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ENZYMATIC REDUCTION OF NIRIDAZOLE BY RAT LIVER MICROSOMES*

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Abstract—Investigations were carried out with liver microsomes to identify the metabolites and to characterize the enzyme responsible for the reduction of niridazole (NDZ) [1-(5-nitro-2-thiazolyl)-2-imidazolidinone]. Data obtained from gas chromatography—mass spectrometry indicated that a major microsomal metabolite of NDZ was the hydroxylamine derivative (hydroxyaminothiamidazol). The metabolite was shown to be extremely susceptible to the presence of oxygen and readily reconverted to NDZ by exposure to air. The following evidence suggests that the conversion of NDZ to hydroxyaminothiamidazol by liver microsomes is mediated by NADPH-cytochrome c reductase: (1) the microsomal nitroreductase was not blocked by carbon monoxide and not associated with the cytochrome P-450; (2) the rate of nitroreduction was proportional to the increase in the activity of NADPH-cytochrome c reductase in liver microsomes from animals pretreated with phenobarbital; (3) both of these reductase activities remained unaffected by prior treatment with 3-methylcholanthrene; (4) the nitroreductase was solubilized by treatment of liver microsomes with steapsin; and (5) similar increases in the activities of NDZ reductase, neotetrazolium diaphorase and NADPH-cytochrome c reductase were noted after partial purification by fractionation with ammonium sulfate.

FAIGLE and Keberle[1,2] reported that NDZ,§ an antischistosomal agent, is metabolized mainly in the liver, although it is also metabolized to a minor extent in a wide variety of tissues. While they suggested that the biotransformation of NDZ involves the reduction of the heterocyclic nitro group, the enzymatic mechanism and metabolic fate of this compound remain unknown.

Liver microsomes contain NADPH-dependent enzymes which catalyze the reduction of azo and nitro compounds to the corresponding amines.[3] These reductive reactions are active under anaerobic conditions and are presumably mediated either by CO-sensitive cytochrome P-450[4] or CO-insensitive NADPH-cytochrome c reductase.[5] In this regard, it was of interest to characterize the enzyme which is responsible for the reduction of NDZ as well as to identify the metabolites.

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§ 1(5-Nitro-2-thiazolyl)-2-imidazolidinone is marketed as Ambilhar by CIBA Limited, Basel.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (NIH colony), weighing 160–220 g, were employed in all experiments and received free access to rat chow and tap water. In some experiments male albino guinea pigs (200–300 g), male New Zealand rabbits (2 kg) and male NIH general purpose mice (20–30 g) were used.

Drug pretreatments. Rats were treated with phenobarbital (80 mg/kg, i.p.) or 3-methylcholanthrene (40 mg/kg in corn oil, i.p.) for 3 consecutive days and were killed by decapitation 24 and 36 hr after the last dose respectively. Groups of control animals were injected with saline or corn oil.

Enzyme preparation. Livers were homogenized with a motor-driven glass-Teflon homogenizer in 4 vol. of 1·15 per cent KCl containing 20 mM tris-HCl buffer, pH 7·4. The homogenate was centrifuged for 20 min at 9000 *g* in a Sorvall centrifuge and the supernatant was carefully decanted and recentrifuged for 1 hr at 105,000 *g* in a Spinco model L preparative ultracentrifuge. The microsomal pellet was suspended in ice-cold 1·15 per cent KCl–20 mM tris buffer, pH 7·4.

NDZ reductase assay. Unless stated otherwise, the incubation mixtures, consisting of 5 mg microsomal protein and 0·625 μmole niridazole in a final volume of 2·5 ml of 20 mM tris-HCl buffer, pH 7·4, were added to an anaerobic Aminco spectrophotometric cell (Al-65085). The mixtures were then gassed for 5 min with N_2 or CO that had been deoxygenated by passage through a solution of 0·05 per cent 2-anthraquinone sodium sulfonate, 0·5 per cent sodium dithionite and 0·1 N NaOH. The plunger assembly containing the NADPH-generating system* (50 μl) or 1 μmole of NADPH was then fitted to the cuvette and gassing was continued for 3 min through the side arm. The cuvette was then sealed and transferred to the sample chamber of a Gilford spectrophotometer (model 2000) attached to a Honeywell recorder. The reaction was initiated by depressing the plunger and followed by measuring the rate of change in absorbancy at 400 mμ on chart paper (90 in./hr). The temperature of the reaction mixture was maintained at 30° throughout the experiment. The reaction velocities, expressed as millimicromoles NDZ disappeared per milligram of protein per minute, were calculated from the initial linear phase of the curve and an extinction coefficient of 10·4 $mM^{-1}cm^{-1}$. Under these assay conditions, the initial rate of NDZ reduction was proportional to the microsomal protein concentration in the range of 0·4–3·0 mg/ml.

