MOLECULAR PHARMACOLOGY, 16, 1019–1030

Aerobic Metabolism of Niridazole by Rat Liver Microsomes

JEFFREY L. BLUMER,[1] RAYMOND F. NOVAK, SAMUEL V. LUCAS,[2] JANNEY M. SIMPSON AND LESLIE T. WEBSTER, JR.

Department of Pharmacology, Northwestern University Medical School, Chicago, Illinois 60611 and the Department of Pharmacology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

(Received February 26, 1979)
(Accepted July 10, 1979)

SUMMARY

BLUMER, JEFFREY L., RAYMOND F. NOVAK, SAMUEL V. LUCAS, JANNEY M. SIMPSON, AND LESLIE T. WEBSTER, JR. Aerobic metabolism of niridazole by rat livers microsomes. *Mol. Pharmacol.* **16**, 1019–1030, (1979).

The metabolism of the anthelmintic nitroheterocyclic compound niridazole by rat liver was studied under aerobic conditions *in vitro*. Metabolism was localized to the microsomal fraction. Four metabolites of niridazole were isolated from these microsomal systems by high pressure liquid chromatography. The metabolites were characterized by mass spectrometry and ^{1}H Fourier transform nuclear magnetic resonance spectroscopy as the 4-hydroxy, 5-hydroxy, 4,5-dihydroxy and the 4,5-dehydro derivatives of the imidazolidinone ring; the nitrothiazole portion of niridazole apparently was unaltered. The aerobic metabolism was shown to require O_2 and exogenous reducing equivalents (NADPH was more effective than NADH), and to be inhibited by CO, suggesting a cytochrome P-450 catalyzed reaction. Inducers of microsomal cytochrome P-450, phenobarbital and 3-methylcholanthrene, were found to enhance niridazole metabolism. Metabolite profiles varied quantitatively with different inducers. Experiments with individual aerobic metabolites showed precursor-product relationships that are consistent with formation of an epoxide intermediate in the overall metabolic sequence.

INTRODUCTION

Despite major advances in medical diagnostics and therapeutics, parasitic diseases remain a major health problem. It is estimated that almost half of the world's population is infected with various helminthic parasites. During the past two decades the nitroheterocycle NDZ[3] (Fig. 1) has been used to treat helminth infections in more than 200,000 people throughout the world. Although the clinical effectiveness of the drug has been established, very little is known concerning its metabolism.

Early studies of NDZ biodisposition in animals and man showed the drug to be extensively metabolized (1). Following oral administration of the ^{14}C-labeled compound, little unchanged drug appeared in the blood or other tissues, whereas radioactivity in the plasma ascribed to polar NDZ metabolites reached levels up to 50

This research was supported in part by grants from the Edna McConnell Clark Foundation and the Rockefeller Foundation to L. T. Webster Jr.

[1] Present address: Department of Pediatrics, Case Western Reserve University, Cleveland, Ohio 44106.

[2] Present address: Battelle Columbus Laboratories, Columbus, Ohio 43201.

[3] Abbreviations used are: DSS, sodium 2,2-dimethyl-2-silapentane sulfonate; NDZ, niridazole, 1(5-nitro-2-thiazolyl)-2-imidazolidinone; PB, phenobarbital; MC, 3-methylcholanthrene; HPLC, high pressure liquid chromatography; ^{1}H FT NMR, ^{1}H Fourier transform nuclear magnetic resonance spectroscopy; TMS, tetramethylsilane.

0026-895X/79/061019-12$02.00/0

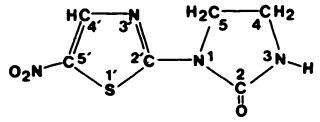


FIG. 1. *Niridazole 1 (5'-nitro-2'-thiazolyl)-2-imidazolidinone*

times those of the parent compound (1). These investigators also incubated various animal tissue homogenates with NDZ under both aerobic and anaerobic conditions. Although no specific reaction products were isolated, they concluded that NDZ was metabolized primarily in the liver via an O_2 inhibited reductive pathway (1). Gillette proposed a nitroreductase pathway to explain these observations and later he and his co-workers characterized NDZ nitroreductase activity in the microsomal and soluble fractions of rat liver homogenates. Nitroreductase activity in microsomes was attributed to NADPH-cytochrome c reductase (2), while that in the soluble fraction was found to be catalyzed by xanthine oxidase (3). In both subcellular fractions the reaction proceeded only under anaerobic conditions and the only metabolite isolated was shown by mass spectral analysis to be the hydroxylamine. However, because this metabolite readily autooxidized back to the parent nitro compound upon exposure to atmospheric oxygen (3), it is unlikely that it contributed significantly to the polar metabolites detected by Faigle and Keberle (1).

The present study was designed to evaluate the metabolism of NDZ under aerobic conditions. We report here the isolation and identification of four NDZ metabolites formed by rat liver microsomal systems under aerobic conditions and describe the role of liver microsomal cytochrome P-450 in their formation.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (80–100 g) were obtained from Holtzman and acclimated for at least one week prior to use.

Chemicals. All solvents employed were ACS reagent grade. Acetonitrile, ethyl acetate, and 4 Å molecular sieves were obtained from Fischer Scientific, Co. NADPH (Type I), β-NADH and 3-methylcholanthrene were purchased from Sigma Chemical Co. Acetone-d_6 and ^2H_2O, both nominally 100% in isotopic purity, were obtained from Aldrich; and TMS and DSS were supplied by Stohler Isotope Chemicals.

We thank Ciba Geigy Corp. for donating niridazole (Ambilhar) and niridazole-4-^{14}C (labeled in the 4 position of the imidazolidinone ring) 3.4 μCi/mg.

Treatment with inducers and preparation of liver microsomes. PB was dissolved in normal saline and rats were injected i.p. with 80 mg/kg/day for three days prior to killing. MC was dissolved in corn oil and rats received 80 mg/kg i.p. 24 hours prior to preparation of microsomes. Rats were killed between 6 and 8 a.m. and livers were removed and placed immediately into ice cold 0.25 M sucrose. Fractionation of rat liver homogenate was performed as described by Mazel (4). Microsomal fractions for routine metabolic assays were prepared as described previously (5). Protein concentrations were determined by the method of Lowry *et al.* (6) with bovine serum albumin used as the standard. The specific content of microsomal cytochrome P-450 was determined with a DW-2 UV-Vis spectrophotometer by the method of Omura and Sato (7).

