Enzyme Induction and Comparative Oxidative Desulfuration of Isothiocyanates to Isocyanates

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Enzyme induction of oxidative metabolism of isothiocyanates to isocyanates by rat liver microsomes and comparative metabolic conversion of some isothiocyanates were investigated. Metabolic activity was assayed by trapping the isocyanate metabolites from isothiocyanates with the inclusion of 2-aminofluorene to form the respective mixed ureas as previously described for the 2-naphthyl isothiocyanate. Male F344 rats were fed either a conventional grain diet for induction with Aroclor 1254 or AIN 76A diet without antioxidant beginning 2 weeks before treatment with Aroclor 1254, β-naphthoflavone, isosafrole, or phenobarbital. Enzymes responsible for the metabolism of 1- and 2-naphthyl isothiocyanate were inducible by all four agents, Aroclor being the best under the current induction protocol and metabolic conversion assay procedure. On the other hand, enzymes responsible for the metabolism of benzyl isothiocyanate were induced only by Aroclor and, to a lesser extent, by phenobarbital. For the comparative metabolic conversion studies, using the microsomes from Aroclor-treated rats fed a conventional grain diet, the rates of metabolic conversion followed the order of 1-naphthyl ≫ phenyl > benzyl and phenethyl ≫ propyl, ethyl, and methyl isothiocyanates.

Introduction

Organic isocyanates (RN═C═O) are used extensively in industries, and production of these compounds is on the rise in the United States (*1*). Apart from direct occupational exposure, exposures can also come from chemotherapeutic dialkylnitrosoureas (*2*) and industrial solvents such as alkylformamides which can be metabolized to isocyanates (*3*). Since isocyanates are reactive electrophilic agents, they are capable of modifying proteins (*4*) and nucleic acids *in vitro* (*5*, *6*) and *in vivo* (*7*); biologically, they can cause chromosome aberrations, sister-chromatid exchanges (*8*−*11*), mutations (*6*, *8*, *12*, *13*) and cancer (*14*, *15*).

Organic isothiocyanates (RN═C═S), the sulfur analogs of isocyanates, possess a wide range of biological activities, and hence, they have been used medicinally (*16*). Moreover, isothiocyanates with diverse structural features can be generated from a variety of vegetables by enzymatic hydrolysis of their glucosinolates with myrosinase (*17*). Several synthetic and dietary isothiocyanates have been used as chemopreventive agents in experimental animals (*18*−*25*) and have been considered for use in humans. A number of isothiocyanates have been shown to be mutagenic for *Salmonella typhimurium* TA100 (*26*, *27*), and mutagenic dietary allyl isothiocyanate has been found to cause bladder tumors in F344 rats (*28*).

Recently, it has been found that reactive isocyanates such as 2-naphthyl isocyanate (*6*) and methyl isocyanate (*5*) formed predominantly N^4-cytosine adducts and exerted mutagenic effects in a bacteriophage system (*6*, *12*). Since we are likely to be exposed to isothiocyanates, it is of interest that their genotoxic effects be evaluated. The results of such an effort have been reported (*29*), in which 2-naphthyl isothiocyanate (2-NITC)[1] was converted to the more reactive and toxic 2-naphthyl isocyanate by a cytochrome P450 system, and the enzymes responsible for this conversion were inducible by Aroclor 1254. Further studies have shown that 1-naphthyl isothiocyanate (1-NITC) and dietary benzyl isothiocyanate (BITC) can also be metabolized to reactive isocyanates (*30*). Although both isomers of naphthyl isothiocyanate were metabolized to a comparable extent by the Aroclor-inducible P450(s), the metabolism of BITC was much lower and Aroclor treatment of rats fed the conventional grain diet did not increase the enzyme activity. The latter observations prompted studies of enzyme induction with three additional common inducers, i.e., β-naphthoflavone, isosafrole, and phenobarbital, to gain insight into the enzymes that are responsible for the metabolic conversion of these compounds. Animals were fed AIN 76A diet without ethoxyquin prior to and during the treatment with the inducing agents in an attempt to minimize the effects of induction due to dietary components, including ethoxyquin which is known to induce P450 2B1, 2B2, 1A2, and 3A2 (*31*).

Metabolic conversion was further studied with aryl, arylalkyl, and aliphatic isothiocyanates using Aroclor-induced liver microsomes.