Cytochrome P-450 content. The assay for cytochrome P-450 content was determined by the method described by Omura and Sato.[6] The amount of cytochrome P-450 was calculated from the absorbance difference ($A_{450} - A_{490}$) and the molar extinction coefficient of 91 $mM^{-1}cm^{-1}$.

NADPH-cytochrome c reductase assay. The reductase activity was measured according to the method of Phillips and Langdon.[7] The reductase activity, expressed as millimicromoles cytochrome c reduced per milligram of protein per minute was calculated, using the molar extinction coefficient of 19·1 $mM^{-1}cm^{-1}$.

Protein concentration. Protein content was determined by the method of Lowry *et al.*[8] Bovine serum albumin was used as the protein standard.

Neotetrazolium diaphorase assay. The method employed was essentially that described by Williams and Kamin.[9] The reaction mixture contained 0·5–1·0 mg protein,

* The NADPH-generating system consisted of: NADPH (1·0 μmole), $MgCl_2$ (15 μmoles), glucose-6-phosphate (15 μmoles) and glucose-6-phosphate dehydrogenase (2 E.U.).

NADPH-generating system, 1·15 μmoles neotetrazolium chloride (NT), and 150 μmoles tris-HCl buffer, pH 7·4, in a total volume of 3 ml. After preincubation for 5 min at 37°, the reaction was initiated by the addition of NT. After 5 min, the reaction was terminated by the addition of 3 ml acidic Triton–formalin (3·6 ml 10 per cent Triton X-100, 5 ml 40 per cent HCHO, 10 ml 1 M formate buffer, pH 3·5, and 40 ml water). The absorbancy of the mixture was measured at 500 mμ and the rate of NT reduction (mμmoles formazan formed/mg protein/min) was calculated, using the molar extinction coefficient of 14 $mM^{-1}cm^{-1}$ as reported by Lester and Smith.[10]

Solubilization and partial purification of NDZ reductase. The procedure employed was essentially that described by Hernandez *et al.*[11] for the solubilization and purification of microsomal azoreductase. Liver microsomes (10–15 mg/ml) suspended in 20 mM tris-HCl buffer, pH 7·4 (10^{-3} M EDTA), were incubated at 0° for 14–20 hr with steapsin (0·07 per cent) under nitrogen. The steapsin digest was centrifuged for 1 hr at 105,000 *g* and the supernatant was fractionated with ammonium sulfate (40–80 per cent saturation). The protein precipitate was redissolved in a small volume of the tris-HCl buffer, pH 7·4, and stored at 4° until use.

Identification of metabolites. For the identification of metabolites, the reaction mixture consisted of 10 mg protein, 2·5 μmoles NDZ and a double amount of the NADPH-generating system in a final volume of 4 ml of 20 mM tris-HCl buffer, pH 7·4. The incubation flasks, placed on ice, were closed with serological stoppers containing a gassing vent and pregassed with nitrogen (2 l./min) for 5 min. The vents were then closed with glass rods and the flasks were transferred to an Aminco metabolic incubator. The flasks were incubated for 25 or 50 min at 37° with shaking (90 oscillations/min) and the reaction was terminated by the addition of 2 ml of 20 per cent TCA. After centrifugation, an aliquot (5 ml) of the acidified supernatant was extracted with an equal volume of ethylene dichloride (EDC). A 4-ml volume of the EDC extract was transferred to a small tube and evaporated to dryness under nitrogen. The residue was then redissolved in 100–200 μl of a 1:1 mixture of acetone and bis(trimethylsilyl)-acetamide (BSA), and allowed to stand overnight under N_2. The extent of metabolism, millimicromoles NDZ reduced, was determined by measuring the change in absorbancy at 360 mμ in the EDC extract. Known amounts of NDZ, carried throughout the procedure, served as standards.

Gas–liquid chromatography. Aliquots of the prepared samples were injected onto the 6 ft glass column (3 per cent OV-22 on 80–100 supelcoport) of a Glowall model 310 gas–liquid chromatograph equipped with a hydrogen flame detector (oven temperature 250°). The column temperature was maintained at 175° and the injection block temperature was 230°. The carrier gas (Ar or N_2) was 20 psi (50 ml/min).

Mass spectra. The mass spectra were recorded on an LKB gas chromatograph—mass spectrometer analyzer unit with an operating voltage of 70 eV and an ion source temperature of 190 ± 10°. The conditions for gas–liquid chromatography were the same as those described above.

