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Report

Evidence for the Involvement of a Nitrenium Ion in the Covalent Binding of Nitrofurazone to DNA

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We have shown that the xanthine oxidase-catalyzed anaerobic reduction of nitrofurazone in the presence of added DNA leads to the formation of covalently bound adducts. Further, by systematically decreasing the pH of the reaction mixture, we have demonstrated that generation of the reactive species is facilitated under mildly acidic conditions. From these observations, we conclude that it is the nitrenium ion formed from nitrofurazone which binds to DNA.

KEY WORDS: nitrofurazone; nitrenium ion; DNA binding; reductive metabolism.

INTRODUCTION

Nitrofurazone is one of the 5-nitrofuran antibiotics used in clinical medicine. There is much circumstantial evidence indicating that reductive metabolism (Fig. 1) of the 5-nitro group is essential not only for the antibiotic activity of these drugs but also for their mutagenic/carcinogenic effects and many, if not all, of the clinically observed toxicities (1). While redox cycling of the nitro group (Fig. 1a) and the nitro anion radical (Fig. 1b) leads to oxidative stress, further reduction probably leads, as proposed by Biaglow *et al.* (2) to the nitroso, the *N*-hydroxylamine, and ultimately, the amine (Figs. 1c–e). It has been speculated that one of these intermediates is the reactive moiety which covalently binds to DNA, resulting in mutagenesis/carcinogenesis. While the radical anion has been detected and the amine isolated for several of the 5-nitrofurans, including nitrofurazone, the reactive species which covalently binds to DNA remains unknown (1). Mason and his colleagues have, however, shown that the radical anion does not bind to DNA or the tripeptide glutathione (3). In addition, the oxygen-insensitive *Escherichia coli* nitroreductase, which does not appear to be able to transfer single electrons, can metabolize nitrofurazone to a species which covalently binds to biomacromolecules (4). This evidence would appear to rule out the involvement of other possible free radical intermediates (such as the hydronitroxide and the amino cation radicals which would be formed by adding a single electron to the nitroso or *N*-hydroxylamine, respectively) in the formation of DNA adducts.

Other aromatic amino and nitro compounds are metabolized to the *N*-hydroxylamine, which, in an acid-catalyzed reaction, loses water to form the nitrenium ion which covalently binds to DNA (5–8). Thus, an increase in the covalent binding with a decrease in pH of the metabolic reaction mixture is taken as prima facie evidence for the participation of a nitrenium ion in the formation of these xenobiotic–DNA adducts (5–8).

We have used xanthine oxidase to catalyze the reduction of nitrofurazone in the presence of DNA to determine if the covalent binding of the metabolically generated, reactive intermediates is pH dependent. Hypoxanthine, which is concomitantly oxidized to xanthine and then to uric acid, was used to provide the reducing equivalents.

MATERIALS AND METHODS

Calf thymus DNA, nitrofurazone, xanthine oxidase, hypoxanthine, xanthine, uric acid, and deoxycytidine monophosphate were purchased from Sigma Chemical Co. (St. Louis, Mo.). [^{14}C]Nitrofurazone (59 mCi/mmol) was prepared as described previously (9) from [^{14}C]-5-nitro-2-furaldehyde diacetate, 59 mCi/mmol, which was purchased from Amersham Corp. (Arlington Heights, Ill.). All other chemicals were of analytical reagent grade or better.

Each nitrogen-purged incubation contained 3 mg of calf thymus DNA and 13 μmol of hypoxanthine dissolved in 3 ml of 16 m*M* potassium phophate buffer and 1 μmol of [^{14}C]nitrofurazone dissolved in 100 μl of dimethyl sulfoxide. After 15 min of preincubation, 1.1 unit of milk xanthine oxidase suspension (100 μl of a suspension in a 2.3 *M* ammonium sulfate, 7 mg of protein) was added. Incubations were carried out (at the pH values indicated in Table I) under a stream of nitrogen at 37°C for 1 hr in amber glass vials with Teflon-coated caps. Samples (100 μl) were taken before and after each incubation for the measurement of the [^{14}C]nitrofurazone concentration using our high-performance liquid chromatographic (HPLC) method (10). Control experiments including incubations without xanthine oxidase and without the N_2 purging resulted in no detectable loss of [^{14}C]nitrofurazone, indicating that metabolic activation had not oc-