Materials and Methods

Caution: *Aromatic amines, isocyanates, isothiocyanates, and Aroclor 1254 are hazardous and should be handled with care.*

Materials. Aroclor 1254 was a gift from Dr. P. Hollenberg, Department of Pharmacology, University of Michigan School of

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[1] Abbreviations: 2-AF, 2-aminofluorene; BFU, *N*-benzyl-*N*′-(2-fluorenyl)urea; BITC, benzyl isothiocyanate; EFU, *N*-ethyl-*N*′-(2-fluorenyl)-urea; EITC, ethyl isothiocyanate; MFU, *N*-methyl-*N*′-(2-fluorenyl)urea; MITC, methyl isothiocyanate; 1-NFU, *N*-(1-naphthyl)-*N*′-(2-fluorenyl)-urea; 1-NITC, 1-naphthyl isothiocyanate; 2-NFU, *N*-(2-naphthyl)-*N*′-(2-fluorenyl)urea; 2-NITC, 2-naphthyl isothiocyanate; PEFU; *N*-phenethyl-*N*′-(2-fluorenyl)urea; PEITC, phenethyl isothiocyanate; PFU, *N*-phenyl-*N*′-(2-fluorenyl)urea; PITC, phenyl isothiocyanate; PrFU, *N*-propyl-*N*′-(2-fluorenyl)urea; PrITC, propyl isothiocyanate; P450, cytochrome P450.

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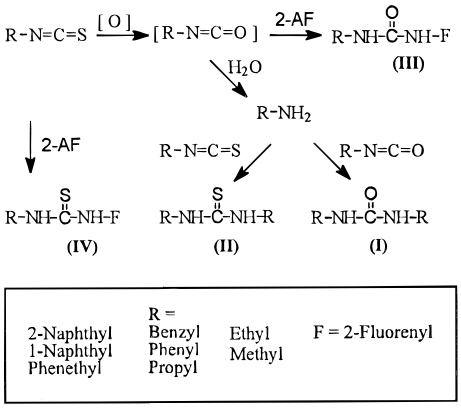
Scheme 1. Scheme for the Metabolic Conversion of Isothiocyanates and Trapping of the Reactive Isocyanate Metabolites with 2-AF



Medicine, Ann Arbor, MI 48109. Trioctanoin was from Pfaltz and Bauer (Stamford, CT). Sodium phenobarbital and NADPH (type III) were from Sigma Chemical Co. (St. Louis, MO). All other chemicals used in this study were either purchased from Aldrich Chemical Co. (Milwaukee, WI) or synthesized in this laboratory.

Instrumentation and General Procedures for Chemical Analysis. UV spectra were recorded using a Hewlett Packard 8452A diode array spectrophotometer. ^{1}H-NMR spectra were recorded with a GE NMR QE-300 instrument. Mass spectra were recorded using a Kratos MS80RFA mass spectrometer. Progress of reactions and purities of compounds were checked by TLC using Whatman PE SIL G/UV silica gel polyester sheets with benzene/95% ethanol = 20/1 or 9/1 as solvent, and chromatograms were viewed under UV light. Reverse-phase HPLC analyses were carried out using a Waters 486 tunable absorbance detector interfaced with a Nelson Model Turbo Chrom Chromatography Software. An LKB Model 2140 diode array detector was used as an in-line device for compound identification. μBondapak C_{18} columns maintained at 35 °C were used throughout the study.

Chemical Syntheses. Di-2-naphthylurea, di-2-naphthylthiourea, *N*-(2-naphthyl)-*N*′-(2-fluorenyl)urea (2NFU), 2-NITC, and *N*-(2-naphthyl)-*N*′-(2-fluorenyl)thiourea were prepared as described previously (*29*). Following similar procedures, all four groups of compounds as depicted in Scheme 1 were either purchased from Aldrich or prepared by reacting isocyanates or isothiocyanates (R = 1-naphthyl, phenyl, benzyl, phenethyl, propyl, ethyl, and methyl) with their respective amines or 2-aminofluorene (2-AF) in inert solvents such as dry ether, acetone, or dimethylformamide. The melting points of the synthetic compounds are listed below. Symmetrically disubstituted ureas, RNHCONHR (**I**): R, 1-naphthyl, 297 °C, [lit. mp 296 °C (*32*)]; benzyl, 168−170 °C [lit. mp 169−170 °C (*33*)]; phenethyl, 137−139 °C [lit. mp 138 °C (*34*)]; propyl, 104−106 °C [lit. mp 105 °C (*34*)]. Symmetrically disubstituted thioureas, RNHCSNHR (**II**): R, 1-naphthyl, 208−209 °C [lit. mp 207−208 °C (*32*)]; benzyl, 146−147 °C [lit. mp 147−148 °C (*33*)]; phenethyl, 94−95 °C [lit. mp 94−95 °C (*35*)]; propyl, 71−72 °C [lit. mp 72 °C (*36*)]. *N*-Substituted-*N*′-(2-fluorenyl)urea, RNHCONHF (**III**): R, 1-naphthyl, 263−278 °C; phenyl, ∼310 °C dec; benzyl, 239−240 °C; phenethyl, 222−223 °C; propyl, 208−209 °C; ethyl, 236−238−310 °C dec; methyl, 219−221 °C. *N*-Substituted-*N*′-(2−fluorenyl)thiourea, RNHCSNHF (**IV**): R, 1-naphthyl, 205−206 °C; phenyl, 215−217 °C; benzyl, 179−180 °C; phenethyl, 214−215 °C; propyl, 171−173 °C; ethyl, 179−181 °C; methyl, 177−179 °C. The exact mass of parent ions or the fragmentation patterns of compounds in mass spectra and signals in NMR spectra of the synthetic compounds are consistent with the structure assignments of these compounds. UV absorptions and molar extinction coefficients of the key compounds for metabolite analysis are tabulated in Table 1.