RESULTS

Intracellular localization and cofactor requirements of NDZ reductase activity in rat liver. The reduction of NDZ was catalyzed by enzyme systems present in the microsomal and 105,000 *g* supernatant fractions (Table 1). The microsomal fraction,

TABLE 1. INTRACELLULAR DISTRIBUTION OF NDZ REDUCTASE ACTIVITY IN RAT LIVER*

Fraction	NADPH-generating system†	Reductase activity (mμmoles/50 mg liver/min)
9000 *g* Supernatant	−	43·2 ± 1·3
	+	52·7 ± 0·8
Microsomes	−	0·0
	+	19·9 ± 0·4
Boiled microsomes‡	+	0·0
Microsomes§	+	0·0
105,000 *g* Supernatant	−	41·2 ± 1·6
Reconstituted 9000 *g*	−	41·7 ± 1·0
supernatant	+	52·3 ± 1·0

* Each fraction, equivalent to 50 mg liver, was placed in an anaerobic cell and gassed with N_2 for 5 min prior to the addition of NDZ. The initial rates of reduction were determined as described in Materials and Methods. Values represent the mean ± S.E.M. of triplicate determinations.

† The plus (+) refers to presence and the minus (−) refers to absence of the system.

‡ Microsomes were boiled for 5 min prior to incubation.

§ Microsomes were incubated with the generating system minus NADP.

inactive by itself, was completely dependent upon the presence of the NADPH-generating system. Omission of NADP from the generating system or boiling the microsomes prior to incubation abolished the enzymatic activity in liver microsomes. In the absence of NADPH, the 9000 *g* supernatant fraction and reconstituted 9000 *g* supernatant fraction possessed reductase activities identical to that present in the 105,000 *g* supernatant fraction.

After dialysis, the 9000 *g* and 105,000 *g* supernatant fractions were unable to reduce NDZ. However, the reductase activities in the dialyzed preparations were restored by the addition of NADPH and NADH (Table 2). In the microsomal fraction, NADPH was six to seven times more effective than NADH as a hydrogen donor, confirming the

TABLE 2. COFACTOR REQUIREMENT FOR THE REDUCTION OF NDZ IN DIALYZED SUBCELLULAR FRACTIONS OF RAT LIVER*

Fraction	Reductase activity (mμmoles/100 mg liver/min)		
	NADPH (0·4 mM)	NADH (0·4 mM)	NADPH + NADH
Microsomes	30·6 ± 1·5	4·7 ± 1·3	29·0 ± 1·6
105,000 *g* Supernatant	0·9 ± 0·4	40·0 ± 2·4	37·8 ± 2·8
Reconstituted 9000 *g* Supernatant	38·7 ± 3·0	40·0 ± 1·5	70·9 ± 2·8

* Each fraction was dialyzed for 20 hr at 4° against 20 mM tris-HCl buffer, pH 7·4. The reductase activities of the dialyzed fractions, equivalent to 100 mg liver, were determined as described in Materials and Methods. The results are expressed as the mean ± S.E.M. of triplicate determinations.

view that NDZ reduction by liver microsomes requires NADPH. In contrast, the reductase activity in the dialyzed 105,000 *g* supernatant fraction was dependent upon the presence of NADH; this enzymatic activity may be attributable to xanthine oxidase.[12] In addition, the reductase activities of the dialyzed fractions were observed to be almost additive in the presence of both cofactors, suggesting that the NADH-dependent soluble enzyme and the NADPH-dependent microsomal enzyme reduced NDZ independently.

Effect of gas phase on the reduction of NDZ. Since the reduction of *p*-nitrobenzoate by liver microsomes is inhibited by carbon monoxide,[4] the influence of the gas phase on the reduction of NDZ was investigated. As shown in Table 3, the presence of air completely blocked NDZ disappearance, whereas carbon monoxide did not affect the reduction of the drug.

It seemed possible that the failure of carbon monoxide to inhibit NDZ reduction might merely be due to a relatively rapid reoxidation of cytochrome P-450 by NDZ. This possibility could be rejected, however, because under a carbon monoxide atmosphere, almost all of the cytochrome P-450 had been converted to the cytochrome

TABLE 3. INFLUENCE OF CARBON MONOXIDE, NITROGEN AND AIR ON THE REDUCTION OF NDZ IN RAT LIVER MICROSOMES*

Gas phase	Reductase activity (mμmoles/mg protein/min)
Air	0·0
Nitrogen	10·9 ± 0·55
Carbon monoxide	11·3 ± 0·58

* Microsomes (5 mg protein) were incubated with NDZ and NADPH-generating system in the designated atmospheres and the initial rates of reduction were determined as described in Materials and Methods. The results are expressed as the mean ± S.E.M. of triplicate determinations.