Enzyme Assay. Unless otherwise specified, NDZ metabolism was assayed in a total volume of 1 ml containing 20 μmol K-phosphate buffer, pH 7.4, 0.25 μmol NDZ, 1 μmol NADPH and 0.6 mg of microsomal protein. After preincubation at 37° for 3 min, reactions were initiated by adding the NADPH and were terminated at 10 min by the addition of 2 ml ice cold ethyl acetate. Samples were extracted twice with 2 volumes of ethyl acetate for 1 min each time and then centrifuged for 5 min at top speed in a clinical centrifuge. The organic layers were withdrawn and filtered (0.4 μ teflon filters, Millipore, FHLB-01300); the filters were washed with 2 ml ethyl acetate and the combined ethyl acetate extracts were evaporated to dryness under reduced pressure. Residues were dissolved in 200 μl of HPLC column elution solvent and aliquots, generally 25 μl were injected for HPLC. For preparation of metabolites for ^1H FT NMR

and mass spectral analysis, reaction mixture volumes were increased 10–20 fold but the rest of the procedure was identical.

Chromatography and metabolite isolation. HPLC was performed with a Waters Associates model ALC/GPC 201 liquid chromatograph. Detection of NDZ and its metabolites was accomplished with a Schoeffel model 770 spectrophotometric detector set at 360 nm. All chromatography was performed isocratically on a Waters Associates μ-Bondpack/C_{18} column (4 mm ID × 30 cm) at a solvent flow rate of 1 ml per min of an acetonitrile:water elution solvent ranging from 18 to 33% acetonitrile. Samples for UV spectroscopy, mass spectrometry and ^1H FT NMR spectroscopy were prepared from purified fractions collected from the HPLC system. Samples for analysis were rechromatographed to insure purity.

NMR spectroscopy. ^1H FT NMR spectra of niridazole and metabolites were recorded on a Varian CFT-20 spectrometer operating at 80 MHz and ambient temperature. The instrument was locked internally on the deuterium signal of the acetone-d_6 or ^2H_2O solvent. Molecular sieves (4 Å) were utilized for water removal from the samples and acetone-d_6 solvent. Either 5 or 1.7 mm NMR sample tubes were used and sample concentrations ranged from 1 mM to 10 μM. Chemical shifts were measured relative to TMS or DSS in the acetone-d^6 and ^2H_2O solvents, respectively. Proton homonuclear decoupling experiments were performed with a Hewlett Packard model 3320 frequency synthesizer interfaced to the Varian CFT-20 computer.

Mass spectrometry. Mass spectra were obtained with Finnigan quadripole mass spectrometers: a model 3200E was used for chemical ionization (methane reagent gas at approximately 0.7 torr) and a model 3000 for electron impact ionization at an ionizing energy of 70 eV. Samples were introduced directly into the mass spectrometer ion source via a variable temperature solid sample insertion probe. Both instruments were interfaced to a Systems Industries model System/150 mass spectrometer data system.

Metabolite extinction coefficient. Because none of the metabolites of NDZ were isolated in sufficient quantity for accurate gravimetric analysis, their extinction coefficients were determined indirectly as follows: NDZ-4-^{14}C was incubated with microsomal protein and the radioactive metabolites were isolated by HPLC (see above). Each metabolite was then mixed with an amount of NDZ-4-^{14}C such that both the NDZ and metabolite peaks were on scale at the same detector response attenuation. Both the metabolite and NDZ fractions were rechromatographed by HPLC, collected, diluted to a reference volume, and the optical densities at 360 nm determined with a Gilford model 2400 spectrophotometer. An aliquot of each fraction was also counted on a Packard Tri Carb liquid scintillation counter with an internal standard used for quench correction. The calculation was as follows:

$$\epsilon \text{ mtb.}/\epsilon \text{ NDZ} = \frac{\text{Abs. (mtb)/DPM (mtb)}}{\text{Abs. (NDZ)/DPM (NDZ)}}$$

where Abs. (mtb) and Abs. (NDZ) are the absorbance at 360 nm of the metabolite and NDZ, respectively, and DPM (mtb) and DPM (NDZ) are ^{14}C disintegrations per minute of equal aliquots of the metabolite and NDZ fractions, respectively. The absolute extinction, $\epsilon_{360\text{ nm}}$, of niridazole was easily determined by dry weight analysis. Hence, the above relationship permitted the indirect determination of the metabolite extinction coefficients because the specific radioactivity of NDZ and all metabolites retaining the ^{14}C-labeled C-4 carbon should be identical. The extinction coefficients, $\epsilon_{360}^{\text{mM}^{-1}}$, employed for metabolite quantitation were 14.0, 13.0, 12.3, 10.4 and 10.7 for NDZ, 4-OH NDZ, 5-OH NDZ, 4,5-glycol and 4,5-dehydro NDZ respectively.

Metabolite quantitation. Metabolites of niridazole were quantitated from HPLC chromatograms monitored at 360 nm. Peak areas of the metabolites were determined by triangulation, and the quantity (pmol) of metabolite corresponding to the chromatographic peak was determined by the equation:

$$\text{pmol} = \frac{\text{Area}}{\epsilon_{360\text{ nm}}} \times 10^{-9}$$

where the area of the peak is expressed in absorbance × ml units and $\epsilon_{360\,nm}$ is the molar extinction coefficient at 360 nm. With this method as little as 5 pmol of NDZ or NDZ metabolite were reproducibly measured.

RESULTS

HPLC separation and identification of NDZ and its aerobic metabolites. Figure 2 shows a high pressure liquid chromatographic analysis of the ethyl acetate extract from a NDZ microsomal incubation. The metabolites have been arbitrarily designated 1 through 4. With the extraction procedure described above, NDZ was routinely recovered from microsomal reaction mixtures in yields of 92% or greater (*i.e.* no more than 8% was metabolized), NDZ and its metabolites were not detected in the aqueous phase following two ethyl acetate extractions.

