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In vitro metabolism of the nephrotoxicant *N*-(3,5-dichlorophenyl)succinimide in the Fischer 344 rat and New Zealand white rabbit

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1. The nephrotoxicant *N*-(3,5-dichlorophenyl)succinimide (NDPS) underwent non-enzymatic hydrolysis to *N*-(3,5-dichlorophenyl)succinamic acid (NDPSA) in buffer, rat liver and kidney homogenates, and rabbit liver homogenates.

2. In the presence of NADPH, rat liver homogenates converted NDPS to NDPSA and *N*-(3,5-dichlorophenyl)-2-hydroxysuccinamic acid (2-NDHSA).

3. Using liver homogenates from the phenobarbital (PB)-pretreated rat, 2-NDHSA production was increased 5-fold, and the metabolites *N*-(3,5-dichlorophenyl)-2-hydroxysuccinimide (NDHS) and *N*-(3,5-dichlorophenyl)-3-hydroxysuccinamic acid (3-NDHSA) were also detected. Formation of these latter metabolites was suppressed by CO or omission of NADPH. No hydroxylated metabolites were detected when NDPSA was incubated with PB-induced rat liver homogenates.

4. Oxidative metabolites were not produced when NDPS was incubated with kidney homogenates from the control or PB-pretreated rat.

5. NDHS underwent rapid hydrolysis in buffer to yield 2-NDHSA and 3-NDHSA.

6. Rabbit liver homogenates converted NDPS to NDPSA, 3,5-dichloroaniline (DCA), and succinic acid (SA). Production of DCA and SA was inhibited by the amidase inhibitor bis-*p*-nitrophenyl phosphate. Oxidative metabolism did not occur in rabbit tissue.

7. These experiments demonstrate that a PB-inducible form of rat liver P450 converts NDPS to NDHS, which then undergoes hydrolysis to 2-NDHSA and 3-NDHSA. An alternative route of production for 2-NDHSA and 3-NDHSA, via hydroxylation of NDPSA, does not occur. In rabbit liver NDPS metabolism was primarily amidase-mediated.

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Introduction

N-(3,5-dichlorophenyl)succinimide (NDPS; figure 1) is one of a series of compounds originally developed in Japan as potential agricultural fungicides (Fujinami *et al.* 1972). On further investigation this analogue was found to be nephrotoxic when administered to the rat (Sugihara *et al.* 1975, Rankin 1982, Rankin *et al.* 1985). Although a role for biotransformation was implicated in its toxicity (Rankin *et al.* 1986–1988), the metabolic profile of NDPS has yet to be fully described. Previously, *in vivo* and *in vitro* metabolites of NDPS were isolated by tlc (Ohkawa *et al.* 1974) or hplc (Nyarko and Harvison 1995). In addition to a trace of NDPS, five metabolites were detected in rat urine, although only three of these could be definitively characterized (Ohkawa *et al.* 1974). Based on gc-ms of their methylated derivatives, these *in vivo* metabolites were identified as *N*-(3,5-dichlorophenyl)succinamic acid (NDPSA), *N*-(3,5-dichlorophenyl)malonamic acid (DMA), and *N*-(3,5-dichlorophenyl)-2-hydroxysuccinamic acid (2-NDHSA). The

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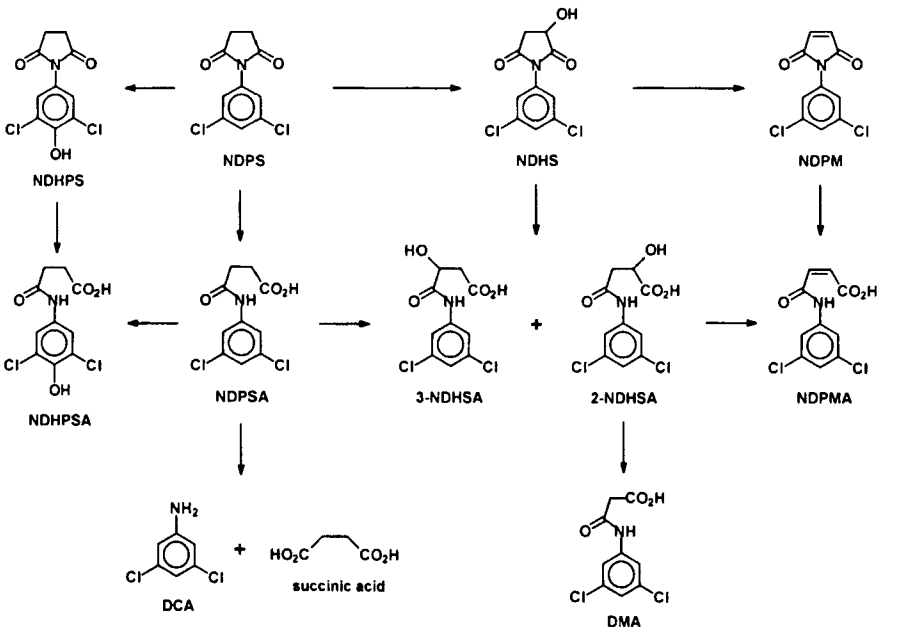

Figure 1. Structures of NDPS, its known and potential metabolites, and their proposed routes of production. Abbreviations used are as follows (left to right): NDHPS, *N*-(3,5-dichloro-4-hydroxyphenyl)succinimide; NDPS, *N*-(3,5-dichlorophenyl)succinimide; NDHS, *N*-(3,5-dichlorophenyl)-2-hydroxysuccinimide; NDPM, *N*-(3,5-dichlorophenyl)maleimide; NDHPSA, *N*-(3,5-dichloro-4-hydroxyphenyl)succinamic acid; NDPSA, *N*-(3,5-dichlorophenyl)succinamic acid; 3- or 2-NDHSA, *N*-(3,5-dichlorophenyl)-3- or -2-hydroxysuccinamic acid; NDPMA, *N*-(3,5-dichlorophenyl)maleamic acid; DCA, 3,5-dichloroaniline; and DMA, *N*-(3,5-dichlorophenyl)malonamic acid.