TABLE 4. COMPARISON OF NDZ REDUCING ACTIVITY IN AN ATMOSPHERE OF NITROGEN OR CARBON MONOXIDE*

Gas phase	Reductase activity (mμmoles/mg protein/min)	Cytochrome P-450:CO complex (mμmoles/mg protein)	
		Before NDZ addition	After NDZ reduction
N_2	11·4 ± 0·24		
CO	11·3 ± 0·33	0·52	0·54

* Microsomes (5 mg protein) were pregassed under an atmosphere of CO or N_2 in the presence of NADPH (1 μmole) and NDZ. The rates of NDZ reduction were measured by recording the change in absorbancy at 400 mμ as described in the text. The amount of the cytochrome P-450:CO complex was measured before the addition of NDZ and after the incubation period. After gassing the reaction mixture with CO, the addition of sodium dithionite did not increase the amount of the cytochrome P-450:CO complex.

P-450:CO complex, even in the presence of NDZ (Table 4). These findings indicate that the reduction of NDZ differs from that of *p*-nitrobenzoate in that it is not catalyzed by microsomal cytochrome P-450.

Effect of prior treatment with phenobarbital or 3-methylcholanthrene on the reduction of NDZ. It is well known that phenobarbital or 3-methylcholanthrene pretreatment in various animals elevates the activities of several drug-metabolizing enzymes in liver microsomes. As shown in Table 5, phenobarbital treatment not only increases the

TABLE 5. EFFECT OF PHENOBARBITAL OR 3-METHYLCHOLANTHRENE PRETREATMENT ON THE NDZ REDUCTASE, NADPH-CYTOCHROME C REDUCTASE AND NEOTETRAZOLIUM DIAPHORASE ACTIVITIES AND CYTOCHROME P-450 CONTENT IN RAT LIVER MICROSOMES*

Treatment	Reductase activity (mμmoles/mg protein/min)			Cytochrome P-450 content (mμmoles/mg protein)
	NDZ	NT	Cyt. c	
Saline control	9·6 ± 0·2	76·5 ± 3·4	76·5 ± 3·5	0·53 ± 0·03
Phenobarbital	16·4 ± 0·2	123·5 ± 1·0	132·4 ± 5·4	1·35 ± 0·03
% Increase	71·8†	61·4†	69·3†	155†
Corn oil control	8·7 ± 0·5	74·4 ± 3·0	61·2 ± 4·0	0·44 ± 0·02
3-Methylcholanthrene	10·1 ± 1·1	84·6 ± 2·5	53·7 ± 2·9	1·04 ± 0·06
% Increase	15·6	13·7‡	−12·2	138†

* All reductase activities were expressed as the initial rates of reduction in the presence of the NADPH-generating system determined as described in the text. Rats were pretreated with phenobarbital and 3-methylcholanthrene as described under Materials and Methods. The results represent the mean of four determinations ± S.E.M.

†$P < 0·01$.

‡$P < 0·05$.

activities of NADPH-cytochrome c reductase, neotetrazolium diaphorase and the amount of cytochrome P-450 in rat liver microsomes, but also enhances the reduction of NDZ. The enhancement of NDZ reductase activity was more closely correlated to the increase in NADPH-cytochrome c reductase and neotetrazolium diaphorase activities than to the elevation in cytochrome P-450 content. On the other hand, prior treatment of the rats with 3-methylcholanthrene did not alter the reductase activities, although the cytochrome P-450 content was increased 138 per cent over controls.

Comparison of NDZ reductase activity in several species. Liver microsomes from all the species studied were capable of reducing NDZ in the presence of the NADPH-generating system (Table 6). The mouse and guinea pig microsomes exhibited the greatest NDZ reductase activity, while the rabbit microsomes showed the lowest activity. The rates of NDZ reduction correlate more closely with the activity of NADPH-cytochrome c reductase than with the cytochrome P-450 content.

Solubilization and partial purification of microsomal NDZ reductase. Azoreductase neotetrazolium diaphorase and NADPH-cytochrome c reductase can be solubilized from liver microsomes by treatment with steapsin and partially purified by ammonium sulfate fractionation.[5,9,11] As shown in Table 7, the NDZ reductase activity in rat liver microsomes was also solubilized by steapsin treatment. Negligible amounts of the reductase activity were retained in the residual particles after the steapsin treatment. After solubilization, NADPH-cytochrome c reductase activity increased about 2-fold,

TABLE 6. COMPARISON OF NDZ REDUCTASE AND NADPH-CYTOCHROME C REDUCTASE ACTIVITIES AND CYTOCHROME P-450 CONTENT IN LIVER MICROSOMES FROM DIFFERENT SPECIES OF ANIMALS*

Species	Reductase activity (mμmoles/mg protein/min)		Cytochrome P-450 content (mμmoles/mg protein)	Relative ratio	
	NDZ	Cytochrome c		Cyt. c/ NDZ	P-450/ NDZ
Mouse	10·1 ± 1·1	129·0 ± 9·3	0·88 ± 0·09	12·8	8·7
Rat	8·6 ± 0·5	93·0 ± 6·6	0·60 ± 0·01	10·8	7·0
Guinea pig	10·8 ± 1·8	132·7 ± 10·3	1·03 ± 0·13	12·3	9·6
Rabbit	6·8 ± 0·4	83·1 ± 5·0	1·21 ± 0·09	12·2	17·8

* The reductase activities were measured as initial rates of reduction in the presence of the NADPH-generating system described in the text. Values represent the mean of three determinations ± S.E.M. obtained from each species.