Treatments of Animals and Preparation of Microsomes. Male F344 rats (300−400 g) from Charles River (Wilmington, MA) were used in this study. The animals were fed either a conventional grain diet (Harlan Teklad 8604, Armada Grain Co., Bartonville, IL) for induction with Aroclor 1254 as described previously (*29*) or the semisynthetic AIN 76A diet without antioxidant (United States Biochemical Corp., Cleveland, OH) starting 2 weeks prior to the first injections for the comparative induction studies with Aroclor and three additional inducers. These compounds were dissolved or suspended in trioctanoin in the following doses (mg inducer/1.3 mL of trioctanoin/kg body weight): Aroclor 1254, 500 (single injection followed by 3 injections with solvent); β-naphthoflavone, 80; isosafrole, 150; sodium phenobarbital, 60. With the exception of Aroclor, the animals in groups of 3−4 were injected once daily for 4 days, and control animals received 4 daily injections with trioctanoin alone. On day 5, after overnight starvation, the animals were sacrificed and the liver microsomes were prepared according to the published procedure (*29*), except that the liver homogenates were centrifuged at 9000*g* for 30 min at 4 °C before the supernatants were centrifuged at 105000*g*. For comparative metabolic conversion studies, the pooled microsomes from 6 of Aroclor-treated animals fed conventional diet were used. The protein content was determined by the method of Bradford (*37*), and the microsomes were stored in small aliquots at −80 °C until use.

Metabolic Conversion Assays. Analyses of the metabolic conversion of isothiocyanates to isocyanates were carried out in the presence of 2-AF to trap the reactive metabolites as mixed ureas RNHCONHF (**III**) as described previously for 2-NITC (*29*). Briefly, for comparative induction studies, 1 mL of the complete incubation mixture contained 0.4 mg of microsomal protein, 0.5 μmol of substrate (2-NITC, 1-NITC, and BITC)/10 μL of DMSO, 5 μmol of NADPH and 0.2 μmol of 2-AF/10 μL of DMSO. All incubations were at 37 °C for 30 min which had produced a time-dependent yield of products when 2-NITC was the substrate (*29*). For comparative metabolic conversion studies, incubation mixtures were proportionally increased to 2 mL to contain 1.0 μmol of substrate [1-NITC, PITC, PrITC (synthetic), BITC, PEITC, EITC, and MITC (naturally occurring)]/20 μL of DMSO. The time course was from 0 up to 60 min. Ethyl acetate extracts of the incubation mixtures (except for EITC, see further treatment below) were evaporated to dryness and redissolved in methanol or acetonitrile containing 10% dimethylformamide for analyses by reverse-phase HPLC. For analysis of EITC metabolite, in order to avoid the interference of product quantitation due to the large neighboring 2-AF peak, the residues were dissolved in 400 μL of acetone and incubated at 45 °C for 1 day with 40 μL of acetone solution of 20 μmol of succinic anhydride containing 10% of triethylamine. The carboxyl-containing amide resulted from the reaction of succinic anhydride with 2-AF was removed by extraction of the ethyl acetate solution with 0.2 N NaOH (2 × 500 μL) and then with water twice. The residue thus obtained was subjected to HPLC analysis.

Determination of the Formation of Thiourea Side Products (IV) and Their Metabolic Conversions to Ureas (III). In order to estimate the possible contribution of urea (**III**) generated from the side product (**IV**) to the urea (**III**) obtained in the metabolism of isothiocyanates, 2-AF was incubated with isothiocyanates without microsomes and NADPH. At the end of incubations, the mixtures were chilled and centrifuged. The products (**IV**) were analyzed by HPLC from the supernatants and the precipitates. Synthetic thioureas (**IV**) in the same amount as the sum of the above analysis were incubated in the same manner as for isothiocyanates metabolism and the urea products (**III**) quantitated by HPLC.

Inhibition Studies. In order to prove that these metabolic conversions were mediated by P450, the CO inhibitions were carried out as described previously (*29*). To prove that flavin-containing monooxygenase was not involved in these reactions, microsomes were heated at 40 °C for 30 min prior to the metabolic incubations.