other two metabolites (34% of total) were not identified and 18% of the urinary radioactivity remained at the origin of the tlc plates. Metabolism of NDPS by rat and rabbit liver and kidney homogenates was limited to the production of NDPSA and succinic acid (Ohkawa *et al.* 1974). Isolated rat hepatocytes (Nyarko and Harvison 1995) converted NDPS to NDPSA, 2-NDHSA, *N*-(3,5-dichlorophenyl)-2-hydroxysuccinimide (NDHS), *N*-(3,5-dichlorophenyl)-3-hydroxysuccinamic acid (3-NDHSA) and *N*-(3,5-dichloro-4-hydroxyphenyl)-succinamic acid (NDHPSA).

One of the major aims of this laboratory is to re-evaluate NDPS metabolism with the eventual goal of determining how biotransformation may relate to toxicity. In the experiments described in this paper the effects of cofactor dependency, enzyme induction and inhibition, tissue differences and species variation on NDPS metabolism were evaluated *in vitro*. The data suggest that rat liver cytochromes P450 can convert NDPS to hydroxylated, potentially nephrotoxic metabolites, whereas metabolism in the rabbit is primarily amidase-mediated.

Materials and methods

Materials

All reagents were of the highest purity commercially available. [2,3-^{14}C]-succinic anhydride (sp. act. 3·6 mCi/mmol, radiochemical purity > 98%) was purchased from Sigma Chemical Co. (St Louis, MO, USA). Male Fischer 344 rats (150–175 g) were obtained from Charles River Breeding Labs, Inc. (Wilmington, MA, USA). Male New Zealand white rabbits (1·5–2·0 kg) were purchased from Myrtle's

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Rabbitry (Memphis, TN, USA). All animals were housed under a 12-h light/dark sequence at 45–50% relative humidity. Room temperatures were maintained at *c.* 22 °C (rat) or 20 °C (rabbit). Food (laboratory rodent diet # 5001 or laboratory rabbit diet HF # 5326, PMI Foods, Inc., St Louis, MO, USA) and water were freely available.

Syntheses

Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. ^{1}H-nmr spectra were obtained on Varian EM-390 or IBM WP-200 instruments and chemical shifts are reported as ppm downfield from tetramethylsilane. The abbreviations used in reporting the data are as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). IR spectra were run on a Perkin-Elmer 710B spectrophotometer and UV spectra were measured on Beckman DU-7 or Hitachi U-3110 instruments. Chemical ionization mass spectra (CIMS) were run on a VG ZAB-E mass spectrometer. High-resolution nmr (200 MHz) and mass spectral data were obtained through the Department of Chemistry at the University of Pennsylvania (Philadelphia, PA, USA). Elemental analyses were performed by Galbraith Laboratories Inc. (Knoxville, TN, USA).

NDPS, NDPSA, *N*-(3,5-dichlorophenyl)maleimide (NDPM), *N*-(3,5-dichlorophenyl)maleamic acid (NDPMA), NDHS, 2-NDHSA, and DMA were synthesized by known methods (Fujinami *et al.* 1972, Shih and Rankin 1989). Although 3-NDHSA could be prepared from NDHS (Shih and Rankin 1989), we found it more convenient to start with trifluoroacetylated malic anhydride (Liesen and Sukenik 1987). Briefly, malic acid (3·0 g, 0·022 mol) was cyclized by refluxing it in trifluoroacetic anhydride (6·9 ml, 0·049 mol) for 1 h. The solvent was removed at reduced pressure and 2·0 g (0·016 mol) of the resulting white crystalline solid was then refluxed with 2·59 g (0·016 mol) of 3,5-dichloroaniline (DCA) in methylene chloride for 2 h. Recrystallization of the crude product with methylene chloride yielded pure 3-NDHSA; m.p. 149–150 °C, lit. m.p. 150–151 °C (Shih and Rankin 1989).