TABLE 7. SOLUBILIZATION AND PARTIAL PURIFICATION OF NDZ REDUCTASE, NT-DIAPHORASE AND NADPH-CYTOCHROME C REDUCTASE*

Fraction	Units (mμmoles/min)			Specific activities (mμmoles/mg protein/min)			Recovery (%)		
	NDZ	NT	Cyt. c	NDZ	NT	Cyt. c	NDZ	NT	Cyt. c
Microsomes	11,140	54,300	79,210	13·9	67·8	98·9	100	100	100
Steapsin supernatant	1731	6435	59,780	5·3	19·7	183·0	16	12	75
Ammonium sulfate (40–80%)	1749	7302	41,020	15·9	66·4	373·0	16	13	52
NADPH-cytochrome c reductase preparation†				27·8		882·5			

* An enzyme unit is defined as that amount of enzyme capable of metabolizing 1 mμmole of substrate/min under the assay conditions described in the text.

† Donated by Dr. P. Mazel, George Washington University.

whereas the activities for NDZ and neotetrazolium decreased considerably. However, after ammonium sulfate fractionation (40–80 per cent) of the steapsin supernatant, the specific activities of all the reductases increased 2- to 3-fold. Furthermore, a preparation of rat liver NADPH-cytochrome c reductase, purified by Dr. P. Mazel, also reduced NDZ.

Kinetic study of the reduction of NDZ. A Lineweaver–Burk plot showed that the K_m and V_{max} values obtained with rat liver microsomes and various concentrations of NDZ were $2{\cdot}7 \times 10^{-4}$ M and 14·2 mμmoles/min/mg of protein respectively.

Extraction and gas–liquid chromatographic separation of NDZ and metabolites. As shown in Table 8, 95 per cent of the NDZ disappeared from the reaction mixture after incubation under nitrogen for 30 min, whereas about 60 per cent of the ^{14}C-label was extracted into an equal volume of ethylene dichloride. Therefore, approximately 55 per cent of the reduced metabolites were recovered in the ethylene dichloride extract, suggesting that the metabolites are more polar than NDZ. Since prolonged incubation under nitrogen did not appreciably alter the partition coefficient of the metabolites, it

TABLE 8. EXTRACTION OF NDZ AND METABOLITES INTO ETHYLENE DICHLORIDE (EDC)*

Time of incubation under nitrogen (min)	Not exposed to air		Exposed to air
	(% Distribution of ^{14}C into EDC)	(% A_{360})	(% A_{360})
0	93·6	100·0	100·0
30	59·6	4·7	97·1
60	63·0	12·6	91·8
90	64·4	12·9	89·8

* Reaction mixtures consisting of 5 mg microsomal protein, 1·25 μmoles ^{14}C-niridazole (0·3 μc/mg) and NADPH-generating system were incubated under nitrogen for various times. Other samples were incubated first in nitrogen for various times and then in air for an additional 30 min. The samples were made acidic and extracted into EDC as described under Materials and Methods. The changes in absorbancy at 360 mμ in the EDC extract were expressed as the per cent absorbancy when compared to known amounts of NDZ in the nonincubated sample. After the extraction of NDZ and metabolites into EDC, aliquots (0·2–0·5 ml) of both phases were removed and dissolved in 15 ml of scintillation mixture (toluene, cellosolve and 2,5-diphenylorazole) and measured for ^{14}C content by liquid scintillation counting (Packard 3375), using an external standardization. The results were expressed as the mean of three determinations.

is likely that they were not further metabolized. Moreover, when the reaction mixtures were exposed to air after incubation at various times under nitrogen, nearly all of the reduced metabolites were auto-oxidized back to NDZ. Even after incubation for 90 min under nitrogen, 90 per cent of the parent compound could be recovered. This reversal of NDZ metabolism by exposure to air not only suggested that the inhibitory effect of air might be due to the reoxidation of the metabolites, but also raised the possibility that the metabolites were hydroxyaminothiamidazol or nitrosothiamidol, rather than the primary amine.

The reduced metabolites in the EDC extract could be stabilized by the formation of trimethylsilyl ether derivatives and separated by gas–liquid chromatography (Fig. 1). The preparation of silylated NDZ from a standard solution and a nonincubated sample gave a single chromatographic peak with a retention time of 9·7 min, while the sample incubated under N_2 for 25 min gave three peaks with retention times of 7·7, 9·7 and 10·4 min. In addition, the sample prepared from a reaction mixture, which was incubated under N_2 for 25 min and then followed with an exposure to air for 25 min, gave a single peak with a retention time identical to that observed for silylated NDZ (9·7 min).

