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# THE METABOLIC ACTIVATION OF RONIDAZOLE [(1-METHYL-5-NITROIMIDAZOLE-2-YL)-METHYL CARBAMATE] TO REACTIVE METABOLITES BY MAMMALIAN, CECAL BACTERIAL AND *T. FOETUS* ENZYMES

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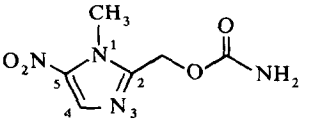
5-Nitroimidazoles are highly effective therapeutic agents against a variety of anaerobic bacteria and protozoa. For this reason the medicinal use of these agents has been explored in humans and in food-producing animals. As a class, however, 5-nitroimidazoles have earned a reputation as mutagens and, therefore, concern exists about their potential carcinogenicity to humans when taken directly as medications or indirectly through the consumption of food products of animals treated with these drugs.

Metronidazole (1-hydroxyethyl-2-methyl-5-nitroimidazole) is perhaps the best known 5-nitroimidazole because of its proven activity against trichomoniasis [1, 2] as well as its prophylactic and therapeutic efficacy against a wide range of gram positive anaerobic bacteria [3–5]. This broad spectrum of activity against anaerobic bacterial and protozoal infections prompted the development of other 5-nitroimidazoles for use in food producing animals.

Ronidazole [(1-methyl-5-nitroimidazole-2-yl)-methyl carbamate, Table 1] is a highly effective drug against *Histomonas meleagridis* and *Treponema hyodysenteriae* protozoa, the chief pathogens responsible for turkey blackhead and swine dysentery, respectively. Both metronidazole and ronidazole are 1,2-disubstituted 5-nitroimidazoles lacking a substituent at $C_4$. Ronidazole has a 2-methylene carbamate moiety which apparently contributes to its approximately 10-fold higher *in vivo* activity, relative to metronidazole, against trichomonal infections. Moreover, ronidazole is more potent against a variety of anaerobic bacteria, *in vitro*.* However, ronidazole is also about 10-fold more mutagenic than metronidazole in Ames *Salmonella typhymurium* strain TA100 and in *Klebsielleae pneumoniae* [6].

Since the toxicity and carcinogenicity of many chemicals have been linked to the formation of reactive metabolites and since enzymatic nitroreduction has been implicated in the mutagenicity of nitroimidazoles [7], we sought to define better the metabolic and chemical basis for reactive metabolite formation from this class of drug. A review of the literature revealed that although significant information was available on the conversion of 5-nitroimidazoles to stable metabolites, little information was available on the mechanism of activation and the properties of reactive metabolites which could be responsible for the selected toxicities to the target organisms and the mutagenicity of these drugs. Consequently, we undertook studies to systematically investigate the mechanism of drug activation and protein alkylation with the assumption that the reactive metabolites characterized by these studies would prove to be informative about the chemical basis for the genotoxicity, specific organism toxicity, and mechanism of drug residue formation in food producing animals.

Table 1. Ronidazole structure and position of radiolabels



| Compound | Position of radiolabel |
|---|---|
| **Ia** | N-$^{14}CH_3$ |
| **Ib** | 2-$^{14}CH_2$ |
| **Ic** | 4,5-$^{14}C$ |
| **Id** | N-$C^3H_3$ |
| **Ie** | 4-$^3H$ |

Earlier studies successfully defined (1) the mammalian enzyme activating system [8, 9], (2) the nucleophilic target alkylated by ronidazole [10], (3) the mutagenicity of the soluble metabolite(s) and derivatives of ronidazole [11], and (4) many of the details of the mechanism of activation and the structure of the protein-bound metabolite [10, 12]. Based on several criteria, recent preliminary studies suggest that rat cecal bacteria catalyze an activation reaction identical to that catalyzed by mammalian hepatic microsomal enzymes while different reaction(s) are catalyzed by the enzymes present in the target organism, *T. foetus*.

## METHODS

Anaerobic incubations and protein alkylation determinations were conducted as described by West *et al.* [8]. The assays for measuring 4-$^3H$ release and ronidazole were those used by Miwa *et al.* [12].

* Unpublished observations.

Anaerobic incubations with rat cecal bacteria were carried out as described by Koch and Goldman [13] for metronidazole except that the $^{14}C$ isotopes, **Ia** through **Ic**, were coincubated with the N-$C^3H_3$ isotopically labeled compound, **Id**. Compound **Id** was used as an internal standard in some experiments, to minimize errors among samples, and the protein binding of each of the $^{14}C$-labeled compounds was normalized to the quantity of N-$C^3H_3$ bound and subsequently expressed relative to the binding of the N-$^{14}C$ compound, **Ia** (Table 2). Studies on the uptake of ronidazole into *T. foetus* were conducted essentially as described by Muller and Lindmark [14] except that compound **Id** was used as an internal standard for determining the relative binding of the $^{14}C$ isotopes, **Ia** through **Ic**.