The two *para*-hydroxy compounds, *N*-(3,5-dichloro-4-hydroxyphenyl)succinimide (NDHPS) and *N*-(3,5-dichloro-4-hydroxyphenyl)succinamic acid (NDHPSA), were synthesized by a modification of the method of Fujinami *et al.* (1972). A mixture of succinic anhydride (3·0 g, 0·03 mol) and 2,6-dichloro-4-aminophenol (5·34 g, 0·03 mol) was heated at reflux in toluene. After cooling, the resulting brown precipitate was added to 200–300 ml boiling water which contained a few crystals of ascorbic acid. Acetonitrile was then added dropwise until dissolution was complete. The hot solution was treated with decolourizing charcoal and filtered through Celite. White crystals began to form immediately. Recrystallization of the crude product from methylene chloride yielded pure NDHPSA; m.p. 201–203 °C; ^{1}H-nmr (DMSO-d_6): δ 2·43 (br s, 4H, CH_2CH_2), 7·48 (br s, 2H, Ar-$H_{2,6}$), 9·83 (br s, 1H, OH or NH), 10·0–11·5 (very br peak, OH or NH); IR (KBr pellet): 1650, 2000–3500 cm^{-1}; UV (acetonitrile): 209 nm (ε 25660 M^{-1} cm^{-1}), 250 (14230 M^{-1} cm^{-1}), 298 (3160 M^{-1} cm^{-1}); CIMS (NH_3), found: 277·9964; $C_{10}H_{10}Cl_2NO_4^+$ requires 277·9987; elemental analysis, found: C, 43·06; H, 3·25; N, 5·17; Cl, 25·41; $C_{10}H_9Cl_2NO_4$ requires C, 43·18; H, 3·26; N, 5·03; Cl, 25·49. NDHPS was prepared by refluxing a mixture of NDHPSA (0·5 g, 0·0018 mol), trifluoroacetic anhydride (3·4 ml, 0·024 mol), and sodium acetate (0·049 g, 0·0006 mol) for 3 h. After evaporation of the trifluoroacetic anhydride, cold water was added. The resulting sticky yellow mass was collected by filtration. This was repeatedly treated with cold water until a pale yellow solid resulted. The crude product was then chromatographed on silica gel with acetonitrile/methylene chloride (1:1). Following recrystallization from methylene chloride, NDHPS was obtained as white crystals; m.p. 211·5–212 °C; ^{1}H-nmr (DMSO-d_6): δ 2·68 (br s, 4H, CH_2CH_2), 7·70 (br s, 2H, Ar-$H_{2,6}$); IR (KBr pellet): 1700, 3350 cm^{-1}; UV (acetonitrile): 205 nm (ε 34400 M^{-1} cm^{-1}), 281 (2700 M^{-1} cm^{-1}), 325 (915 M^{-1} cm^{-1}); CIMS (NH_3), found: 258·9827; $C_{10}H_7Cl_2NO_3^+$ requires 258·9803; elemental analysis, found: C, 45·99; H, 2·71; N, 5·33; Cl, 27·14; $C_{10}H_7Cl_2NO_3$ requires C, 46·18; H, 2·71; N, 5·39; Cl, 27·27.

The hplc internal standard ethyl *N*-(3,5-dichlorophenyl)succinamate (NDPSA-Et) was synthesized by stirring a solution of DCA (1·0 g, 0·006 mol), ethyl succinyl chloride (0·85 ml, 0·006 mol), and triethylamine (0·83 ml, 0·006 mol) in acetonitrile (50 ml) at room temperature. After 1 h the reaction mixture was filtered and then concentrated *in vacuo*. The resulting solid was dissolved in methylene chloride and the solution filtered to remove any remaining salts. Recrystallization of the crude product from 95% aqueous ethanol provided 0·61 g (35·0% yield) of NDPSA-Et as white plates; m.p. 134–136 °C; ^{1}H-nmr ($CHCl_3$-d_1): δ 1·29 (t, CH_3, J = 7·14 Hz), 2·69 (m, CH_2CH_2), 4·17 (q, CO_2CH_2, J = 7·14 Hz), 7·06 (t, Ar-H_4, J = 1·82 Hz), 7·46 (d, Ar-$H_{2,6}$, J = 1·82 Hz), 8·12 (br s, NH); IR (KBr pellet): 1590, 1690, 3350 cm^{-1}; UV (acetonitrile): 216 nm (ε 37300 M^{-1} cm^{-1}), 249 (18000 M^{-1} cm^{-1}); CIMS (NH_3), found: 290·0347; $C_{12}H_{14}Cl_2NO_3^+$ requires 290·0350; elemental analysis, found: C, 49·74; H, 4·52; N, 4·81; Cl, 24·66; $C_{12}H_{13}Cl_2NO_3$ requires C, 49·67; H, 4·51; N, 4·82; Cl, 24·44.

^{14}C-NDPS was synthesized by the method of Fujinami *et al.* (1972). The crude product was purified by preparative tlc (100% chloroform) on 1000-μm thick silica gel GF plates to provide 140 mg (62·0% yield) ^{14}C-NDPS (sp. act. 0·65 mCi/mmol). Final chemical and radiochemical purity (99%) were established using the hplc assay described below.

Hplc equipment

The hplc system consisted of a Beckman model 421 system controller, a model 340 organizer, two model 114M solvent delivery modules, and a model 165 variable wavelength detector (operating at 254 nm). Samples were injected with an Altex 210A valve or with a Beckman model 502 autosampler. In some analyses, a Raytest Ramona-5-LS radiochemical detector was used. EcoLume scintillation fluid (ICN, Costa Mesa, CA, USA) was pumped through the radiochemical flow cell at 5·1 ml/min. The chromatograms were obtained from a Hewlett Packard model 3396A integrator or from an IBM-compatible computer using the Chromasoft software package (RSM Analytische Instrumente GmbH, Germany).

Chromatographic conditions

Separation of a mixture of 12 potential metabolites was achieved on a Beckman 5-μm C-18 column (4·2 mm × 25 cm) using a gradient between acetonitrile containing 3% THF (solvent A) and water containing 2% acetic acid (solvent B). The mobile phase flow rate was 1·7 ml/min. Immediately following injection, an 8-min linear gradient was run from 29 to 27% solvent A. Between 8 and 17 min conditions remained isocratic at 27% A and then another linear gradient was run over 5 min from 27 to 55% solvent A. From 22 to 26 min the mobile phase was pumped isocratically at 55% A, at which point a 4-min linear gradient increased solvent A to 100%. The column was flushed with 100% A for 3 min and then a final 4-min gradient restored the system to starting conditions (29% A). Peaks were identified by comparison of their retention times on the UV chromatograms to synthetic standards. A retention time for succinic acid (SA), which has no UV absorbance at 254 nm, was determined by hydrolyzing a small amount of [2,3-^{14}C]succinic anhydride in water. When this reaction mixture was analyzed by hplc a peak appeared at 1·9 min on the radiochromatogram.