HPLC Analysis of Products. Identifications of metabolite-derived mixed ureas RNHCONHF (**III**) and isothiocyanate-derived side products RNHCSNHF (**IV**) were based on their UV

Table 1. UV Absorptions and Molar Extinction Coefficients (ϵ) of the Key Compounds

		RNHCONHF (**III**)		RNHCSNHF (**IV**)	
compound, R	solvent	λ_{max} (nm)	ϵ	λ_{max} (nm)	ϵ
1-naphthyl	methanol	288	31 340	284	25 480
		308	31 310	294	25 950
				304	25 400
phenyl	0.5% dimethylformamide in acetonitrile	280 (sh)	36 000	282	30 580
		290	40 940	302	28 410
		318 (sh)	17 240		
benzyl	methanol	278 (sh)	29 590	264 (sh)	19 580
		286	31 610	282	22 350
		318 (sh)	11 090	292	22 730
				302	22 440
phenethyl	1% dimethylformamide in acetonitrile	288	30 300	264 (sh)	19 990
		320	10 700	282	24 100
				290	24 630
				302	23 850
propyl	1% dimethylformamide in acetonitrile	286	32 690	262	19 580
		320 (sh)	12 240	282	24 120
				292	24 690
				302	23 730
ethyl	1% dimethylformamide in acetonitrile	286	33 340	264 (sh)	18 880
		318 (sh)	13 180	282	23 340
				290	23 940
				302	23 020
methyl	1% dimethylformamide in acetonitrile	286	29 070	260 (sh)	18 700
		320	9 510	280	24 650
				292	25 520
				302	24 490

Table 2. HPLC Retention Times (min) of Compounds Used in the Analysis of Metabolic Conversion Products

R	solvent systems[a]	RNH_2	2-AF	RN=C=S	RNHCONHR (**I**)	RNHCSNHR (**II**)	RNHCONHF (**III**)	RNHCSNHF (**IV**)
2-naphthyl	A	9.8	11.7	24.1	19.9	16.4	29.1	22.0
1-naphthyl	B	11.7	13.7	27.4	17.3	15.0	26.1	20.5
phenyl	C	6.6	18.6	27.2	12.0	11.0	37.4	33.1
benzyl	D		15.8	19.6	12.0	15.6	21.9	26.8
phenethyl	E		15.3	19.7	13.7	15.3	19.7	27.6
propyl	F		23.9	21.8	5.7	9.1	30.6	42.6
ethyl	G		27.2	14.7	~3.5	4.7	24.9	34.4
methyl	H		40.2	13.2	3.3	4.3	26.7	33.6

[a] Solvent systems are as described in Materials and Methods.

characteristics and retention times in HPLC profiles (Tables 1 and 2). Quantitation of these products was carried out by using the external standard calibration curves constructed from the synthetic standard compounds for each substrate. Solvent systems used for HPLC analysis of reaction mixture from each substrate are as follows: System A for 2-NITC: 30% methanol/water to 68% methanol linearly in 5 min, at 68% for 27 min, to 100% linearly in 3 min; the flow rate was 1.3 mL/min and detection was at 254 or 286 nm. System B for 1-NITC: 30% methanol/water to 70% methanol linearly in 5 min, at 70% for 25 min, to 100% linearly in 3 min; the flow rate was 1 mL/min and detection was at 284 nm. System C for PITC: 40% acetonitrile/water to 44% acetonitrile linearly in 10 min, at 44% for 30 min, to 100% linearly in 6 min; the flow rate was 1 mL/min and detection was at 290 nm. System D for BITC: 30% acetonitrile/water to 51% acetonitrile linearly in 5 min, at 51% for 20 min, to 100% linearly in 5 min; the flow rate was 1 mL/min and the detection was at 286 nm. System E for PEITC: 30% acetonitrile/water to 53% acetonitrile linearly in 5 min, at 53% for 25 min, to 100% linearly in 5 min; the flow rate was 1 mL/min and detection was at 288 nm. System F for PrITC: 30% acetonitrile/water to 40% acetonitrile linearly in 10 min, at 40% for 25 min, to 100% linearly in 6 min, at 100% for 3 min; the flow rate was 1 mL/min and detection was at 286 nm. System G for EITC: 30% acetonitrile/water to 37% acetonitrile linearly in 5 min, at 37% for 25 min, to 100% linearly in 7 min; the flow rate was 1 mL/min and detection was at 286 nm. System H for MITC: 10% acetonitrile/water to 34% acetonitrile linearly in 5 min, at 34% for 30 min, to 100% linearly in 7 min, at 100% for 2 min; the flow rate was 1 mL/min and detection was at 286 nm. Data were analyzed statistically by Student's *t*-test to evaluate differences between the untreated group and treated groups in the comparative enzyme induction studies. Probability values ≤ 0.05 were considered significant.

