphere ODS column (30 cm × 4.6mm). The metabolites were separated by isocratic elution with 65% methanol for 25 min and then by a linear gradient to 100% methanol in 5 min (for organic phase) and by isocratic elution with 25% methanol for 35 min and then a linear gradient to 100% methanol in 5 min (for aqueous phase). A flow rate of 1 ml/min was maintained and the absorbance was monitored at 254 nm.

## RESULTS

*Establishment of optimal conditions*

It is most important that the reaction measured was being carried out at optimal conditions. The enzymatic assay should be performed under conditions in which the concentration of the substrate and cofactors are not rate-limiting, and product formation should be linear with time during the incubation period. The rate of metabolite formation in rat liver microsomes was nearly linear for at least 15 min, between 15 and 30 min of incubation the slope was curvilinear. The cofactor requirements for hepatic drug metabolizing enzyme as determined in the absence of G6P, G6P-dehydrogenase and $NADP^+$, the oxidative rate decreased to 4%, 2% and 0.5%, respectively, of the rate determined in the complete incubation system.

*The influence of P1NA and P2NA on monooxygenase of rat tissues*

The influence of P1NA and P2NA after 1-8 days i. p. administration or by gastric intubation on monooxygenase of rat tissues was investigated. As shown in Table 1 and 2, the relative rates of microsomal metabolism were not affected by P1NA and P2NA after 1-4 days i. p. administration. However, the relative rates of microsomal metabolism by the 8 days gastric intubation with P1NA or P2NA increased (Table 1). In the effect of animal

pretreatment with the inducers on monooxygenase activity experiments, treatment of male rats were done with single or repeated (4 days) dose, and only weak to moderate induction of hepatic microsomal cytochrome P-450, the activities of aldrin epoxidation and benzo ($\alpha$) pyrene hydroxylation were observed, while the activity of ethylmorphine demethylation increased significantly. Female rats received repeated intragastric doses and showed a vigorous response, in particular, benzo ($\alpha$) pyrene hydroxylation was strongly increased (Table 2).

**Table 1 Relative rates of microsomal metabolism of P1NA and P2NA by various treatment**

| Experiment | Pretreatment | Dose, mg/kg | Number of treatment | Sex | Rate, nmoles/min/mg protein | |
|---|---|---|---|---|---|---|
| | | | | | P1NA | P2NA |
| | Control | | | Male | 0.18 ±0.03 | 0.21 ±0.01 |
| 1 | PB | 40 | 1 | Male | 0.29 ±0.02** | 0.21 ±0.01* |
| | MC | 20 | 2 | Male | 0.30 ±0.04** | 0.44 ±0.05** |
| | Control | | | Male | 0.42 ±0.01 | 0.37 ±0.01 |
| 2 | P1NA | 200 | 4 | Male | 0.37 ±0.04* | 0.35 ±0.03* |
| | P2NA | 200 | 4 | Male | 0.42 ±0.02* | 0.38 ±0.02* |
| | Control | (olive oil) | | Famale | 0.24 ±0.10 | 0.29 ±0.10 |
| 3 | P1NA | 200 | 8[a] | Famale | 0.60 ±0.10** | 0.55 ±0.10** |
| | P2NA | 200 | 8[a] | Famale | 0.30 ±0.10* | 0.34 ±0.10* |

Rates were determined from a modification of the colorimetric reducing equivalent assay of Kadlubar *et al.* (1976).

The data are mean of four single determinations $\pm SD$.

a. 3 days / week for female rats by gastric intubation.

* $P > 0.05$, ** $P < 0.05$

*Identification of major P1NA and P2NA metabolites*

In order to obtain sufficient amounts of metabolites for structural identification, large-scale incubations with hepatocytes were necessary. The incubation mixture was extracted with ethyl acetate and the individual metabolites were separated by HPLC and TLC. In all cases, one major, metabolite (I) was detected in P1NA incubation mixture and two major metabolites (I and II) in P2NA incubation mixture; representative chromatograms from rat hepatocyte are shown in Fig. 1 and 3. The chromatogram of HPLC of P2NA incubation mixture was consistent with the report of Anderson and Beland (1982). Two major metabolites were detected, 6-hydroxy-P2NA and 4'-hydroxy-P2NA. Confirmation of this assignment was made by comparing the UV spectrum and HPLC retention time of the metabolite with those of an authentic standard. They were identical with each other. The mass spectrum of peak I of the hepatocyte incubations with P1NA is consistent with a phenolic metabolite,

as indicated by the molecular peak representing the acetyl-derivative (Fig. 2).