Preparation of tissue homogenates

A group of rats were pretreated with phenobarbital (80 mg/kg for 3 days, i.p. in saline). Control animals received saline only (1·8 ml/kg). The rats were killed (24 h after the last injection) by cervical dislocation and the livers and kidneys rapidly removed and placed in ice-cold 20 mM Tris–1·15% KCl buffer, pH 7·4. The organs were weighed and homogenized in 3 vols Tris–KCl buffer with three strokes of a motor-driven Potter-Elvehjem homogenizer. The tissue homogenates were obtained following centrifugation of the crude homogenates at 2500*g* and were diluted to a protein concentration of 10 mg/ml. Liver tissue from male, New Zealand white rabbits was homogenized as described above. All tissue homogenates were used on the day of preparation. Protein concentrations were determined by the method of Lowry *et al.* (1951) or with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, CA, USA).

Incubations

Stock solutions of ^{14}C-NDPS or ^{14}C-NDPSA (100 mM, 0·442–0·569 mCi/mmol) were prepared in dimethyl sulphoxide. The NADPH-generating system contained $NADP^+$ (sodium salt, 8·0 mg), glucose 6-phosphate (disodium salt, 80 mg), and glucose 6-phosphate dehydrogenase (20 units) in 5·0 ml 50 mM Tris buffer (pH 7·4) to which was added 1·0 M $MgCl_2$ (0·2 ml). Complete incubations (1·0 ml total volume) contained 0·02 ml of the ^{14}C-NDPS stock solution (final concentration, 2·0 mM); 0·2 ml tissue homogenate (final concentration, 2·0 mg/ml); 0·167 ml of the NADPH-generating system (final concentrations; $NADP^+$, 0·35 mM; glucose 6-phosphate, 8·4 mM; glucose 6-phosphate dehydrogenase, 0·64 units), and 0·613 ml 50 mM Tris buffer (pH 7·4). In control incubations the NADPH-generating system was replaced with an equal volume of buffer. The spontaneous hydrolysis of NDPS was measured in incubations in which protein was omitted. All experiments were conducted in 20-ml glass scintillation vials at 37 °C. Following a 3-min preincubation, the reactions were initiated by addition of the substrate. For the P450 inhibition studies, carbon monoxide was bubbled through the incubations for 3 min prior to initiating the reactions; these reactions remained sealed for the duration of the 3-h incubation. In some experiments, the amidase inhibitor bis-*p*-nitrophenyl phosphate (0·5 mM) was preincubated with the homogenates before addition of substrate. All incubations were allowed to proceed for up to 3 h. Time course measurements were conducted by removing aliquots (0·1 ml) at the appropriate time points. Incubations were terminated by adding 0·1 ml of the reaction mixture to 0·3 ml ice-cold acetonitrile containing the internal standard, ethyl *N*-(3,5-dichlorophenyl)succinamate (NDPSA-Et, 0·133 mM). The samples were centrifuged for 15 min and the supernatants stored at −20 °C.

The oxidative metabolites 2-NDHSA and 3-NDHSA could be produced either by direct hydroxylation of NDPSA or by non-enzymatic hydrolysis of NDHS (figure 1). To address the first possibility, ^{14}C-NDPSA (0·16 or 2·0 mM) was incubated with phenobarbital-induced rat liver homogenates as described for NDPS. The hydrolysis of NDHS (0·08 or 2·0 mM) was monitored in Tris buffer (50 mM, pH 7·4, 37 °C) over the course of 1 h. Aliquots (0·1 ml) were removed from the reactions at different time points and were prepared for hplc analysis as above.

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Quantitation of metabolites

Aliquots (20–25 μl) of the supernatants were analyzed by the hplc system described above. Metabolites were quantitated radiochemically or by comparison of peak area ratios (metabolite/internal standard) at 254 nm to standard curves. In some experiments peaks were collected at 30-s intervals following elution from the hplc column using an ISCO Retriever III fraction collector. Each fraction was then mixed with 10 ml EcoLume scintillation cocktail and counted in a Pharmacia Wallac 1410 Scintillation Counter using internal quench correction. All counts were corrected for background radioactivity. Mean recoveries of radioactivity from the hplc column were > 94%.

Statistics

Statistical comparisons were made using an unpaired *t*-test or a one-way ANOVA. The level of significance was set at $p < 0.05$. Results are presented as mean ± SD and were obtained from four-to-eight separate homogenate preparations for each experiment.

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Results

Figure 2 shows the separation of a mixture of NDPS from its known and proposed metabolites that was achieved via reversed-phase hplc. The known metabolites 2-NDHSA (peak c), DMA (peak f), and NDPSA (peak h) are well separated from each other and from NDPS (peak g). Furthermore, 3-NDHSA (peak e) is resolved from its isomer 2-NDHSA and probable precursor NDHS (peak d).

Typical hplc chromatograms resulting from incubations of ^{14}C-NDPS with rat liver homogenates are shown in figure 3. In the absence of NADPH only peaks corresponding to NDPS, NDPSA, and NDPSA-Et (the internal standard) are visible (figure 3A). When NADPH was present in the incubations, a small peak corresponding to 2-NDHSA was also apparent (figure 3B). These peaks were quantitated by liquid scintillation counting and the results are summarized in table 1. Production of the major metabolite, NDPSA, was the same in the absence or presence of NADPH (161·3 ± 24·2 and 154·1 ± 13·7 nmol/incubation respectively, data recalculated from table 1) and did not differ significantly from the amount formed (170·3 ± 26·7 nmol/incubation) when the liver homogenate was omitted from the incubations (buffer-only controls).