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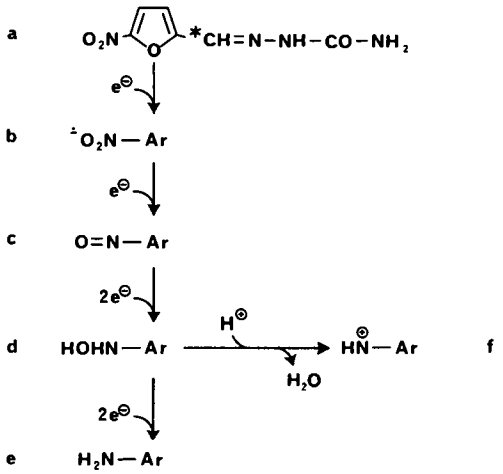

Fig. 1. Reductive metabolism of NFZ (a). (*) Radiolabeled carbon atom. The metabolites shown are the nitro anion radical (b), the nitroso (c), the *N*-hydroxylamine (d), the amine (e), and the nitrenium ion (f).

curred. In these cases no DNA binding was detectable. Additional samples (140 μl) were taken before and after each incubation and added to 60 μl of the 16 m*M* potassium phosphate, pH 7.4, buffer containing 0.12 μmol of deoxycytidine monophosphate, the internal standard, for the HPLC determination of uric acid, hypoxanthine, and xanthine. The retention times were 3.6, 5.3, 6.0, and 7.5 min, respectively, on an Alltech C18 column (10 μ, 25 × 0.45 cm) (Deerfield, Ill.) with a mobile phase of 0.01 *M* potassium dihydrogen phosphate (adjusted to pH 2.5 using orthophosphoric acid) pumped at 2 ml/min. Standard curves of peak height ratios of absorbances at 280 nm were linear over the entire range ($r^2 > 0.98$). At the conclusion of the incubation period, the contents of each vial were placed inside lengths of dialysis tubing (Spectra-Por 2, Spectrum, Los Angeles, Calif.) and dialyzed against 9 successive vol of 1500 ml of distilled water at 4°C. Each dialyzed sample was chromatographed on a 3-g hydroxylapatite (DNA-grade, Bio-Gel HTP, Bio-Rad, Richmond, Calif.) column as described by Beland *et al.*

Table I. Nitofurazone and Hypoxanthine Metabolism at Various Incubation pH's

pH	Time (hr)	NFZ (m*M*)	Hypoxanthine (m*M*)	Xanthine (m*M*)	Uric acid (m*M*)
5.79	0	0.653	4.20	0.00	0.00
	1	0.008	1.78	1.68	0.20
5.82	0	0.658	4.26	0.00	0.00
	1	0.014	1.74	1.70	0.23
5.97	0	0.674	4.14	0.00	0.00
	1	0.004	1.82	1.75	0.24
6.13	0	0.652	4.05	0.00	0.00
	1	0.000	1.63	1.74	0.29
6.44	0	0.649	4.03	0.00	0.00
	1	0.000	1.76	1.65	0.24
6.86	0	0.706	4.10	0.00	0.00
	1	0.000	1.63	1.68	0.33
7.23	0	0.653	4.16	0.00	0.00
	1	0.001	1.61	1.67	0.39
7.40	0	0.629	4.10	0.00	0.00
	1	0.007	1.68	1.62	0.41

(11). Fractions of about 3 ml were collected and the DNA content (by UV absorbance at 260 nm) and radioactivity (by liquid scintillation counting) were measured. The recovery of the DNA initially added to the incubations was 62.9 ± 6.3% (mean ± SD). For estimation of the specific activity bound to DNA the mean values from the fractions containing >200 μg were used (i.e., two or three per incubation) and the results are expressed as the number of drug residues bound per 10^6 base pairs of DNA.

RESULTS AND DISCUSSION

The results of these experiments are presented in Table 1 and Fig. 2. At the end of the 60-min incubation, 99.4 ± 0.8% of the [^{14}C]nitrofurazone had been metabolized. A total of 89.2 ± 2.0% of the hypoxanthine was accounted for as hypoxanthine, xanthine, or uric acid. Moreover, only 41.4 ± 1.9% of the hypoxanthine had been oxidized. Therefore, differences in the extent of binding of [^{14}C]nitrofurazone to DNA were not due to incomplete reduction of the antibiotic or to differences in the extent of hypoxanthine metabolism over the pH range studied.