**Table 2 Effect of animal pretreatment with inducers on monooxygenase activity**

| Experi-ment | Pretreat-ment | Dose, mg/kg | Number of treatment | Sex | Cytochrome P-450, nmoles/mg protein | Rate, nmoles/min/mg protein | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Ethylmorphine demethylation | Aldrin epoxidation | Benzo(α) pyrene hydroxylation |
| | Control | | | Male | 0.72±0.08 | 7.40±1.13 | 2.88±0.36 | 0.24±0.02 |
| 1 | PB | 40 | 1 | Male | 1.50±0.11** | 14.20±2.20* | 5.18±1.00* | 0.42±0.03** |
| | MC | 20 | 2 | Male | 0.84±0.07* | 7.20±0.70* | 2.52+0.40* | 0.76±0.13** |
| | Control | | | Male | 0.89±0.05 | 5.65±0.63 | 2.82±0.21 | 0.24±0.01 |
| 2 | P1NA | 200 | 1 | Male | 0.95±0.06* | 8.50±1.60* | 3.10±0.16* | 0.22±0.03* |
| | | 500 | 1 | Male | 1.12±0.05** | 11.90±0.13*** | 4.54+0.07*** | 0.25±0.04* |
| | | 1000 | 1 | Male | 1.25±0.03** | 12.20±0.80*** | 4.39±0.20** | 0.23±0.02* |
| | Control | | | Male | 0.97±0.07 | 8.31±0.75 | 4.70±0.36 | 0.37±0.02 |
| 3 | P2NA | 250 | 1 | Male | 1.11±0.17* | 13.14±2.17* | 2.00±0.12* | 0.31±0.04* |
| | | 500 | 1 | Male | 1.13±0.15* | 13.22±1.06* | 1.98±0.56* | 0.36±0.05* |
| | | 750 | 1 | Male | 0.91±0.11* | 10.64±1.98* | 4.35±0.76* | 0.37±0.07* |
| | Control | | | Male | 0.87±0.04 | 8.61±0.69 | 7.48±0.47 | 0.22±0.04 |
| 4 | P1NA | 200 | 4 | Male | 1.03±0.03** | 13.41±1.56* | 5.38±0.45* | 0.30±0.03* |
| | P2NA | 200 | 4 | Male | 0.96±0.04* | 12.17±0.55* | 5.60±0.42* | 0.28±0.06* |
| | Control | (olive oil) | | Famale | 0.90±0.12 | 0.71±0.20 | 0.27±0.04 | 0.02±0.01 |
| 5 | P1NA | 200 | 8[a] | Famale | 0.92±0.09* | 2.85±0.41*** | 0.47±0.08* | 0.10±0.02*** |
| | P2NA | 200 | 8[a] | Famale | 1.03±0.22* | 2.49±0.44*** | 0.25±0.04* | 0.32±0.06*** |

Microsomes from male or female rats weighing 150 g were assayed. The data are mean of four single determinations ± *SD*.

a. 3 days / week by gastric intubation. $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$

The HPLC chromatograms of the P2NA and P1NA hepatocyte incubations were carefully examined for the presence of 2-naphthylamine and 1-naphthylamine, 2-naphthol, 1, 4-naphthoquinone, 2-hydroxy-1, 4-naphthoquinone, 5-hydroxy-1, 4-naphthoquinone and 1, 4-benzoquinone, an expected by-product of the dephenylation reaction. However, UV absorbance coincident with its retention time was not detected.

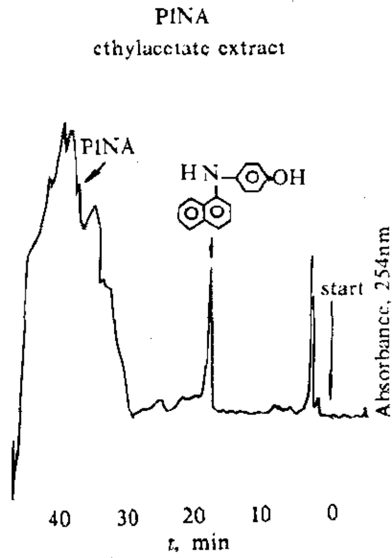


Fig. 1 HPLC profiles of metabolites obtained from incubating N-phenyl-1-naphthylamine with rat hepatocytes