Results

Quantitation of Products from Metabolism of 2-NITC, 1-NITC, and BITC by Liver Microsomes from Untreated and Inducer-Treated Rats Fed Semisynthetic AIN 76A Diet without Ethoxyquin or a Conventional Grain Diet. For the sake of comparison, the published data from 2- and 1-NITC and BITC metabolized by microsomes from rats fed conventional grain diet (*30*) are shown as part of Table 3. 1-NITC and 2-NITC were metabolized to comparable extent and the enzymes responsible for their metabolism were Aroclor-inducible; the metabolism of BITC was much lower than that of the other two compounds, and the microsomes from Aroclor-treated rats did not increase the production of BFU (*30*). Further studies now showed that although the side products, **IV** in Scheme 1, did form from chemical reactions of 1-NITC or BITC with 2-AF during the 30 min incubations, their possible maximal contributions to the mixed urea products (1-NFU and BFU) were estimated to be <5% when 0.2 μmol of the mixed thioureas (**IV**) (possible maximal amount of side product generated in the metabolic incubation mixtures) were incubated with NADPH and microsomes in the similar manner.

Comparative induction studies were carried out with 4 inducers using rats fed semisynthetic diet AIN 76A

Table 3. Metabolic Formation of Isocyanates from Isothiocyanates in the Presence of 2-AF by Induced or Uninduced Liver Microsomes from Rats Fed Conventional Grain Diet or AIN 76A Diet without Ethoxyquin[a]

source of microsomes: inducer (no. of rats)	diet	incubation conditions	product (nmol/0.4 mg of protein/30 min) 2-NFU	1-NFU	BFU
none (2)	conventional grain[b]	complete system	<0.1	1.17, 1.15	2.10, 2.14
		−NADPH	<0.1	1.12, 0.38	0.65, 0.61
none (4)	AIN 76A[c]	complete system	1.19 ± 0.29	1.11 ± 0.10	1.39 ± 0.10
		−NADPH	0.38 ± 0.32	0.26 ± 0.08	0.32 ± 0.15
Aroclor (3)	conventional grain[b]	complete system	11.60 ± 2.73	11.65 ± 1.65	1.95 ± 0.17
		−NADPH	0.07 ± 0.12	0.27 ± 0.22	0.35 ± 0.08
Aroclor (3)	AIN 76A[c]	complete system	9.07 ± 0.83[d]	11.12 ± 1.15[f]	2.30 ± 0.35[g]
		−NADPH	0.26 ± 0.28	0.36 ± 0.19	0.26 ± 0.13
β-naphthoflavone (3)	AIN 76A[c]	complete system	3.36 ± 0.14[d]	5.50 ± 0.85[e]	1.17 ± 0.14
		−NADPH	0.21 ± 0.20	0.27 ± 0.05	0.29 ± 0.14
isosafrole (3)	AIN 76A[c]	complete system	2.65 ± 0.70[e]	1.88 ± 0.30[g]	1.58 ± 0.22
		−NADPH	0.31 ± 0.36	0.22 ± 0.05	0.31 ± 0.02
phenobarbital (3)	AIN 76A[c]	complete system	3.57 ± 0.58[d]	2.78 ± 0.07[d]	1.85 ± 0.17[f]
		−NADPH	0.34 ± 0.41	0.24 ± 0.07	0.32 ± 0.05

[a] Complete system consisted of 0.5 μmol of substrate, 0.4 mg of microsomal protein, 5 μmol of NADPH, and 0.2 μmol of 2-AF in 1 mL of solution. 2-NFU, 1-NFU, and BFU are products from metabolic conversion of 2-NITC, 1-NITC, and BITC, respectively. [b] Incubation mixtures of each substrate with microsomes from rats fed the conventional grain diet were analyzed by HPLC. Data are mean ± standard deviation obtained from microsomes from 3 rats or the numbers from microsomes from 2 rats (*30*). [c] Incubation mixtures of each substrate with microsomes from rats fed AIN 76A without ethoxyquin were analyzed by HPLC using systems A, B, and D, respectively, as described in Materials and Methods. Data are mean ± standard deviation obtained from microsomes from 3 rats. [d] The values are significantly higher than the uninduced control values, since the p values of the Student's t-test on the values of the complete system are <0.0008.[e] p <0.013. [f] p <0.006. [g] $p \leq 0.05$.

without ethoxyquin. As shown in Table 3, all four inducing agents significantly increased the formation of 2-NFU from metabolism of 2-NITC in the order of Aroclor ≫ phenobarbital ≥ β-naphthoflavone > isosafrole under the present protocol. The formation of 1-NFU from the metabolism of 1-NITC was also increased by the four inducers in the order of Aroclor ≫ β-naphthoflavone > phenobarbital > isosafrole. Aroclor increased the production of BFU from the metabolism of BITC ~2-fold, and only a slight increase was observed with the phenobarbital-induced microsomes. Treatment of these rats with β-naphthoflavone or isosafrole did not increase the production of BFU. Consistent with experiments with 2-NITC (*29*), BFU formation from the metabolism of BITC was inhibited by CO ($CO:O_2 = 80:20$) by ~40% as compared to the experiments carried out in air when Aroclor-induced microsomes were used (data not shown).