HPLC purified metabolites were identified by combined mass spectra and ^{1}H FT NMR analysis.[4] Chemical ionization mass spectra of NDZ and the four metabolites gave quasi-molecular ions with the correct masses corresponding to the addition of one oxygen atom for Metabolites 1 and 2, the addition of two oxygen atoms for Metabolite 3 and the loss of two hydrogen atoms for Metabolite 4, relative to NDZ. In each case the metabolic alteration occurred at the C-4 and/or C-5 positions of the imidazolidinone ring (see corroborative ^{1}H FT NMR data below). Features of the mass spectra which aided metabolite identification are summarized in Table 1.

The electron impact mass spectrum of NDZ shows a base peak at m/Z 158 (see Table 1 for fragmentation scheme). Feller *et al.* (2) have reported a partial mass spectrum of the monotrimethylsilyl derivative of NDZ in which the analogue of the ion at m/Z 158 was a prominent ion. The 70 eV electron impact mass spectra of the niridazole metabolites reported here (Table 1) are in excellent agreement with what one would expect for a fragmentation pathway analogous to that proposed for NDZ, and they lend strong support to the structural assignments deduced from the ^{1}H FT NMR data (see below).

A representative ^{1}H FT NMR spectrum of NDZ in acetone-d_6 is shown in Figure 3. The signal occurring δ = 8.39 ppm downfield from TMS (δ = 0.0 ppm) was readily ascribable to the proton on the 4′ carbon of the nitrothiazole ring because it occurred in the region for aromatic protons (8) and displayed no coupling. The broad signal at $\delta \cong$ 7.0 ppm (Fig. 3) may be assigned to the amide proton (3-N*H*) in the imidazolidinone ring. The position of the N*H* signal and linewidth at half-maximal intensity may vary substantially however, depending upon the NDZ concentration, solvent composition and the concentration of residual water. The two sets of triplets centered at 4.27 and 3.75 ppm were assigned to the

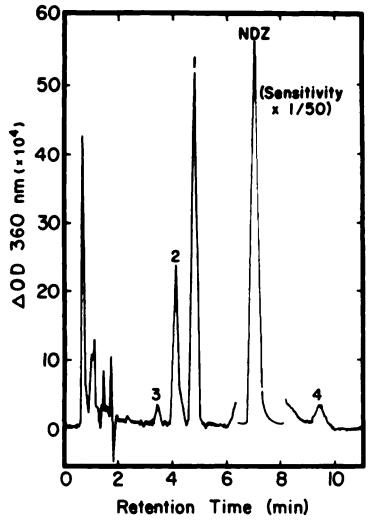


FIG. 2. *HPLC chromatogram of an ethyl acetate extract of a microsomal reaction mixture*

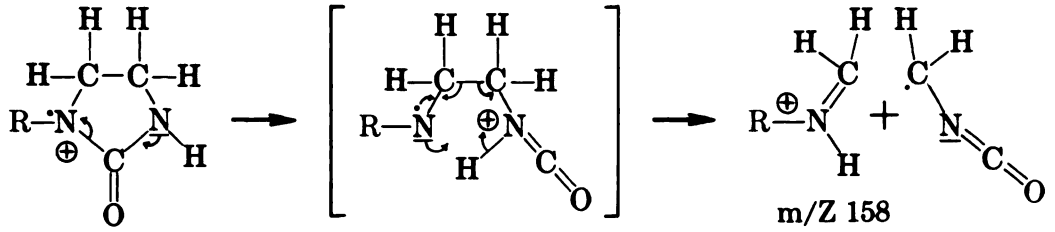
Rat liver microsomes were incubated in 20 mM K phosphate buffer, pH 7.4, with NDZ and NADPH, extracted with ethyl acetate and extracts chromatographed by HPLC, as described under MATERIALS AND METHODS. Peaks designated 1–4 correspond to Metabolites 1–4; the peak due to NDZ is presented at an attenuation of 1/50 compared to the metabolite peaks. With an elution solvent composed of 18% acetonitrile, k′ values were 6.2, 8.2, 9.4, 13.9 and 20.0 for Metabolite 3,2,1 NDZ and Metabolite 4, respectively. Peaks which appeared before the solvent front (biphasic peak) were variable in amplitude and were observed in microsomal extracts in the absence of NDZ; they were not characterized further.

[4] A complete set of chemical ionization and electron impact mass spectra and 80 MHz ^{1}H FT NMR spectra are available from the authors on request.

TABLE 1

Relevant Ions in the Mass Spectra of Niridazole and its Metabolites

Niridazole and its metabolites were purified from microsomal systems by HPLC. Following rechromatography of the individually isolated peaks, the samples were evaporated under reduced pressure onto a solid probe cartridge. In most instances the samples were first analyzed by ^{1}H FT NMR spectroscopy and then retrieved, divided in half and evaporated for mass spectral analysis. The electron impact mass spectrum shows a base peak at m/Z 158 which probably results from decomposition of the molecule ion by the scheme



Designation	Metabolic alteration	Electron impact		Chemical Ionization
		Base Peak	Molecule Ion	Quasi-Molecule Ion
		(m/Z)	*(m/Z)*	*(m/Z)*
NDZ	None	158	214	215
MTB 1	4-Hydroxy	158	230	231
MTB 2	5-Hydroxy	174	230	231
MTB 3	4,5-Dihydroxy	174	246	247
MTB 4	4,5-Dehydro	94	212	213

methylene groups at the C-5 and C-4 positions, respectively, of the imidazolidinone ring. These assignments are supported by the work of Vail *et al.* (9) who reported a chemical shift of 3.51 ppm for the methylene protons of 2-imidazolidinone in ^2H_2O, and the electron withdrawing influence of the nitrothiazole ring of NDZ should result in a greater deshielding of the C-5 protons as compared to the C-4 protons. This conclusion is supported by spectral data for other nitro-containing nitrogen heterocycles (pyrazole, triazole, etc.) (9). In these cases, the nitro-containing substituent produces a downfield shift in the signal of protons adjacent to the N-atom to which the substituent is attached.