## RESULTS AND DISCUSSION

### *Liver microsomal enzymes catalyze the reductive activation of ronidazole*

Monitoring reactive metabolite formation through irreversible protein alkylation, the enzymic nature of the activation could be traced through inhibition and induction studies principally to hepatic microsomal NADPH-cytochrome P-450 reductase and cytochrome P-450 [8, 9]. Rat liver microsomes are an abundant source of these enzymes [15, 16] and have been shown to catalyze the activation of ronidazole [8, 9] and other nitroaromatic substrates [17, 18]. Consequently, rat liver microsomes were chosen as a model mammalian activation system for mechanistic studies.

Protein alkylation paralleled factors implicating a reductive rather than an oxidative mechanism of activation. For example, (1) NADPH was required [8], (2) alkylation was diminished rather than increased in the presence of oxygen [8], (3) purified NADPH-cytochrome P-450 reductase optimally generated protein-bound products under anaerobic, reductive conditions [9], (4) sodium dithionite chemically produced intermediates that could alkylate cysteine [19], and (5) unequivocal evidence was provided by the identification of (1-methyl-4-*S*-cysteinyl-5-amino)-2-yl-*S*-methylcysteinylimidazole from microsomal incubations of ronidazole with excess cysteine and an NADPH-generating system [19].

### *Protein cysteine thiol is the principal target of alkylation by reactive metabolite(s) of ronidazole*

Several lines of evidence implicate cysteine thiol as the site of ronidazole alkylation on proteins. (1) Only cysteine, of several amino acids added to microsomal incubations, inhibited protein alkylation. Moreover, the inhibition was noncompetitive in accord with that expected for competition between microsomal and exogenous cysteine for a reactive metabolite [10]. (2) Immobilized thiols, such as agarose-cysteine and agarose-ethanethiol were capable of trapping reactive metabolites while agarose-methionine was no more effective than agarose alone in trapping insignificant quantities of ronidazole metabolites [10]. (3) The alkylation of bovine serum albumen could be blocked by the cysteine thiol specific agent methyl methanethiosulfonate [10]. (4) A dicysteinyl adduct was identified in microsomal incubations of ronidazole with cysteine and an NADPH-generating system [19]. (5) Unequivocal evidence for protein cysteine addition to the 2-methylene carbon of ronidazole was obtained from the protein-bound metabolites of 2-[$^{14}CH_2$] labeled ronidazole, **Ib**, obtained from both *in vitro* and *in vivo* sources. Acid hydrolysis of these samples yielded $^{14}C$-labeled carboxymethylcysteine.*

### *The mechanism of ronidazole activation*

There is evidence that a variety of reductants such as dihydroflavins [20], NADPH-cytochrome P-450 reductase [17], xanthine oxidase [21], and dithionite [22] reduce nitroaromatic compounds, including 5-nitroimidazoles, by four-electrons. Moreover, the generation of an unstable hydroxylaminoimidazole from 1-methyl-2-nitroimidazole following a four-electron reduction has been reported [23]. Accord-

* Unpublished observations.

Table 2. Relative protein-binding* of specifically labeled ronidazole substrates catalyzed by various enzyme sources

| | Rat liver | | Pig† | | | |
|---|---|---|---|---|---|---|
| Compound‡ | Microsomes§ | *In vivo*‖ | Liver | Muscle | Cecal bacteria¶ | *T. foetus*** |
| **Ia** | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| **Ib** | 0.99 | 1.17 | 1.00 | 0.96 | 1.12 | 1.40 |
| **Ic** | 1.09 | 1.00 | 1.07 | 1.00 | 1.18 | 1.30 |
| **Ie** 4-$^3H$ release/metabolism | 0.90 | 0.88 | 0.94 | 1.04 | 0.88 | 0.49 |

* Protein alkylation is expressed for each of the $^{14}C$-labelled substrates relative to **Ia**.

† A mixture of the indicated $^{14}C$-labeled drug and **Id** internal standard were dosed to animals in a small portion of feed 6 hrs before sacrifice. Liver and muscle samples were analyzed for protein binding and are expressed, relative to **Ia**, after correction for the $^3H$ internal standard.

‡ The position and identity of the radiolabel is given in Table 1.

§ Data obtained from [10].

‖ Data obtained from [12].

¶ Cecal bacteria were anaerobically incubated with ronidazole isotopes (0.5 mM) for 2 hr as described in Methods and protein-alkylation determined.