Quantitation of Products from Comparative Metabolic Conversion of Isothiocyanates to Isocyanates. Since Aroclor was the best inducer so far tested under the current induction protocol and the metabolic conversion assay procedure, the pooled microsomes from 6 Aroclor-treated rats fed conventional grain diet were used to study the metabolic potentials of 1-NITC, PITC, BITC, PEITC, PrITC, EITC, and MITC. The time course was from 0 up to 30 or 60 min using the procedure described in Materials and Methods. Figure 1 shows the results from 1-NITC as an example. The linear velocity in the complete reaction reached maximum at 5 min in all cases. The controls without NADPH showed very little or no activity. Table 4 shows the comparative metabolic conversion of these 7 compounds based on the initial linear rates (5 min) of the formation of the ureas (**III**) as a measure of the extent of isocyanate metabolite formation. Metabolic potentials followed the order of 1-NITC > PITC > BITC, PEIT > PrITC, EITC, and MITC. In the case of MITC, the HPLC profile was more complicated and the urea (MFU) formation was so little that the result from only one good HPLC run was shown in Table 4.

Possible Contribution of Ureas (III) due to the Formation of the Side Product Thioureas (IV) in the Comparative Metabolic Conversion of Isothiocyanates to Isocyanates. Except for the 3 aliphatic isothiocyanates, at the end of 5 min incubations, some side product (**IV**) did form when NADPH and microsomes were omitted from the complete metabolic incubation mixtures (~105 nmol from 1-NITC, ~15 nmol from PITC, BITC, and PEITC, and none from PrITC, EITC, and

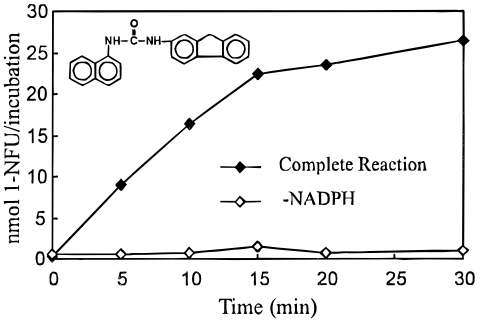


Figure 1. Time course of metabolic conversion of 1-naphthyl isothiocyanate. Metabolic conversion of 1-NITC to 1-naphthyl isocyanate; trapping by 2-AF to form 1-NFU and HPLC analysis of 1-NFU was as described in Materials and Methods.

Table 4. Comparative Reactivities of Metabolic Conversion of Isothiocyanates to Isocyanates[a]

substrates (0.5 mM)	2-AF-derived urea product RNHCONHF (**III**)	nmol of product/(mg of protein·min) complete reaction	−NADPH control
1-NITC	1-NFU	2.27 ± 0.06[b]	0.15 ± 0.02
PITC	PFU	1.26 ± 0.20	~0.12[c]
BITC	BFU	0.82 ± 0.06	0.04 ± 0.005
PEITC	PEFU	0.73 ± 0.02	0.03 ± 0.005
PrITC	PrFU	0.07 ± 0.03	~0.01[c]
EITC	EFU	0.13[d]	0 ± 0
MITC	MFU	0.03[e]	0[e]

[a] The reactivities are based on initial linear reaction rates at 5 min for the conversion of isothiocyanates to isocyanates with liver microsomes from Aroclor-treated rats. The metabolites were trapped by formation of ureas with 2-aminofluorene (0.2 mM). [b] Data are from HPLC analysis of the 2-AF-derived ureas at 5 min using solvent systems as described in Materials and Methods. Standard deviations are shown for data from 3 or more samples. [c] This is taken from the −NADPH control time course curve at 5 min. [d] This is the average result from 2 samples. [e] This is the result from 1 sample.

Table 5. Effects of CO and Heating of Microsomes on the Production of RNHCONHF (III) from Metabolism of Isothiocyanates[a]

	RNHCONHF (%)			
isothiocyanate	N_2:O_2 = 9:1	CO:O_2 = 9:1	unheated microsomes	heated microsomes
1-NITC	100	40 ± 2	100	96 ± 5
PITC	100	39 ± 4	100	95 ± 3
BITC	100	47 ± 3	100	100 ± 12
PEITC	100	29 ± 11	100	112 ± 8
EITC	100	51 ± 2	100	106, 91

[a] Experiments were carried out using liver microsomes from Aroclor-treated rats as described in Materials and Methods. Results of CO inhibition in the atmosphere of CO:O_2 = 9:1 were compared with that from the controls in N_2:O_2 = 9:1. Results from heated microsomes were compared with those from unheated microsomes as controls. Data are from triplicates (with standard deviations) or from duplicates.