Characteristic changes in the ^{1}H FT NMR spectrum of NDZ (Fig. 3) resulting from metabolic alteration of the molecule are listed in the legend to Figure 3. In each case the spectrum was in complete agreement with that predicted by mass spectral analysis (see above), and confirmed by selective homonuclear decoupling experiments.[4] Thus substitutions at the C-4, C-5 and C-4 and C-5 positions were found for Metabolites 1, 2 and 3 respectively; characteristic doublets easily ascribable to olefinic protons characterized the spectrum for Metabolite 4. Recently, the structures of Metabolites 1 and 4 have been confirmed by mass spectral and high resolution ^{1}H FT NMR analysis of synthetic standards.[5]

Thus the combined analytical procedures described above permit the identification of Metabolites 1 through 4 as the 4-hydroxy, 5-hydroxy, 4,5-dihydroxy and the 4,5-dehydro metabolites respectively. Further investigations were directed toward the elucidation of the biological basis for their formation.

Subcellular distribution of aerobic NDZ metabolism. The aerobic metabolism NDZ was assessed in the presence of various fractions of tissue homogenate prepared from livers of untreated rats. More than 90% of the total activity was found in the microsomal fraction. The specific oxidase activity of the microsomal fraction was 3–9-fold greater (depending on the metabolite of interest) than in the whole liver homogenate. Apparent differences in the extent of purification of the various metabolite spe-

[5] Dr. E. M. Fairchild, personal communication.

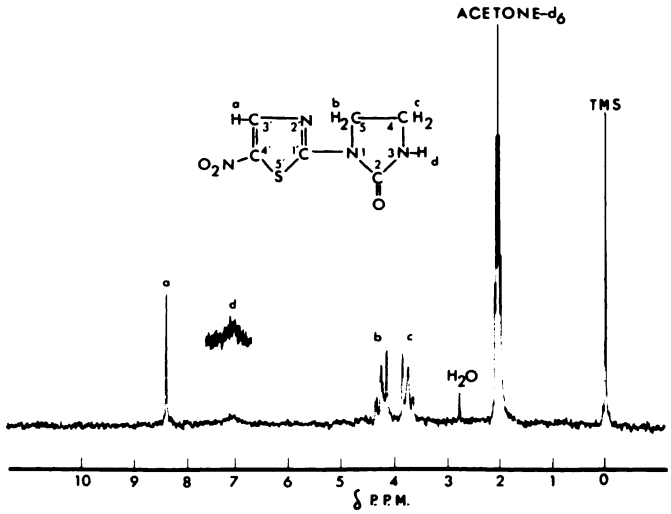


FIG. 3. *^1H NMR spectrum of ~5 mM Niridazole in Acetone-d_6*

The spectrum was recorded at 80 MHz under ambient conditions. Sweep width was 1000 Hz, acquisition time 4.095 sec, number of transients 200, pulse width 200 μsec and data words 8192. Chemical shift values were measured with respect to TMS (δ = 0.0 ppm). Signal assignments were made as described in th text. The signals designated *a* through *d* correspond to similarly labeled protons on the included structural formula. Alterations in the various signals attributed to microsomal metabolism are summarized as follows:

Designation	Metabolic alteration	Solvent	Signal δ^a (Multiplicity)			
			C4'	N-3-H	C-5	C-4
MTB 1	4-Hydroxy	A[b]	8.32(s)	N	3.91–4.47 (m)	5.60 (d)
MTB 2	5-Hydroxy	B	8.42(s)	N	6.19 (t)	N
MTB 3	4,5-Dihydroxy	A, B	8.41(s)	N	5.83 (s) (Broad)	5.27(s) (Broad)
MTB 4	4,5-Dehydro	B	8.40(s)	N	7.23–7.19 (d)	6.84–6.80 (d)

[a] Chemical shift, δ, in parts per million from TMS (acetone-d_6) or DSS (^2H_2O).

[b] Abbreviations: A, acetone-d_6; B, ^2H_2O; N, not observed; s, singlet; d, doublet; t, triplet; m, multiplet.

cific activities might be related to differences in metabolite stability, differences in the kinetics of their formation and/or differences in the rates and extents of their reutilization by microsomal enzyme systems (see below and DISCUSSION).

When NDZ-4^{14}C was employed as a substrate, either with whole liver homogenate or with the microsomal fraction, about 98% of the total radioactivity added could be recovered as NDZ plus a mixture of the four metabolites. HPLC chromatography of ethyl acetate extractable radioactivity revealed only those peaks which corresponded to NDZ or one of the metabolites. After a 10 min incubation, approximately 8% of the total radioactivity was extracted as the NDZ metabolites identified in this study.

Requirements of the microsomal NDZ metabolizing system. Table 2 lists some of the requirements for the microsomal NDZ metabolizing system. When the complete reaction mixture was incubated in an atmosphere of N_2, virtually no metabolite formation was detected, suggesting an absolute requirement for molecular oxygen. Similarly, the replacement of 90% of the O_2 by CO resulted in essentially complete inhibition of aerobic product formation. In the absence of NADPH no activity was observed, and when NADH was quantitatively substituted for NADPH, the activity was depressed 40 to 83%, depending on the metabolite considered. The findings demonstrated a requirement for exogenous reducing equivalents, which was satisfied preferentially by NADPH. Metabolite for-

TABLE 2

Requirements of the microsomal niridazole metabolizing system

The complete reaction mixture contained 0.25 μmol of NDZ, 20 μmols of K phosphate buffer pH 7.4, 0.6 mg of microsomal protein and 1 μmol of NADPH in a total volume of 1 m. Reactions were run for 10 min after initiation by the addition of reduced pyridine nucleotide. The rates of metabolite formation in the complete system were 160 and 75 pmols/min/mg microsomal protein for the 4-OH and 5-OH metabolites respectively and 74 and 271 pmol/min/ng microsomal protein for the glycol and dehydro metabolites respectively. Experimental values represent the average of duplicate determinations.