Mass spectra of the trimethylsilyl derivatives. The mass spectrum of silylated NDZ gave a molecular ion at *m/e* 289 (Fig. 2). The increase of 72 mass units over the molecular weight of NDZ (214) represents the addition of one trimethylsilyl group (TMSi). Formation of a prominent peak at *m/e* 271 suggests the loss of a methyl group from the molecular ion, a well established fragmentation reaction observed with trimethylsilyl ethers. Other fragments at *m/e* 240 and *m/e* 256 may represent the loss of $-NO_2$ and $-NO$ from the molecular ion, a conclusion supported by the presence of metastables at *m/e* 201 and *m/e* 229 respectively. This evidence indicates that the nitro group remained intact and the formation of the trimethylsilyl ether occurred elsewhere on the parent compound. The formation of a peak at *m/e* 196 may be due to the loss of trimethylsilanol (TMSiOH) from *m/e* 286. However, the position of the TMSi group is

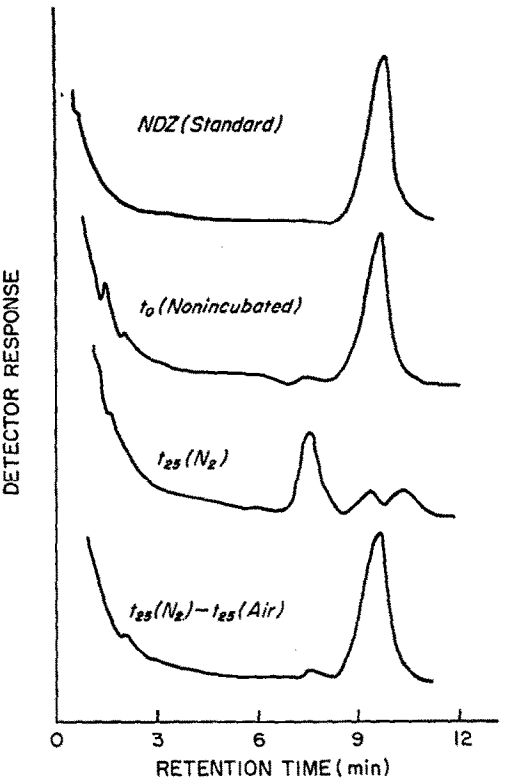

FIG. 1. Gas–liquid chromatographic separation of the trimethylsilyl ethers of NDZ and metabolites. The top trace indicates a sample prepared from a standard solution of NDZ; the second trace, a non-incubated sample of NDZ (t_0); the third trace, a sample incubated under nitrogen for 25 min (t_{25} nitrogen); and the bottom trace, a sample treated as in the third trace, but exposed to air for 25 min (t_{25} nitrogen → t_{25} air).

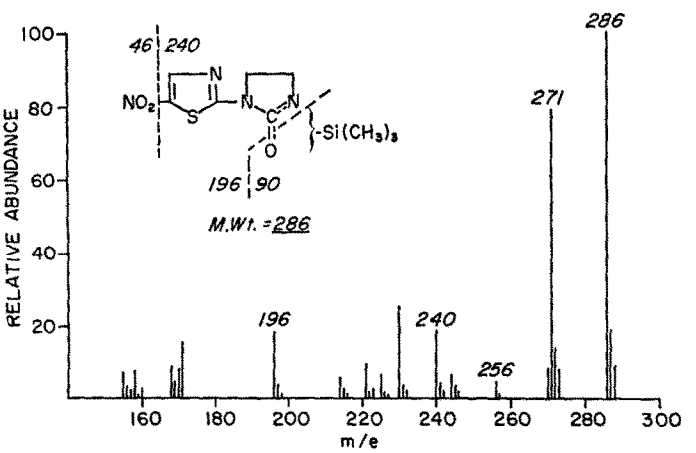

FIG. 2. Partial mass spectrum of the monosilylated ether of NDZ (nitrothiamidazol).

not assigned, since a rearrangement of TMSi from the N to the O may have occurred under electron impact. In addition, the molecular ion and fragmentation pattern obtained for the single chromatographic peak in sample D was identical in all respects to that of silylated NDZ. This finding confirms the view that the metabolites are converted to NDZ by exposure to air.