The amount of covalently bound ^{14}C label was found to increase with decreasing pH. Least-squares linear analysis of the plot of drug residues bound per 10^6 DNA base pairs resulted in a line described by the equation $y = -70.5X + 677$ ($r^2 = 0.94$). This increase in the extent of binding as a function of decreasing pH is typical of the acid-catalyzed loss of water from an *N*-hydroxylamine to form a nitrenium ion which then reacts with DNA (5–8). However, the increase in binding of nitrofurazone to DNA was less than twofold between pH 7.5 and pH 5.8. With each unit decrease in pH there should, theoretically, be a 10-fold increase in the amount of nitrenium ion formed. Even with *N*-hydroxylarylamines, however, this binding increases only about fivefold (8). We believe that the relative reactivity of the *N*-hydroxylamines may account for both the current and

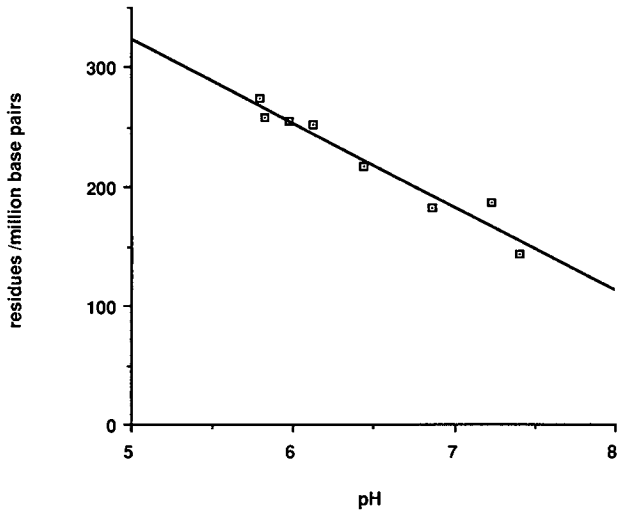

Fig. 2. A plot of the residues of nitrofurazone bound per 10^6 base pairs of DNA versus the pH of the incubation. The [^{14}C]nitrofurazone was metabolically activated under reductive conditions by xanthine oxidase and residues bound to added DNA were determined as described in the text. The line fitted through the points by least-squares regression analysis has an $r^2 = 0.94$.

the previous results (8). While the *N*-hydroxylamines and amines of aromatic hydrocarbons can be isolated and have finite shelf lives, the *N*-hydroxylamines of furans have never been isolated. Only the end product of the 6-electron reduction, the amino or the isomeric cyano, has been reported. Thus, the relative reactivity of the *N*-hydroxyl precursor of the nitrenium ion may explain the differences in the extent of DNA binding. Thus, it is possible that, like other aromatic nitro and amino compounds, nitrofurazone forms an adduct with the C-8 of deoxyguanosine or with other nucleophilic centers on DNA (12,13). We could also speculate that a glutathione conjugate of nitrofurazone (14) is formed by interaction of this nitrenium ion with the sulfhydryl function.

Under oxidative conditions 2-amino-4-(5-nitro-2-furyl)-thiazole, a 5-nitrofuran which is a bladder carcinogen, forms a glutathione conjugate via an S–C bond with the thiazole ring of the side chain (15). Also under oxidative conditions, furans without a nitro group, such as 4-ipomeanol and furosemide, form electrophilic species, presumably epoxides, which covalently bind to DNA and proteins (16). It is possible that nitrofurazone could also form adducts that do not involve the generation of the nitrenium ion. Our work did not test for binding under these conditions and does not, therefore, preclude such adducts. However, it has been shown that the covalent binding of nitrofurantoin (another 5-nitrofuran antibiotic) to rat lung or liver microsomes is favored under reductive conditions, while that by 4-ipomeanol is favored under oxidative conditions (17). Nevertheless, we report here the first evidence which indicates that an aryl nitrenium ion of nitrofurazone binds covalently to DNA.

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