System	Glycol	5-OH	4-OH	Dehydro
		(% Activity)		
Complete	100	100	100	100
−NADPH	3	8	8	ND[a]
+CO[b]	ND	4	ND	ND
+N_2[b]	ND	6	8	11
−NADPH + NADH[c]	60	28	17	ND
Boiled[b] microsomes	ND	3	ND	ND

[a] ND—Not detectable.

[b] Reactions in which the atmosphere was CO:O_2 (9:1) or N_2 were carried out in Thunberg tubes. These were alternatively evacuated and flushed with either CO:O_2 or N_2 three times before the NADPH was tipped in from the side arm to initiate the reaction.

[c] NADH was added to achieve a final concentration of 1 mM.

[d] Samples of microsomal protein were immersed in boiling water for 5 min and then cooled to room temperature.

mation appeared to be enzymatic because no metabolism was observed when heat inactivated microsomal protein was substituted in the complete system.

The time courses for the formation of the four NDZ metabolites are depicted in Figs. 4A and 4B. Formation of the 4-OH and 5-OH NDZ metabolites was linear with time up to 10 min. The initial rate of formation of the 4-OH metabolite was approximately twice that of the 5-OH NDZ metabolite and formation of both products continued to increase with time for at least 45 min without reaching a plateau.

In contrast, formation of the glycol and dehydro metabolites was detectable only after an apparent lag phase of about two minutes (Fig. 4B). From 15 to 30 min the rates of formation of both glycol and dehydro metabolites appeared to be nearly linear with time and at all times the amount of dehydro NDZ exceeded the amount of glycol.

The dependence of the rate of product formation on pH is depicted in Figure 5 for the 4-OH, 5-OH and glycol metabolites. The rate of formation of the dehydro metabolite varied little (~25%) over a pH range of 6.2 to 9.4. The pH optimum for the generation of either of the monohydroxylated metabolites was 7.5. In contrast, the pH optimum for glycol formation was found to be 8.3.

Effect of inducing agents. Since microsomal cytochrome P-450 appeared to be

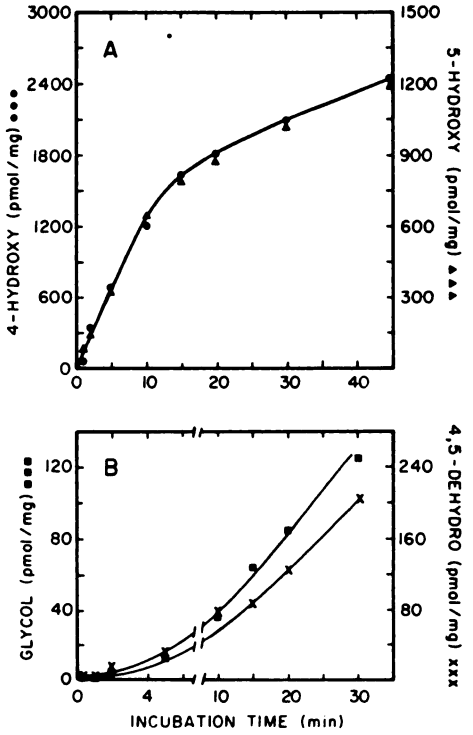


FIG. 4. *Time course for niridazole metabolite formation by rat liver microsomes*

Liver microsomes were incubated with NDZ and NADPH for various time intervals and metabolite formation was assessed by HPLC. (A) Time course for the formation of 4-OH (●) and 5-OH (▲) NDZ; (B) Time course for the formation of glycol (■) and 4,5-dehydro (×) NDZ. Each point represents the mean of duplicate determinations on each of three different microsomal preparations. The standard deviation never exceeded 15% of the mean.

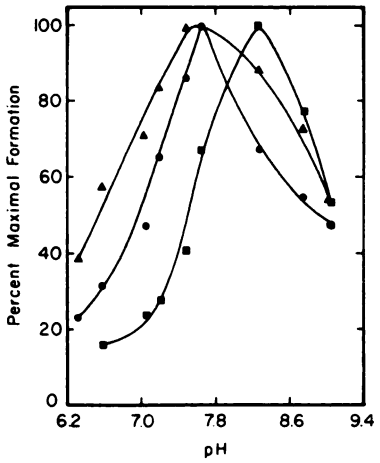


FIG. 5. *pH Profile for niridazole metabolite formation*

Niridazole oxidation by rat liver microsomes was measured in 20 mM K phosphate buffer at the pH values shown. Each 1 ml reaction mixture contained 0.25 μmol of NDZ and 0.6 mg of microsomal protein. Rates are reported as percentages of the rate of product formation at the pH optimum for the 4-OH (●), 5-OH (▲) and glycol (■). Maximal rates for the the 4-OH and 5-OH at their pH optima (7.5) were 158 and 81 pmol/min/mg, respectively. The analogous rate of glycol formation at pH 8.3 was 186 pmol/10 min/mg. Each point represents the mean of triplicate determinations. Values did not vary more than 13% from the mean.

involved in NDZ metabolism, the metabolism was investigated further in liver microsomes from rats treated with PB or MC, prototypic inducers of the cytochrome P-450 monoxygenase system. Table 3A shows that the increase in the rate of formation of both monohydroxylated metabolites after PB pretreatment was approximately proportional to the increase in microsomal cytochrome P-450 specific content. In contrast, MC pretreatment resulted in a selective increase in the rate of formation of the 4-OH metabolite with little or no effect on the rate of formation of the 5-OH metabolite.

The amount of monohydroxylated product formed was always greater than the amount of glycol or 4,5-dehydro NDZ. PB treatment resulted in an 80% increase in the amount of dehydro NDZ formed, while with MC pretreatment the increase was only about 28%. Glycol formation in microsomes from rats treated with either inducer was enhanced by about 140% (Table 3B).