Derivative 1, which has a retention time of 7·7 min, gave a molecular ion at *m/e* 344 and probably is the disilylated derivative of hydroxyaminothiamidazol, the *N*-hydroxy metabolite of NDZ. As shown in Fig. 3, the labile molecular ion undergoes cleavage

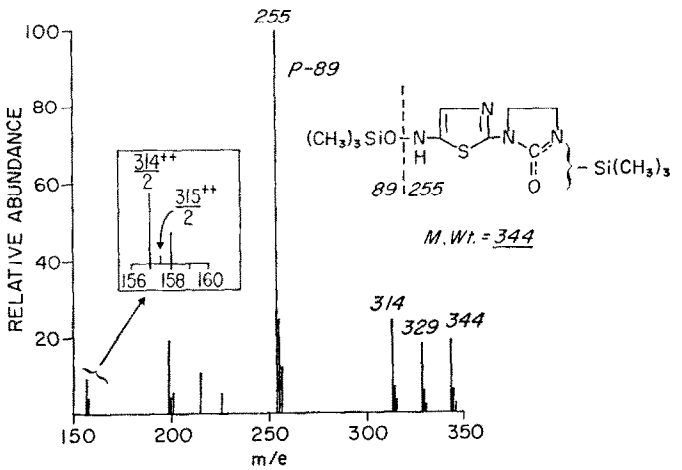

FIG. 3. Partial mass spectrum of the disilylated ether of hydroxyaminothiamidazol.

to form the base peak at *m/e* 255, which may represent the loss of trimethylsiloxy (TMSiO) from the oxime. It seems reasonable to suggest that this group could be assigned to a different position from that of the parent compound, since in the latter case a loss of 90 mass units was noted. Prominent peaks at *m/e* 329 and *m/e* 314 are most likely derived by the consecutive loss of methyl groups from the trimethylsilyl groups, a conclusion supported by the presence of metastables at *m/e* 314 and *m/e* 299 respectively. The presence of a doubly charged ion at *m/e* 157, indicated by the presence of the ^{13}C isotope at *m/e* 157·5, suggests the loss of two methyl groups from remote positions of the molecular ion. If both ethers were attached to the active hydrogens of the hydroxylamine, the formation of the doubly charged fragment would be less probable. Thus the TMSi groups are probably at opposite ends of the molecule.

Derivative 2, which has a retention time of 10·4 min, gave a molecular ion at *m/e* 416 (Fig. 4). It is probable that derivative 2 represents the trisilylated ether of hydroxyaminothiamidazol, since the difference in mass from the molecular ion of *m/e* 344 for derivative 1 is 72 mass units, the equivalent of an additional TMSi group. In this regard, the loss of 73 mass units from the labile molecular ion to the base peak at *m/e* 343 may be attributed to a TMSi group. A difference of 88 mass units between *m/e* 343 and *m/e* 255 may be due to the loss of a trimethylsiloxy group (TMSiO-H). The formation of this fragment peak at *m/e* 255 then is analogous to that observed with derivative

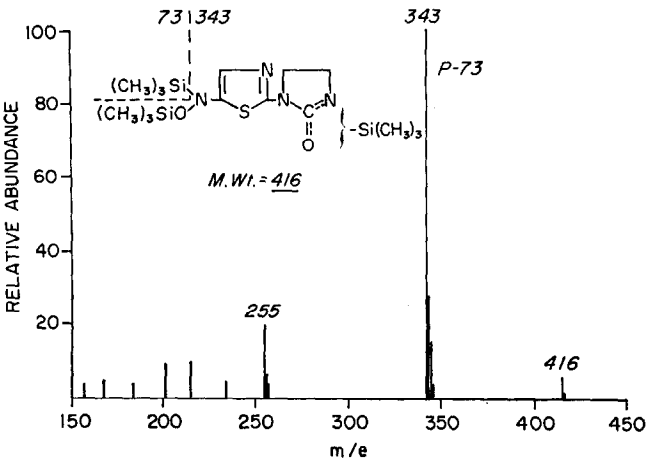

FIG. 4. Partial mass spectrum of the trisilylated ether of hydroxyaminothiamidazol.

1. It is conceivable therefore that these metabolite derivatives represent the disilylated and trisilylated ethers of hydroxyaminothiamidazol. These interpretations remain tentative, however, until the metabolite is synthesized and the spectra are analyzed.

Effect of NDZ on NADPH oxidation in the steapsin-treated supernatant fraction of liver microsomes. Since the metabolites of NDZ are extremely susceptible to oxidation, it seemed possible that a simultaneous reduction of NDZ and oxidation of the metabolite occurred under aerobic conditions. Indirect evidence for this view was obtained by investigating the effect of NDZ on NADPH oxidation in the solubilized microsomal preparation. As shown in Table 9, NDZ stimulates the rate of NADPH oxidation under

TABLE 9. EFFECT OF NDZ ON THE NADPH OXIDATION ASSOCIATED WITH THE SUPERNATANT OF STEAPSIN-TREATED RAT LIVER MICROSOMES*

NDZ added (mμmoles)	NADPH oxidized (mμmoles/mg protein/min)	Net increase/mμmole NDZ
0	0·98 ± 0·05	
125·0	2·88 ± 0·13	0·052
312·5	5·63 ± 0·16	0·049

* Reaction mixture consisted of the supernatant of steapsin-treated microsomes (5 mg protein) and various amounts of NDZ in a total volume of 2·5 ml. After the reaction was initiated by the addition of NADPH (1 μmole), the change in absorbancy at 340 mμ was followed in a Gilford recording spectrophotometer and the results were expressed as the initial rate of NADPH oxidation. Each value represents the mean of triplicate determinations ± S.E.M.

aerobic incubation and this rate is directly proportional to the amount of the drug added. No apparent decrease in the concentration of NDZ was observed under these conditions.