MITC). These quantities of synthetic thioureas from these four aryl and arylalkyl isothiocyanates were enzymatically incubated in the same manner as for the isothiocyanates and the urea (**III**) products analyzed. These results were compared with the amounts of urea (**III**) obtained from enzymatic incubation of isothiocyanates in each case, and their contributions are no more than 4.6−7.2%.

Inhibition Studies. As shown in Table 5, the metabolic conversion of these isothiocyanates (PrITC and MITC were not studied due to their low metabolic potentials) was inhibited by CO, and heating of microsomes did not diminish the product formation.

Discussion

Aroclor 1254 is known to induce substantial amounts of P450 1A1, 1A2, 2A1, 2B1, and 2B2 (*38*). In the initial study of the oxidative desulfuration of 2-NITC to 2-naphthyl isocyanate (*29*), liver microsomes used were prepared from rats fed a conventional grain diet before and during the Aroclor induction period. There was an increase in the production of 2-NFU of ∼10-fold with the induced microsomes. Extension of this metabolic conversion study to 1-NITC and BITC using microsomes from rats fed conventional grain diet (*30*; part of Table 3) suggested that Aroclor-induced enzymes increased metabolism of both 1-NITC and 2-NITC but was without effect on BITC. It was reasoned that some component(s) of the grain diet might have acted as an inducer(s) in the untreated animals, and since the metabolism of BITC was low, that any additional induction effect due to Aroclor was not manifested in the treated animals. Alternatively, another enzyme system, such as flavin-containing monooxygenase, might be responsible for the metabolism of BITC. The purpose of the present comparative induction study was threefold: The first was to find out if the enzyme(s) for metabolic conversion of BITC was inducible; the second was to gain insight into which enzyme(s) is most likely responsible for the metabolic conversion of these compounds; the third was to find a good inducer to screen for the metabolic potentials of more isothiocyanates. To minimize the possible induction effects on the control animals from the conventional grain diet, the animals were fed semisynthetic AIN 76A diet that had been prepared without ethoxyquin, an agent which induces P450 2B1, 2B2, 1A2, and 3A2 (*31*), that is usually added to serve as an antioxidant to protect the corn oil of the diet.

Results of BITC metabolism by microsomes from rats fed AIN 76A diet without antioxidant show that, indeed, formation of BFU from the uninduced microsomes decreased as compared to that from rats fed conventional grain diet and the use of the Aroclor-induced microsomes now gave approximately twice the amount of BFU as compared to that of the uninduced control. Phenobarbital-induced microsomes also slightly increased the formation of BFU, suggesting that P450 2B1 and 2B2 may be involved in the metabolism of BITC. This is consistent with the finding that the microsomes from the untreated rat fed the conventional grain diet gave the same amount of BFU as that treated with Aroclor (see Table 3), since ethoxyquin in the diet served as inducing agent for P450 2B1 and 2B2 (*31*) and probably P450 3A2 induced by the same agent may also catalyze the metabolic conversion of BITC. These data suggest that Aroclor was the only effective inducer for the metabolism of BITC and feeding animals with semisynthetic diets may be required in future studies where the identification of inducing agents capable of increasing the metabolism of isothiocyanates is sought. Results from this study have established that metabolism of BITC is mediated by P450(s) and not by the flavin-containing monooxygenase.

β-Naphthoflavone, isosafrole, and phenobarbital are known to induce primarily P450 1A1, P450 1A2, and P450 2B1 and 2B2, respectively (*38*). As shown in Table 3, the formation of 1-NFU and 2-NFU from Aroclor-induced microsomes may involve all of these P450s, since each of these agents increased the metabolism of 1-NITC and 2-NITC. P450 1A1 may be more important for metabolism of 1-NITC than for 2-NITC, since β-naphthoflavone-induced microsomes gave higher amount of 1-NFU than 2-NFU.