Preliminary evaluation of precursor-product relationships. The apparent lag in the formation of the dehydro and glycol metabolites from NDZ and their small quantities relative to the monohydroxylated metabolites suggested that the dehydro and glycol compounds might be derived from the other NDZ metabolites rather than directly from the parent compound. To evaluate this possibility 4-OH, 5-OH and 4,5-dehydro NDZ, isolated from microsomal reaction mixtures and purified by HPLC, were used as substrates in microsomal reaction mixtures (Table 4). When

TABLE 3

Effect of Microsomal Enzyme Inducers on Niridazole Metabolism by Rat Liver Microsomes

Microsomes were prepared from untreated, PB-treated or MC-treated rats as described under MATERIALS AND METHODS. Cytochrome P-450 specific content was determined from the CO-reduced minus reduced difference spectrum of microsomes suspended to 1 mg/ml in 0.1 M K phosphate buffer, pH 7.4, 0.6 mg of microsomal protein and 1 μmol of NADPH is a total volume of 1 ml. All values represent the mean ± S.E. of triplicate determinations on a single microsomal isolate.

A. Treatment	P-450	Niridazole metabolism	
		4-OH	5-OH
	(nmol/mg)	*(pmol/min/mg)*	
Untreated	1.02	153 ± 16	79 ± 11
Phenobarbital	1.84	292 ± 32	182 ± 10
Methylcholanthrene	1.63	256 ± 21	90 ± 12
B. Treatment		**Niridazole metabolism**	
		Glycol	Dehydro
		(pmol/10 min/mg)	
Untreated	1.02	82 ± 19	262 ± 21
Phenobarbital	1.84	195 ± 15	472 ± 18
Methylcholanthrene	1.63	197 ± 24	335 ± 63

[a] The rates of glycol and dehydro NDZ formation were determined as the amount formed within the first 10 min following the addition of NADPH. These determinations probably underestimate the maximal rate of formation, but the time period was chosen to permit the determination of all four metabolites in single sample (See Fig. 4).

TABLE 4

Metabolite Metabolism in Rat Liver Microsomes

The final substrate concentration in each reaction mixture was 0.25 mM, 0.08 mM and 0.07 mM for the 4-OH, 5-OH and 4,5-dehydro metabolites respectively. Otherwise reaction conditions were identical to those described under Table 3. Each value represents the mean of duplicate determinations.

Substrate	Glycol	Product		NDZ	Dehydro
		5-OH	4-OH		
	(pmol/10 min/mg)				
Dehydro	1250	80	300	50	—
4-OH NDZ	170	180	—	80	660
5-OH NDZ	ND[a]	—	440	140	80

[a] ND denotes none detected.

the 4-OH metabolite was employed as a substrate, the primary product found was the dehydro metabolite; smaller amounts of glycol, 5-OH and NDZ were also detected.[6] With the 5-OH metabolite the only appreciable product formed was the 4-OH metabolite.[6] Finally, use of the 4,5-dehydro metabolite as substrate resulted in a striking formation of the glycol. These observations are consistent with the metabolic scheme presented in Figure 6 where the predominant pathway proposed is NDZ oxidation to the 4-OH derivative which then is converted to the dehydro metabolite. The dehydro compound in turn forms the glycol via an epoxide intermediate.

DISCUSSION

Many toxic effects of NDZ may be related to its extensive and varied metabolism. Examples include niridazole's antiparasitic and antimicrobial actions as well as its central nervous system toxicity, immunosuppressive activity and putative mutagenicity/carcinogenicity (10–13). Interest in NDZ metabolism has focused on its nitro group because this group is required for antischistosomal activity (14); reduction of the nitro group to reactive intermediates has been postulated to account for much of the toxicity of the parent molecule (3). However only a single reductive metabolite of NDZ has been isolated and that was generated under anaerobic conditions (3). In air, this hydroxylamine compound is rapidly oxidized back to NDZ. Hence it is unlikely that it, either by itself or by reaction with endogenous molecular species, could account for the large unidentified metabolite pool found in the tissues, plasma, feces and urine of animals or patients treated with NDZ (1).

During a search for NDZ-induced immunosuppressive factor (10), we found four niridazole metabolites which were present in significant quantities in both urine and plasma. These compounds, which could also be produced by mild permanganate oxidation of NDZ, turned out to be identical to the microsomal oxidation products described herein.[7] Following extraction by ethyl acetate, isolation by HPLC and analysis by a combination of ^{1}H FT NMR and mass spectroscopy, it was shown that all four products resulted from metabolic alterations at the C-4, C-5 regions of the imidazolidinone ring. The NMR and mass spectral analyses complemented each other in providing their unambiguous identification as the 4-OH, 5-OH, 4,5-dehydro and 4,5-glycol derivatives of NDZ.

The primary localization of NDZ oxidase activity to the microsomal fraction, along with the requirements for atmospheric oxygen and exogenous reducing equivalents, especially from NADPH, are properties ascribable to the cytochrome P-450 monoxygenase system (15). That the increase in 4-OH NDZ formation induced by PB pretreatment was directly proportional to the increase in cytochrome P-450 specific content further supports this contention (15).

With rat liver microsomes hydroxylation on the C-4 position of the imidazolidinone ring was the predominant oxidative pathway regardless of animal pretreatment with inducers of drug metabolism; with microsomes from untreated rats the ratio of 4-

[6] The mechanism of NDZ formation in these systems has not been elucidated. Chromatography of all metabolite samples used as substrates showed that no NDZ was present before addition to the reaction mixtures. The appearance of NDZ among the reaction products suggests that certain of the reactions depicted in Figure 6 may be reversible. This prospect is currently under investigation.

[7] J. L. Blumer, R. F. Novak, S. V. Lucas, J. M. Simpson and L. T. Webster, Jr., unpublished observation.

OH to 5-OH, the next most abundant metabolite, was approximately 2:1.

Unlike PB, MC pretreatment produced little or no change in microsomal C-5 hydroxylation as compared to untreated controls while C-4 hydroxylation was increased by 67%; the increase in C-4 hydroxylation was again directly proportional to the increase in cytochrome P-450 specific content. Similar selective increases in the formation of a specific metabolite following MC have been reported for rat and mouse liver microsomes with respect to the hydroxylation of biphenyl (18). As with PB, these selective alterations might be related to the enrichment of microsomes with a specific form of P-450.

The interpretation of the effects of PB and MC on the formation of the glycol and dehydro metabolites is more difficult. Both the time course of their formation (Fig. 4) and the observation that each apparently derives from another metabolic product (Table 4) suggest that different mechanisms and/or different enzymes are responsible for their formation.