DISCUSSION

Recent evidence indicates that liver microsomes contain at least two types of NADPH-dependent enzymes which catalyze the reduction of nitro and azo compounds.[3] Gillette *et al.*[4] have shown that the conversion of *p*-nitrobenzoate to *p*-aminobenzoate in liver microsomes requires the CO-sensitive cytochrome P-450. In addition, it has been demonstrated that azo compounds are reduced by both the cytochrome P-450 and NADPH-cytochrome c reductase systems.[5,11]

In the present study, it has been shown that the nitro reduction of NDZ by liver microsomes is catalyzed by an enzyme which is similar, if not identical, to NADPH-cytochrome c reductase. Accordingly, the nitro-reducing enzyme for NDZ in rat liver microsomes was not sensitive to CO and its activity paralleled the increase in the activity of NADPH-cytochrome c reductase after pretreatment with phenobarbital, while 3-methylcholanthrene pretreatment did not modify either of the enzyme activities. No correlation was observed between the NDZ reductase activity and the cytochrome P-450 content. The fact that the microsomal reduction in several species correlated well with the relative activity of NADPH-cytochrome c reductase rather than the cytochrome P-450 content provides additional support for the role of NADPH-cytochrome c reductase in the reduction of NDZ.

During the treatment of microsomes with steapsin, the total activities for neotetrazolium and NDZ decreased in the steapsin supernatant, while that of the NADPH-cytochrome c reductase increased. After solubilization, however, the changes in NDZ reductase activity during partial purification paralleled the changes observed with neotetrazolium diaphorase. This observation suggests that the reductive pathway for NDZ may be related to the neotetrazolium diaphorase system. In this regard, Williams and Kamin[9] reported a similar phenomenon during the solubilization of neotetrazolium diaphorase from liver microsomes. Even so, they suggested that the diaphorase activity was dependent upon NADPH-cytochrome c reductase, since only gradual changes in these reductase activities were noted in subsequent purification steps. Although little evidence is available which clearly explains the loss of reductase activity during solubilization, it may be attributed to a partial destruction of the enzyme, removal of an unidentified carrier or activator, or an alteration in a binding site.

Evidence for the tentative identification of the hydroxylamine analogue (hydroxyaminothiamidazol) as a major microsomal metabolite of NDZ was obtained through the use of combination gas chromatograph–mass spectrometry (Figs. 2–4). In the mass spectra, the molecular ions obtained for the silylated derivatives were particularly important, since they were consistent with the postulated structures. The appearance of several peaks in the fragmentation patterns were derived from well established fragmentations of trimethylsilyl ethers and have provided satisfactory evidence for the existence of the molecular ions. Moreover, the mass spectra were able to confirm the reconversion of the auto-oxidizable metabolite, hydroxyaminothiamidazol, to NDZ, after exposure of the metabolites to air. This observation suggests that NDZ is not reduced directly to the corresponding amine, since its reoxidation would be highly improbable. Thus far, however, we have been unable to synthesize either hydroxyaminothiaminothiamidazol or aminothiamidazol, and thus have not been able to confirm the absence of the amine analogue by known chemical tests.

The extreme sensitivity of NDZ metabolite to air may account for the apparent

blockade of NDZ reduction in air. Indeed, the rate of NADPH oxidation in the steapsin-treated supernatant was stimulated in direct proportion to the amount of substrate added, and provides indirect evidence for the view that NDZ is reduced even under aerobic conditions. During the course of this reaction, NDZ is apparently converted to the nitroso- or to the hydroxylamine derivative, both of which undergo reoxidation. It is also possible that this type of cyclization process may explain the oxygen sensitivities previously noted in the reduction of azo and neotetrazolium compounds.

It is noteworthy that the formation of nitroso- and hydroxylamine derivatives from other compounds, whether derived from *N*-hydroxylation or nitroreduction, has been implicated in a variety of toxicities,[13,14] including drug-induced hemolytic anemia in patients with glucose 6-phosphate dehydrogenase (G6PD) deficiency. The metabolic information obtained in this study may thus be useful in providing an explanation of the schistosomicidal and/or toxicological actions of NDZ. In fact, NDZ has been reported to cause hemolytic anemia in patients with G6PD deficiency.[15]

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