** The uptake of total radioactivity and protein-binding were determined after a 30 min incubation with 0.05 mM ronidazole as described in Methods.

ingly, we postulate the initial enzymatic four-electron reduction of ronidazole (Fig. 1) to the hydroxylamine intermediate, **II**, although other intermediates, such as the nitrosoimidazole, cannot be completely ruled out.

The hydroxylamine activates position $C_4$ of the imidazole to nucleophilic attack by protein thiols or water, producing imines **IIIa** and **IIIb**. Products identified from the hydrolysis of a 2-hydroxylaminoimidazole [23] are compatible with the analogous reactions proposed for **II**. The competition of thiols and water for a common reactive intermediate, such as **II**, is supported by kinetic evidence [10]. The loss of the $C_4$-proton, when a potential leaving group is not present at the 2-methylene position, could yield a 4-substituted 5-aminoimidazole and such a compound (1-hydroxyethyl-2-methyl-4-*S*-cysteinyl-5-aminoimidazole) has been identified from microsomal incubations of metronidazole with cysteine.* Moreover, precedence exists for an analogous reaction of phenylhydroxylamine to yield substituted anilines [24].

Loss of the $C_4$-proton from the labile intermediates **IIIa** or **IIIb** followed by elimination of the carbamate results in the formation of the Michael-like acceptors **IVa** and **IVb**. This is supported by the fact that the 4-$^3$H of **Ie** (Table 2) and the carbamate [10] are absent in the protein-bound metabolite while neither ronidazole nor *N*-acetyl ronidazole spontaneously eliminate their carbamoyl groups in neutral, aqueous solution. Moreover, the dicysteinylamino adduct of ronidazole, identified from microsomal incubations with excess, exogenous cysteine, is exactly the product expected from the stepwise nucleophilic attack of **II** and **IVa** by cysteine. During protein alkylation, however, **IVa** would be immobilized by the protein, R'SH, and, consequently, water might be a more reasonable alternative nucleophile yielding the putative 4-cysteinyl-aminoimidazole, **Va**.

The Michael-like acceptor **IVb**, which results from the initial attack of **II** by water, could undergo nucleophilic addition with protein thiols to yield the protein-bound imidazolinone, **Vb**, while reactions with water would yield various hydrolysis products. Structure-activity studies† and the previously observed carboxymethylcysteine, identified as an acid hydrolysis product from protein-bound ronidazole metabolites, suggest that **Vb** is the major protein-bound product.

The protein-bound adduct, **Vb**, retains the radiolabels in the *N*-methyl, 2-methylene, and 4,5-ring carbons (Table 2) but lacks the carbamate, as has been observed *in vitro* [10]. The observed 1:1 ratio between the $C_4$-proton release and total metabolism (Table 2) dictates that the loss of this proton occurs during all protein-binding and hydrolytic reactions. Since the quantity of ronidazole metabolized was approximately equal to the quantity of $C_4$-proton released, and since *in vitro* titration experiments have shown that the total quantity of reactive electrophilic metabolite formed was comparable to the quantity of 4-$^3$H released [8, 10, 12], a single reactive intermediate, such as **II**, must be common to both

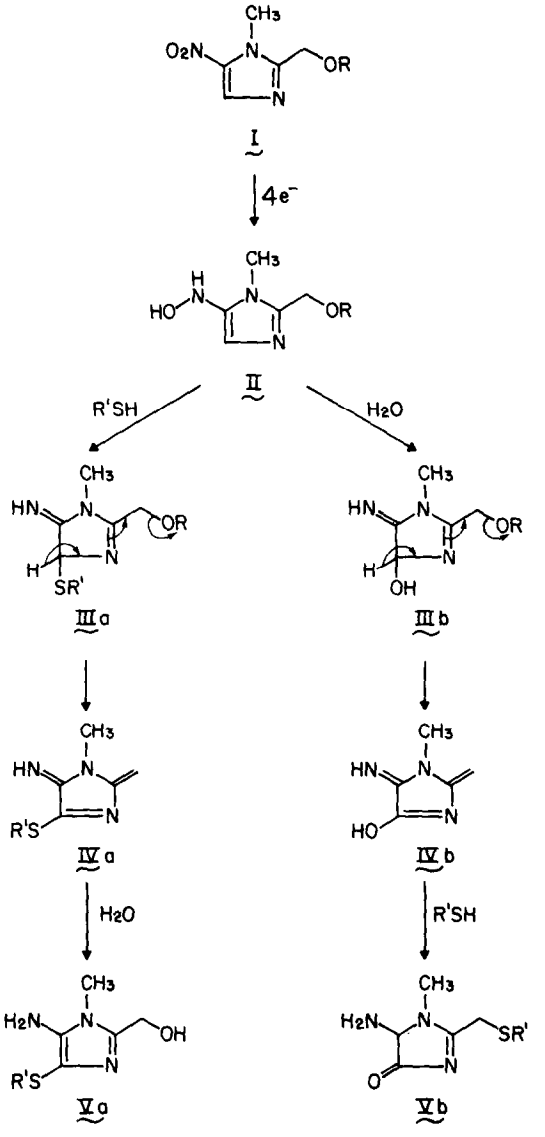



Fig. 1. Postulated mechanism for ronidazole activation.