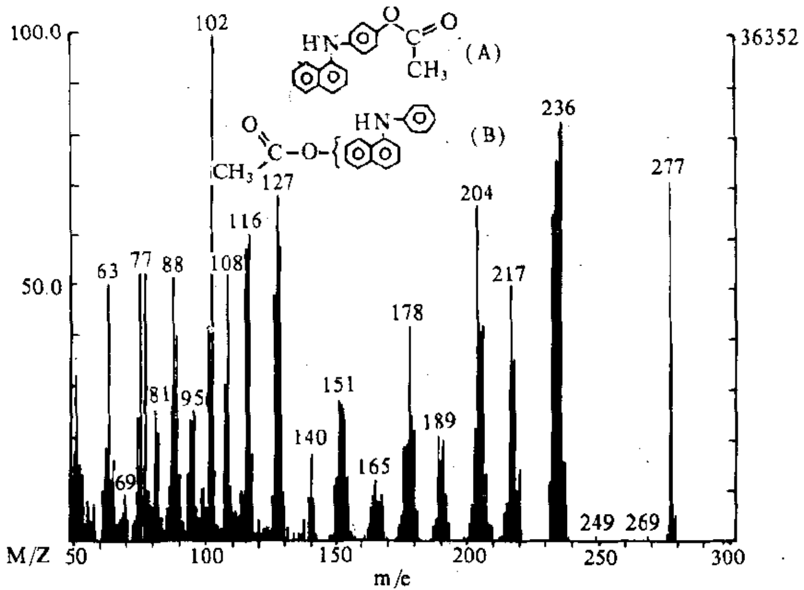


Fig .2 Mass spectrum of metabolite 1 of P1NA

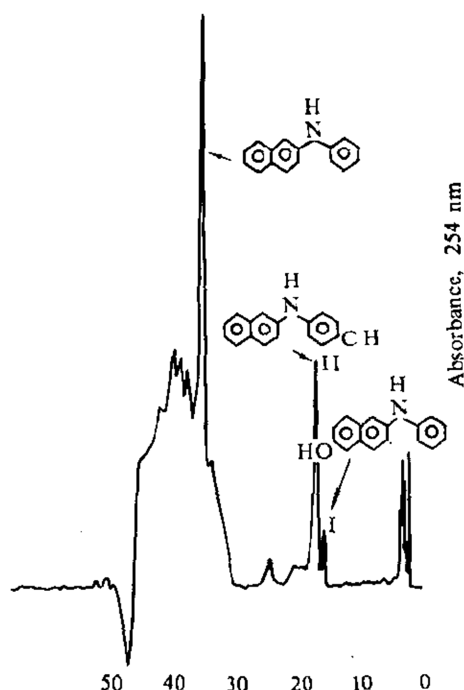


Fig. 3 HPLC profiles of metabolites obtained from incubating N-phenyl-2-naphthylamine with rat hepatocytes Metabolites were separated with ODS reverse phase column by isocratic elution with 65% methanol at 1 ml/min for 25 min and then a linear gradient to 100% methanol in 5 min.

## DISCUSSION

The study of Anderson *et al.* showed that hepatic microsomes convert P2NA to 2 phenolic metabolites, 4'-hydroxy-P2NA and 6-hydroxy-P2NA. This finding is confirmed by the results of this study performed under conditions more related to the in vivo situation. Freshly isolated hepatocyte containing monooxigenases, conjugating enzymes and the appropriate cofactors converted P2NA to the same phenolic metabolites as observed in microsomal incubations.

P1NA formed only one phenolic metabolite in hepatocyte incubations. The mass spectrum of the acetylated derivative was consistent with the structure of a phenolic derivative of P1NA. At present we cannot decide, whether the hydroxylation occurs in the phenylring or at the naphthalene moiety. Regarding the metabolism of P2NA, hydroxylation in the phenylring

at the para (4')-position seems to be a likely reaction, since the position, where the phenylring is attached to the naphthalene system should not have a major influence on the probability for both compounds that oxidation occurs at the para-position.

Hepatic microsomes were used to examine the involvement of cytochrome P-450 in the formation of the phenols. The effect of SKF-525 A, the general inhibitor of various P-450 reactions, and the increase of the reaction rate observed after pretreatment with the P-450 inducers, PB and MC, indicate that the phenols are formed by a cytochrome P-450 dependent reaction. Since both, PB and MC, inducing different families of cytochrome P-450 enzymes had similar inducing effects, several P-450 enzymes seem to be involved in the hydroxylation reaction.

1-Naphthylamine and 2-naphthylamine, which according to Anderson *et al.* might be formed as a consequence of N-dephenylation of P1NA and P2NA, respectively, were not detectable in neither incubation. Likewise, metabolites that may be formed as secondary oxidation products of the naphthylamines could not be detected (please check for accurate conditions of detection). These observations suggest that the major route of oxidative metabolism proceeds via the epoxide pathway which leads to the formation of phenols as stable reaction products. It is likely therefore, that the carcinogenicity of P2NA in the rat is not primarily due to the formation of the carcinogenic intermediate, 2-naphthylamine, a pathway suggested for humans and dogs. A similar statement can be made for P1NA.

However, it is well possible that the epoxide pathway provides DNA-adduct forming species as a first critical event leading to tumor formation. Various carcinogenic aromatic hydrocarbons generate DNA-binding epoxides during metabolic oxidation. At present, we do not know whether epoxides of P1NA and P2NA eventually formed are stable enough to reach the cell nucleus to form DNA adducts or are detoxified before. The fact that the tumors were found in tissues other than liver may be interpreted in the way that the epoxides formed in the liver cells are effectively detoxified there. Regarding tumor formation in the lung and the kidney as the major target tissues, it is possible that the tumor initiating metabolites are generated in situation, possibly by a different pathway. But it should also be considered that both compounds may primarily act via an epigenetic mechanism, i. e. they may be tumor promoters favoring tumors initiated by a yet unknown process. Which of the 2 mechanisms is more likely to occur deserves further investigation.

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