Figure 6 depicts a proposed scheme for microsomal NDZ metabolism. The two monohydroxylated metabolites are shown as products of cytochrome P-450 catalyzed oxidations of NDZ with the 4-OH pathway predominating (Table 3). The dehydro metabolite is most likely produced by subsequent dehydration of the 4-OH derivative. This hypothesis is supported by the lag phase seen in formation of the dehydro metabolite from NDZ (Fig. 4) and by the data in Table 4 showing that the 4-OH derivative is a good precursor substrate for the dehydro compound whereas the 5-OH metabolite is not. Although some spontaneous reaction occurs, dehydration of the 4-OH compound to the dehydro derivative is probably enzyme-catalyzed because incubation of the 4-OH compound with microsomes and NADPH at 37° resulted in 1000-fold more dehydro NDZ than was formed by heating an aqueous solution of the 4-OH NDZ for a week under similar conditions.[8] This reaction's kinetics, pH optimum, dependence on reducing equivalents and/or O_2 have yet to be determined.

Although the glycol product could be produced either from a monohydroxy NDZ metabolite or from the dehydro metabolite as shown in Figure 6, the data of Table 4 support the dehydro metabolite as the major substrate for glycol formation. The rate of glycol formation was enhanced 2.4-fold and 2.6-fold by PB and MC, respectively, suggesting that cytochrome P-450 is involved in this pathway, too.

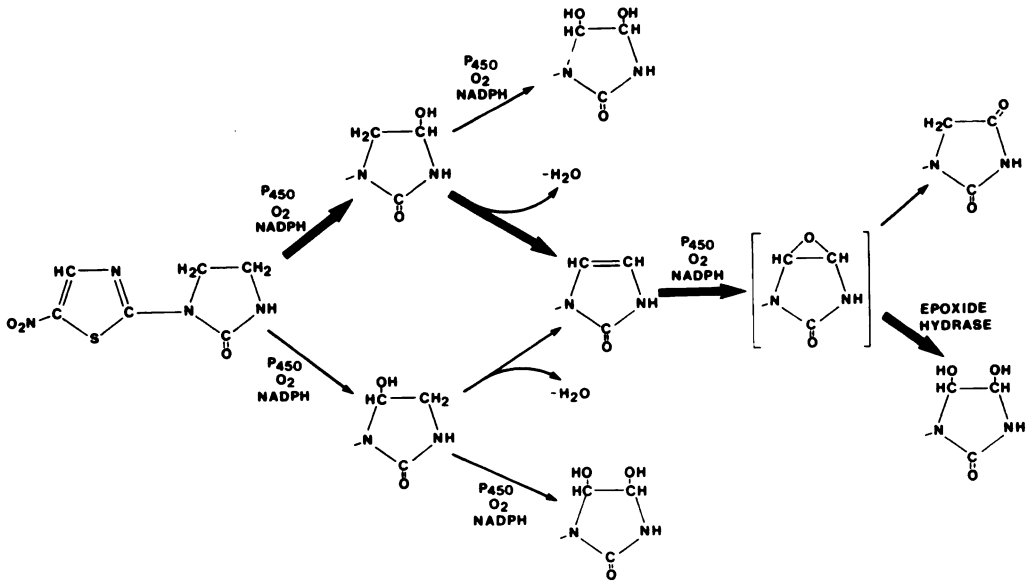


FIG. 6. *Proposed pathway for microsomal niridazole metabolism*

Production of the glycol from the dehydro compound suggests the formation of an epoxide intermediate (Fig. 6). Conversion of this alkene oxide intermediate to the glycol would be expected to be catalyzed by another microsomal enzyme, epoxide hydrase (19). Although there are some examples of alkene oxide metabolites which are stable enough for isolation (20–22), the product usually observed following oxidation of an olefinic substrate is the glycol (23–25). Two additional observations favor the involvement of an epoxide intermediate in NDZ glycol formation. First, a fifth NDZ metabolite, tentatively identified as the 4-keto derivative,[8] has been isolated by HPLC from the urine of NDZ-treated animals. By analogy to the arene oxide systems (26), the 4-keto would be the expected spontaneous isomerization product of the NDZ epoxide. Second, the pH optimum of 8.3 observed for glycol formation (Fig. 5) is similar to the optimum reported by various investigators for solubilized forms of epoxide hydrase (27). Attempts to demonstrate specific inhibition of the formation of NDZ glycol from NDZ itself with either phenylimidazole or 3,3,3-trichloroprophylene oxide ("specific" epoxide hydrase inhibitors) were unsuccessful because their addition inhibited the formation of the monohydroxylated metabolites as well;[7] similar results have recently been reported with respect to benzpyrene metabolism (28).

An important question is whether the putative NDZ epoxide could cause NDZ toxicity. In toxicity studies related to metabolic activation, arene oxides have received more attention than alkene oxides such as would be formed from NDZ. However, an alkene oxide intermediate produced by microsomal cytochrome P-450 may be involved in hepatoxicity produced by trichloroethylene (29).

The implication of an epoxide in the oxidative metabolism of NDZ provides an alternative mechanism for NDZ toxic activation which does not involve the nitro group. The relative contribution of both pathways to NDZ metabolism and toxicity needs to be assessed under physiological conditions in both the host and parasite. For example, antiparasitic activity, mutagenicity and carcinogenicity, all probably require the metabolic acivation of NDZ. The oxidation products described in this study all have modifications of the imidazolidinone portion of the NDZ molecule which, unlike the nitro group, is relatively insensitive to chemical modification for retention of antiparasitic activity (13). If such oxidative metabolites are shown to participate in the central nervous system, mutagenic or carcinogenic sequellae of NDZ treatment, it may be possible to design an antihelminthic analogue of NDZ which cannot be metabolized in like manner and would therefore retain antiparasitic activity and be less toxic to the host.