hydrolytic and protein-binding reactions. Based on these results and the fact that the protein-bound products from *in vitro* and *in vivo* samples are identical, as assessed by identical imidazole ring fragments following acid hydrolysis, the ratio of 4-$^3$H release/total ronidazole metabolized may permit an estimation of the fraction of nitroimidazole metabolized which forms a unique electrophilic intermediate.

This technique has been applied to experiments in the intact rat and pig as well as *in vitro* experiments with cecal bacteria and *T. foetus* (Table 2). These data clearly demonstrate that the metabolism observed in the rat and pig, *in vivo*, and with rat liver microsomes or cecal bacteria, *in vitro*, share the common feature of essentially quantitative liberation of the 4-$^3$H from **Ie**, suggesting that ronidazole is almost completely metabolized to a common electrophilic intermediate by all these systems. In addition, experiments with various positional labels demonstrate that protein alkylation occurs

* P. Wislocki and E. Bagan, personal communication.

† Unpublished observations.

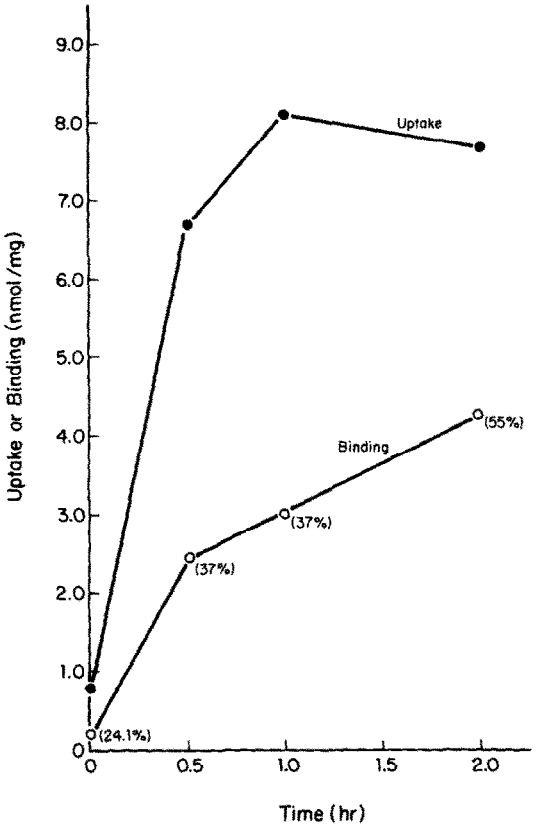



Fig. 2. Uptake and protein-binding of [N-$^{14}CH_3$]ronidazole in *T. foetus*. Total uptake of radioactivity into cells (●——●) and protein-binding (○——○) were determined as described in Methods. Numbers in parenthesis indicate fraction of total radioactivity that is protein-bound.

with retention of the imidazole carbon framework (Table 2, compounds **Ia** through **Ic**). Loss of the carbamate during protein alkylation has been demonstrated *in vitro* with rat liver microsomes [10] while direct evidence from *in vivo* and *in vitro* samples has already demonstrated the replacement of the carbamate with a protein cysteine thiol on the 2-methylene carbon of ronidazole [12].

These data are in contrast to similar studies with *T. foetus*. The preliminary evidence suggests that these organisms metabolize ronidazole by a pathway dissimilar to that catalyzed by mammalian and gut bacterial enzymes since only about 50% of the 4-$^3H$ was released (Table 2) during protein-binding. Moreover, binding may not have occurred with retention of an intact imidazole carbon skeleton as the relative binding of the 4,5-ring and 2-methylene radiolabels exceeded that for the *N*-methyl label. It is also remarkable that, under the conditions of the study, approximately 95% of the ronidazole taken up by the protozoa had been metabolized, resulting in a substantial fraction (24% to 55%) of the total radioactivity being protein-bound (Fig. 2). These observations indicate that *T. foetus* avidly metabolizes ronidazole by pathways different from mammalian and gut bacterial enzymes and suggest opportunities of more clearly defining the mechanism underlying the specific trichomonal activity of 5-nitroimidazoles. Such studies are currently being pursued in our laboratory.

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