Phenobarbital (PB)-pretreatment enhanced oxidative metabolism of NDPS by rat liver homogenates. UV peaks corresponding to 2-NDHSA and 3-NDHSA are evident in the chromatogram from an incubation fortified with NADPH (figure 4B), whereas these peaks are absent in incubations in which the cofactor was omitted (figure 4A). Owing to its low UV absorbance at 254 nm (approximately 33-fold lower than the hydroxy acids) no NDHS peak is seen in figure 4B. However, radioactivity was present in the fractions that corresponded to its retention time on hplc. In contrast, radioactivity was not associated with any of the fractions corresponding to the hydroxylated metabolites when NADPH was omitted from the incubations. The effects of PB-pretreatment on metabolism by the hepatic homogenates are summarized in table 1. When compared with NADPH-fortified homogenate incubations from the control rat, a five-fold increase in 2-NDHSA was noted. In addition, 2-NDHSA was formed in an approximately 7:1 excess over 3-NDHSA. Production of all three oxidative metabolites was suppressed by carbon monoxide or omission of the cofactor, but levels of NDPSA were not affected by NADPH or CO (*c.* 168·4 nmol/incubation, data recalculated from table 1) and were not significantly different from buffer-only incubations (170·3 ± 26·7 nmol/incubation). Owing to the limited amount of oxidative metabolism that occurred in 1 h, some incubations were allowed to proceed for longer time periods. However, as

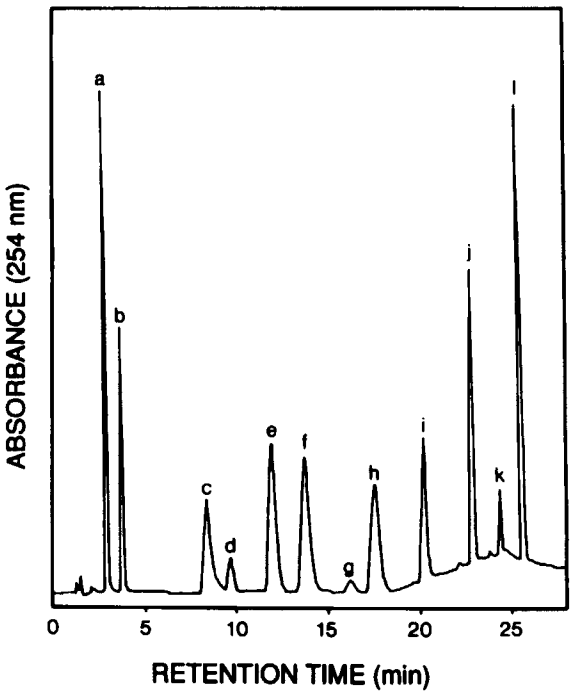

Figure 2. Hplc chromatogram for NDPS and its known and potential metabolites. Separation was achieved on a C18 column using a gradient between water and acetonitrile with UV detection at 254 nm (see Materials and methods for details). Peaks are labelled as follows (retention times (min) in parentheses): a, NDHPSA (2·9); b, NDHPS (3·8); c, 2-NDHSA (8·4); d, NDHS (9·7); e, 3-NDHSA (11·9); f, DMA (13·7); g, NDPS (16·1); h, NDPSA (17·6); i, NDPMA (20·3); j, DCA (22·9); k, NDPM (24·4); and l, NDPSA-Et (25·6). The injection contained 12·5 nmol NDHPS, NDHS, NDPS, and NDPM; and 2·5 nmol of the other compounds. The absorbance scale is 0·1 AUFS.

shown in figure 5, the predominant reaction at all time points was the hydrolysis of NDPS to NDPSA. Only NDPSA was produced when the cofactor was omitted from the incubations (figure 5).

NDHS hydrolysis was initially investigated at the approximate concentration (0·08 mM) at which it was produced from NDPS by phenobarbital-induced rat liver homogenates. This concentration was extrapolated from the data for the oxidative metabolites (i.e. NDHS + 2-NDHSA + 3-NDHSA) in table 1. Under these conditions, only 2-NDHSA could be detected after 1 h. When repeated at a higher concentration (2·0 mM), both hydroxysuccinamic acids were produced (2-NDHSA/3-NDHSA = 5·5 : 1). NDPSA was incubated with phenobarbital-induced rat liver homogenates at two different concentrations, 0·16 mM (estimated concentration of NDPSA that was generated in NDPS-containing incubations as calculated from the data in table 1), and 2·0 mM (same concentration as NDPS). There was no evidence for the production of 2-NDHSA or 3-NDHSA from NDPSA in any of these incubations even after 3 h (data not shown).

NDPS was also incubated with kidney homogenates from the control and phenobarbital-pretreated rat. In contrast with the liver homogenates, the only metabolite detected under all incubation conditions was NDPSA (data not shown). There were no significant differences in the amount of NDPSA produced

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Table 1. Metabolism of NDPS by rat liver homogenates. ^{14}C-NDPS (2·0 mM) and tissue (2·0 mg/ml) were incubated for 1 h at 37 °C as described in the Materials and methods. Results are expressed as mean ± SD from separate homogenate preparations ($n = 4$, unless otherwise stated). Values for each metabolite that are significantly different from each other ($p < 0·05$) are indicated by an asterisk.