REFERENCES

1. Faigle, J. W., and H. Keberle. Metabolism of niridazole in various species including man. *Ann. N.Y. Acad. Sci.* **160:** 544–557, 1969.
2. Feller, D. R., M. Morita, and J. R. Gillette. Enzymatic reduction of niridazole by rat liver microsomes. *Biochem. Pharmacol.* **20:** 203–215, 1971.
3. Morita, M., D. R. Feller, and J. R. Gillette. Reduction of niridazole by rat liver xanthine oxidase. *Biochem. Pharmacol.* **20:** 217–226, 1971.
4. Mazel, P. Experiments illustrating drug metabolism *In vitro*, in *Fundamentals of Drug Metabolism and Drug Disposition*, edited by LaDu, B. N., H. G. Mandel, and E. L. Way. Williams and Wilkins, Baltimore, 1971, 551–556.
5. Blumer, J. L., and J. J. Mieyal. Genetic expression of microsomal electron transport in mice: different patterns of dependence of constitutive cytochrome P-450 reductase activity on pyridine nucleotide concentration. *J. Biol. Chem.* **253:** 1159–1166, 1978.
6. Lowry, O. H., J. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the folin phenol regent. *J. Biol. Chem.* **193:** 265–275, 1951.
7. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. I. evidence for its hemoprotein nature. *J. Biol. Chem.* **239:** 2370–2378, 1964.
8. Jackman, L. M., and S. Sternell. Chapter 3–6 in *Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry*, ed. 2. Pergamon Press, Oxford, 1969, 201–214.
9. Vail, S. L., R. H. Barker, and P. G. Mennitt. Formation and identification of *cis*- and *trans*-dihydroxyimidazolones from urea and glyoxals. *J. Org. Chem.* **30:** 2179–2182, 1965.
10. Webster, L. T. Jr., A. E. Butterworth, A. A. F. Mahmoud, E. N. Mngola, and K. S. Warren.

Suppression of delayed hypersensitivity in schistosome-infected patients by niridazole. *N. Engl. J. Med.* **292:** 1144–1147, 1975.

11. Legator, M. S., T. H. Connor, and M. Stoeckel. Detection of mutagenic activity of metronidazole and niridazole in body fluid of humans and mice. *Science* **188:** 1118–1119, 1975.
12. Urman, H. K., O. Bulay, D. B. Clayson, and P. Shubik. Carcinogenic effects of niridazole. *Cancer Lett.* **1:** 69–74, 1975.
13. Collins, F. M. Effect of oral niridazole treatment on some bacterial infections in mice. *Antimicrob. Agents Chemother.* **7:** 453–456, 1975.
14. Robinson, C. H., E. Bueding, and J. Fisher. Relationship between structure, conformation and antischistosomal activity of nitroheterocyclic compounds. *Mol. Pharmacol.* **6:** 604–616, 1971.
15. Gillette, J. R., D. C. Davis, and H. A. Sasame. Cytochrome P-450 and its Role in Drug Metabolism. *Ann. Rev. Biochem.* **12:** 57–84, 1972.
16. Orrenius, S., J. E. Ericsson, and L. Ernster. Phenobarbital-induced synthesis of the microsomal drug-metabolizing enzyme system and its relationship to the proliferation of endoplasmic membranes. *J. Cell. Biol.* **25:** 627–639, 1965
17. Haugen, D. A., T. A. van der Hoeven, and M. J. Coon. Purified microsomal cytochrome P-450: separation and characterization of multiple forms. *J. Biol. Chem.* **250:** 3567–3570, 1975.
18. Atlas, S. A., and D. W. Nebert. Genetic association of increases in naphthalene, acetanilide and biphenyl hydroxylations with inducible aryl hydrocarbon hydroxylase in mice. *Arch. Biochem. Biophys.* **175:** 495–506, 1976.
19. Oesch, F. Mammalian Epoxide Hydrases: inducible enzymes catalysing the inctivation of carcinogenic and cytotoxic metabolites derived from aromatic and olefinic compounds. *Xenobiotica* **3:** 305–340, 1972.
20. Davidow, B., and J. L. Radomski. Isolation of an epoxide metabolite from fat tissues of dogs fed hepatochlor. *J. Pharmacol. Exp. Ther.* **107:** 259–264, 1953.
21. Sims, P. Metabolism of polycyclic compounds: the metabolism of 1,4-epoxy-1,4-dihydronaphthalene in rats. *Biochem. J.* **95:** 608–611, 1965.
22. Bruer, H., and R. Knuppen. The formation and hydrolysis of 16α, 17α-epoxy-oestratriene-3-ol by rat liver tissue. *Biochem. Biophys. Acta* **49:** 620–621, 1961.
23. Boyland, E., and P. Sims. Metabolism of polycyclic compounds. 16. The Metabolism of 1:2-Dihydronaphthalene and 1:2-Epoxy-1:2:3:4-Tetrahydronaphthalene. *Biochem. J.* **77:** 175–182, 1960.
24. Fukami, J.-I., I. Yamamoto, and J. E. Casida. Metabolism of rotenone *in vitro* by tissue homogenates from mammals and insects. *Science* **155:** 713–717, 1967.
25. Sisenwine, S. F., C. O. Tio, S. R. Shrader, and H. W. Ruelius. The biotransformation of protriptyline in man, pig and dog. *J. Pharmacol. Exp. Ther.* **175:** 51–59, 1970.
26. Jerina, D. M., and J. W. Daly. Arene oxides: a new aspect of drug metabolism. *Science* **185:** 573–582, 1974.
27. Lu, A. Y. H., D. Ryan, D. M. Jerina, J. W. Daly, and W. Levin. Liver microsomal epoxide hydrase solubilization, purification and characterization. *J. Biol. Chem.* **250:** 8283–8288, 1975.
28. Fahl, W. E., S. Nesnow, and C. R. Jefcoate. Microsomal metabolism of benzo(α)pyrene: multiple effects of epoxide hydrase inhibitors. *Arch. Biochem. Biophys.* **181:** 649–664, 1977.
29. Allemand, H., D. Pessayre, V. Descatoire, C. Degott, G. Feldmann, and J.-P. Benhamon. Metabolic activation of trichloroethylene into a chemically reactive metabolite toxic to the liver. *J. Pharmacol. Exp. Ther.* **204:** 714–723, 1978.