The experimental approach of trapping the reactive isocyanate metabolites with 2-AF is a complex chemical system and, although useful, is not perfect, and cannot serve as a measure of the absolute activity of the microsomal proteins toward these isothiocyanate substrates since there are factors that may affect the estimation of the total amount of the isocyanate metabolites formed. First, hydrolysis of isocyanates leads to the formation of symmetrically disubstituted ureas (**I**) and thioureas (**II**) in the cases of 2-NITC and 1-NITC, which occurred even in the presence of 2-AF. However, in the absence of 2-AF, neither dibenzylurea nor dibenzylthiourea was detected in the conversion of BITC to its isocyanate. This is due to their low molar extinction coefficients (this applies to all arylalkyl and aliphatic isothiocyanates). Therefore, for comparison purposes, this trapping method was adopted in order to be able to detect benzyl isocyanate formed in the metabolism of BITC as BFU. Second, 2-AF is also known to be the substrate for rat liver microsomes and purified P450s from rats treated with β-naphthoflavone, isosafrole, and phenobarbital (*39*). Hence, the trapping agent, 2-AF (0.2 mM, with the maximal trapping capability in the ranges of 0.05−0.6 mM of 2-AF tested in 1-NITC metabolism using Aroclor-induced microsomes), may compete for P450 with the isothiocyanate substrates. Since isosafrole-induced P450 (1A2) had the highest activity in the metabolism of 2-AF (*38*), the activity toward the isothiocyanates may be more affected in this case. Another factor that may reduce the activity of isosafrole-induced microsomes is the formation of a P450 complex with the metabolites of isosafrole causing the partial loss of the

enzyme activity (*40*). Removal of the metabolites of isosafrole from possible P450 complexes was not attempted in the present study. Furthermore, 2- and 1-aminonaphthalenes may compete for enzymes once they are formed in the incubation mixtures of 2- and 1-NITC through hydrolysis of isocyanate metabolites, since these amines are also substrates for P450s induced by these agents, particularly, β-naphthoflavone and isosafrole (*39*).

Results from the comparative metabolic conversion studies using microsomes from Aroclor-treated rats fed conventional grain diet (Table 4) reveal that the highly aromatic 1-NITC had the highest metabolic potential followed by the monocyclic aromatic PITC, the metabolic activities were futher diminished for BITC and PEITC, and aliphatic PrITC, EITC, and MITC were only very weakly metabolized. However, the low reactivity of the aliphatic isothiocyanates may be partly due to the relatively volatile nature of their isocyanate metabolites, possible decomposition in the incubations, and more importantly, the major enzymes required for the metabolic conversion of these compounds may not be present in the Aroclor-induced microsomes used in these experiments. Metabolic potentials of isothiocyanates will have to be explored further with other families of P450 enzymes. Experiments using reconstituted specific P450 enzymes and/or specific antibodies against P450 enzymes are required to identify the specific enzymes involved in the metabolism of these compounds.

Although the formation of side products, RNHCSNHF (**IV**), increased from aliphatic isothiocyanates (none) to the highly aromatic naphthyl isothiocyanates during the incubations, the rates of conversion of these side products to the ureas RNHCONHF (**III**) had a reverse order.[2] Therefore, these ureas detected as a rough measure of metabolic activity were judged to come primarily from the reaction of 2-AF and the isocyanate metabolites and not from the direct metabolic conversion of these thiourea side products (**IV**). The oxidative desulfuration of these disubstituted thioureas is consistent with the report that the relatively nontoxic diarylthioureas (diphenyl and 4-hydroxydiphenyl) desulfurated slightly *in vivo*, whereas the monoarylthioureas desulfurated to a considerable extent (*41*).

The conversion of isothiocyanates to isocyanates is mediated by P450 enzymes and not by the flavin-containing monooxygenase, since these reactions are inhibitable by CO and heating of microsomes did not decrease the product formations (Table 5). The mechanism of conversion of RN=C=S to RN=C=O is presumably the same as that proposed for the conversion of CS_2 to CSO (*42*). It was proposed that the enzyme-bound singlet oxygen atom reacts with one of the unshared electron pairs of a sulfur. This is followed by a rearrangement to form a hypothetical cyclic CSO intermediate which then rearranges to release sulfur to form carbonyl sulfide. The sulfur atom thus released is very reactive and can bind to P450 protein covalently to cause the loss of enzyme activity. This was proved by the excess binding of ^{35}S over ^{14}C radioactivity to protein using ^{14}C- and ^{35}S-labeled CS_2 (*43*).

Several isothiocyanates have been found to inhibit the carcinogenicity of a wide variety of agents in animal models as mentioned in the Introduction. In recent years, the mechanism of the inhibition of chemical carcinogenesis by isothiocyanates has been extensively studied with nitrosamines (reviewed in refs 44−46). The inhibitions of tumorigenicity and DNA adduct formation of these procarcinogens are due to the chemical inactivation and competitive inhibition of the reqired P450 enzymes. This is consistent with the fact that isothiocyanates can react with protein and may be metabolized by P450 enzymes as described in this report, thus, competing for the activation enzymes required by the carcinogens. However, the possible genotoxic consequences of the activation of isothiocyanates by P450's remain largely unexplored.

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[2] Unpublished observations.

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