	Metabolite production (nmol/mg protein/h)				
	Control		Phenobarbital-induced		
Metabolite	−NADPH	+NADPH	−NADPH	+NADPH[a]	+NADPH/+CO
NDPSA	80·7 ± 12·1	77·1 ± 6·9	87·2 ± 20·5	81·8 ± 19·2	83·7 ± 14·3
2-NDHSA	nd[b]	4·6 ± 1·7*	nd	23·7 ± 7·1*	2·4[c]
3-NDHSA	nd	nd	nd	3·2 ± 0·5[d]	nd
NDHS	nd	nd	nd	12·2 ± 3·5	nd

[a] Results were obtained from eight separate homogenate preparations.
[b] nd, Not detected.
[c] 2-NDHSA was detected in one out of four incubations.
[d] 3-NDHSA was detected in four out of eight incubations. Mean and SD were calculated from those four values only.

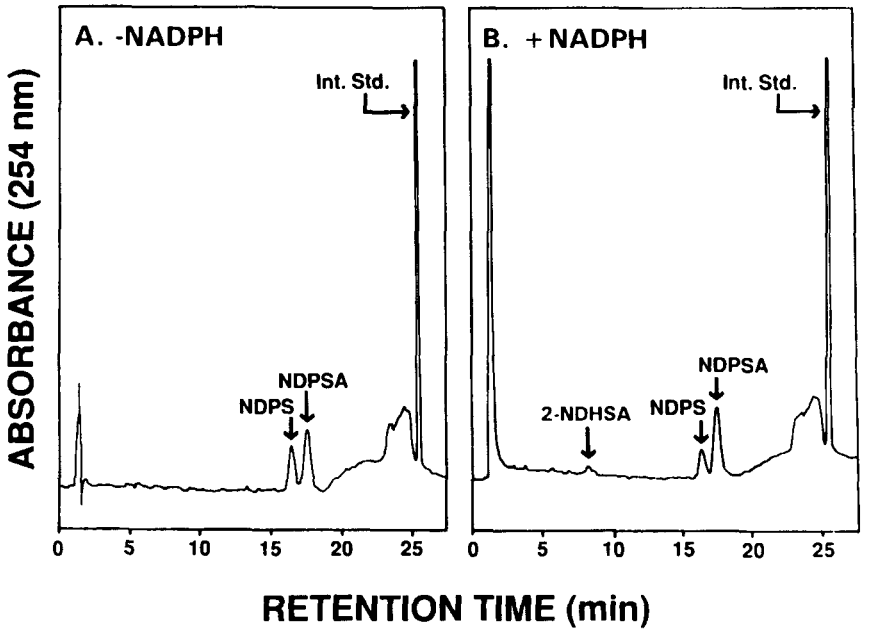

Figure 3. Hplc chromatograms obtained from incubations of NDPS with liver homogenates isolated from control rats in the (A) absence or (B) presence of NADPH. Incubations contained ^{14}C-NDPS (2·0 mM) and rat liver homogenate (2 mg/ml) in 50 mM Tris buffer (pH 7·4, 37 °C) and were terminated after 1 h. 'Int. Std.' represents the internal standard (NDPSA-Et). The absorbance scale is 0·1 AUFS.

(*c.* 162 nmol/incubation) in the presence or absence of NADPH or from incubations in which renal protein was omitted (170·3 ± 26·7 nmol/incubation).

Incubation of NDPS with uninduced rabbit liver homogenates for 1 h resulted in the formation of different metabolites than were produced by rat liver homogenates (figure 6). Peaks for NDPSA and 3,5-dichloroaniline (DCA) were evident on the UV chromatogram (figure 6, top) and a peak corresponding to the

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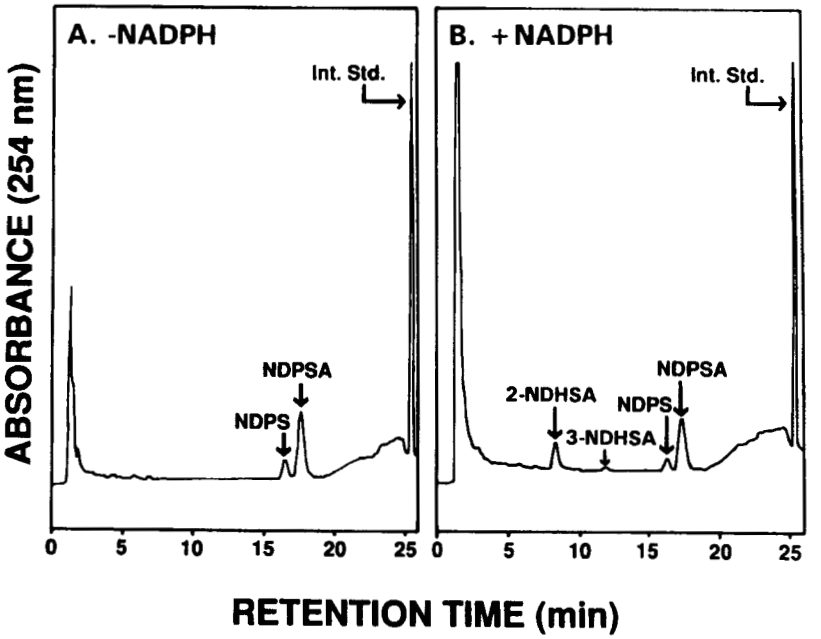

Figure 4. Hplc chromatograms obtained from incubations of NDPS with liver homogenates isolated from the phenobarbital-pretreated rat in the (A) absence or (B) presence of NADPH. Incubations contained ^{14}C-NDPS (2·0 mM) and rat liver homogenate (2 mg/ml) in 50 mM Tris buffer (pH 7·4, 37 °C) and were terminated after 1 h. 'Int. Std.' represents the internal standard (NDPSA-Et). The absorbance scale is 0·1 AUFS.

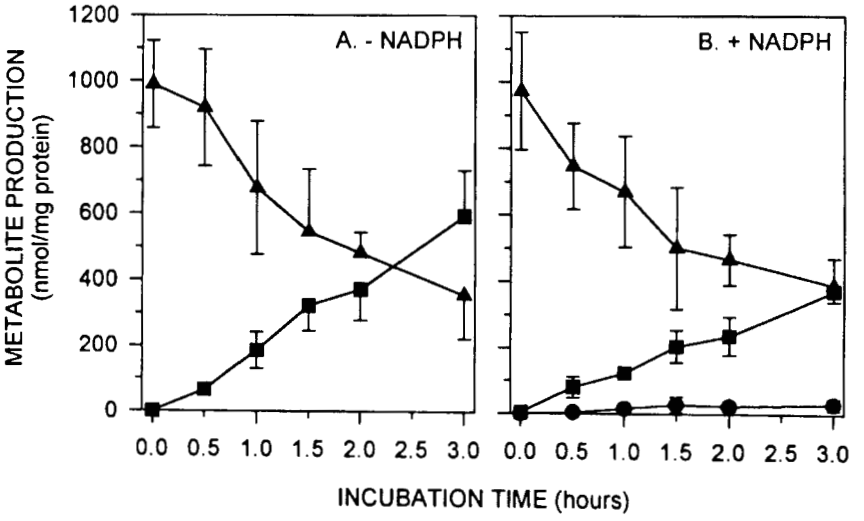

Figure 5. Time course for metabolism of NDPS by liver homogenates isolated from the phenobarbital-pretreated rat in the (A) absence or (B) presence of NADPH. Incubations contained ^{14}C-NDPS (2·0 mM) and rat liver homogenate (2 mg/ml) in 50 mM Tris buffer (pH 7·4, 37 °C) and were terminated at the indicated time points. Symbols represent NDPS (triangles), NDPSA (squares), and 2-NDHSA (circles).

Table 2. Metabolism of NDPS or NDPSA by rabbit liver homogenates. ^{14}C-NDPS or ^{14}C-NDPSA (2·0 mM) and tissue (2·0 mg/ml) were incubated for 1 h at 37 °C as described in the Materials and methods. Results are expressed as mean ± SD from separate homogenate preparations ($n = 5$). Values for each metabolite that are significantly different from each other ($p < 0·05$) are indicated by an asterisk.

	Metabolite production (nmol/mg protein/h)			
	From NDPS		From NDPSA	
Metabolite	+NADPH	+BNPP	+NADPH	+BNPP
SA	193·4 ± 59·6	nd[a]	559·1 ± 307·8*	8·6 ± 10·8*
DCA	200·4 ± 46·6	nd	454·3 ± 69·5	nd
NDPSA	85·9 ± 27·9*	169·8 ± 44·5*	507·8 ± 179·0*	1550·8 ± 657·3*
2-NDHSA	nd	11·8 ± 10·7	nd	nd
3-NDHSA	nd	nd	nd	nd
NDHS	nd	nd	nd	nd

[a] nd, Not detected.

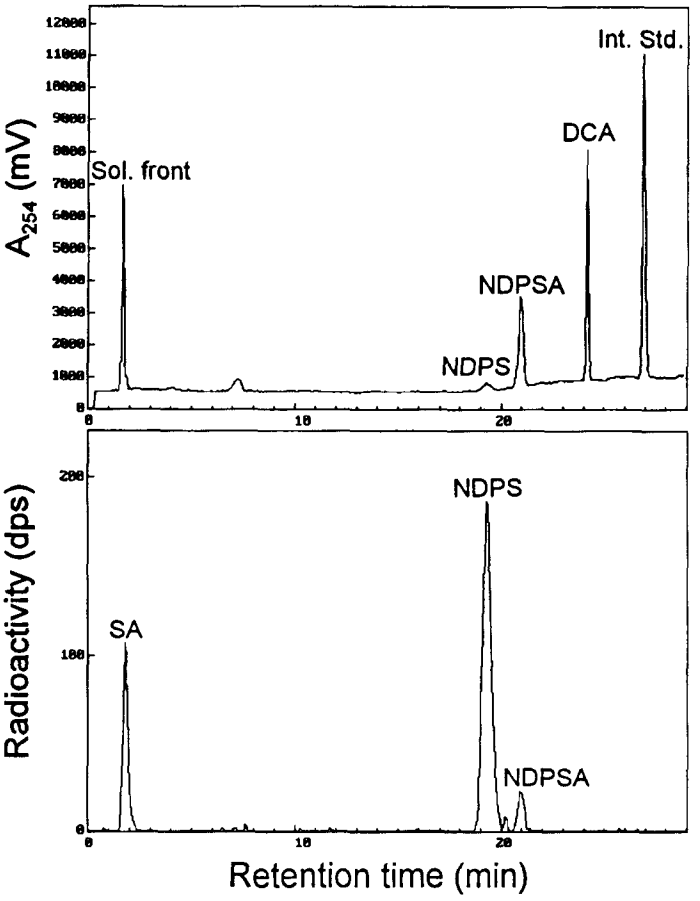

Figure 6. Hplc chromatogram obtained from an incubation of ^{14}C-NDPS (2·0 mM) with liver homogenates (2 mg/ml) isolated from the control rabbit. Incubations contained ^{14}C-NDPS (2·0 mM) and rabbit liver homogenate (2 mg/ml) in 50 mM Tris buffer (pH 7·4, 37 °C) and were terminated after 1 h. 'Sol. front' and 'Int. Std.' represents the solvent front and internal standard (NDPSA-Et) respectively.

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retention time of succinic acid (SA, 1·9 min) could be seen on the radiochromatogram (figure 6, bottom). DCA and SA were produced in a 1:1 ratio. Production of DCA and SA were abolished by the inclusion of the amidase inhibitor bis-*p*-nitrophenyl phosphate (BNPP) in the incubations (chromatograms not shown). There was no evidence for the formation of any hydroxylated metabolites. The results of these experiments are summarized in table 2. Similar results were obtained when NDPSA was incubated with rabbit liver homogenates (table 2).

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Discussion

NDPSA, the major NDPS metabolite, was produced to a similar extent under all incubation conditions, even when protein was omitted. Therefore, it seems likely that NDPSA is actually generated by non-enzymatic hydrolysis of NDPS rather than by an enzyme-mediated process. This is consistent with the fact that *N*-phenylsuccinimides hydrolyze in buffer in a pH-dependent manner (Herd *et al.* 1966, Hargreaves *et al.* 1970). Our results do not completely agree with those reported by Ohkawa *et al.* (1974) who found that SA was also produced to a limited extent from NDPS by rat liver and kidney homogenates. 3,5-Dichloroaniline (DCA), which would be the other product of this reaction (figure 1), was not detected in our incubations. Unlike Ohkawa *et al.* (1974), we also found that oxidative metabolites were produced by rat liver homogenates. Furthermore, the results presented here are consistent, at least qualitatively, with those we obtained with freshly isolated rat liver cells (Nyarko and Harvison 1995). The reason for the discrepancies between our findings and those of Ohkawa *et al.* are not clear, although they could be due to differences in assay methods, rat strains, incubation conditions, etc. BNPP, an amidase inhibitor, was not used in the rat liver experiments since the conversion of NDPSA to NDPS was non-enzymatic.

Production of 2-NDHSA, 3-NDHSA, and NDHS by rat liver homogenates was NADPH-dependent, carbon monoxide-inhibitable (Testa and Jenner 1981) and phenobarbital-inducible, suggesting that cytochrome P450 is involved in this process. Similar metabolites were found when NDPS was incubated with isolated rat hepatocytes (Nyarko and Harvison 1995), although we were unable to detect NDHPSA in the homogenates. This metabolite was produced to a very limited extent by the liver cells and may be below our detection limits in the current experiments. Hydroxysuccinimide metabolites are known for other compounds, including succinimide itself (Schulte *et al.* 1978, Lawson and Chalmers 1980). Based on what is known about the hydrolytic behaviour of hydroxysuccinimides (Paulssen *et al.* 1968, Shih and Rankin 1989), detection of both 2-NDHSA and 3-NDHSA is not surprising. Furthermore, the predominance of the 2-hydroxy isomer is also not unexpected. For example, we found that NDHS hydrolyzes in buffer to a mixture of 2-NDHSA and 3-NDHSA in a 5·5:1 ratio. An alternative pathway for production of 2- and 3-NDHSA could involve initial hydrolysis of NDPS to NDPSA, followed by hydroxylation to the isomeric acids (figure 1). Since we could find no evidence that NDPSA was converted directly to 2-NDHSA and 3-NDHSA, it is more likely that these metabolites are produced by hydrolysis of NDHS (figure 1). In contrast with the liver homogenates, renal homogenates did not produce oxidative metabolites, even following phenobarbital pretreatment. This is not surprising, however, since the kidney is known to be much lower in P450 activity than the liver and renal P450 is resistant to phenobarbital induction (Tarloff *et al.* 1990).

In contrast with the results obtained with rat liver, formation of SA and DCA was the predominant reaction in rabbit liver homogenates. Since these metabolites were produced in a stoichiometric ratio, they are probably derived from a common precursor, i.e. NDPSA (figure 1). The potent inhibition of this reaction by BNPP suggests that this process is amidase-mediated in the rabbit. Similar conclusions were reached by Ohkawa *et al.* (1974) who found that this reaction could be suppressed by arylamidase inhibitors such as sumioxon.

Our results provide an explanation for prior observations on the relationship between NDPS metabolism and its toxicity in rat. For example, phenobarbital pretreatment was previously shown to potentiate NDPS-induced renal damage in the rat (Rankin *et al.* 1987). Furthermore, the oxidative metabolites, NDHS, 2-NDHSA, and 3-NDHSA, produce kidney damage at doses as low as 0·2 mmol/kg, whereas nephrotoxicity from NDPS occurs at doses $\geqslant$ 0·4 mmol/kg (Rankin *et al.* 1988, 1989). Our results demonstrate that phenobarbital pretreatment enhances production of these same toxic metabolites, providing a metabolic basis for the enhancement of nephrotoxicity by PB. The kidney itself cannot generate the hydroxylated metabolites in detectable amounts, which suggests that hepatic metabolism must be involved, as previously proposed by Rankin *et al.* (1987). Unlike the rat, NDPS metabolism in the rabbit was predominantly amidase-mediated. The relevance of this observation to NDPS toxicity in the rabbit is unknown, since NDPS nephrotoxicity has not been examined in this species.

In conclusion, the results of these experiments are in general agreement with our recent findings in rat hepatocytes and additionally provide evidence that NDPS is directly metabolized to NDHS by a phenobarbital-inducible P450 isozyme in rat liver. NDHS then undergoes non-enzymatic hydrolysis to 2-NDHSA and 3-NDHSA. The major metabolite, NDPSA, is produced by hydrolysis of NDPS and is not hydroxylated in the liver. There was no evidence that NDPS is metabolically activated in the kidney confirming that extrarenal metabolism is necessary for nephrotoxicity to occur, provided it is caused by NDHS, 2-NDHSA and/or 3-NDHSA.

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